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INDIAN PHARMACOPOEIA 2022

Volume III



Government of India
Ministry of Health & Family Welfare

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THE INDIAN PHARMACOPOEIA COMMISSION, GHAZIABAD

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General Notices

General Statements

The General Notices provide the basic guidelines for the interpretation and application of the standards, tests, assays, and other specifications of the Indian Pharmacopoeia (IP), as well as to the statements made in the monographs and other texts of the Pharmacopoeia.

A monograph is to be constructed in accordance with any general monograph or notice or any appendix, note or other explanatory material that is contained in this Pharmacopoeia and that is applicable to that monograph. All statements contained in the monograph, except where a specific general notice indicates otherwise and with the exceptions given hereafter, constitute standards for the official articles. An article is not of pharmacopoeial quality unless it complies with all of the requirements stated.

Exceptions to the General Notices do exist, and where they do, the wording in the individual monograph or an appendix takes precedence and specifically indicates directions or the intent. Thus, the specific wording of standards, tests, assays and other specifications is binding wherever deviations from the General Notices exist. Likewise, where there is no specific mention to the contrary, the General Notices apply.

Name. The full name or title of this book, including addenda thereto, is Indian Pharmacopoeia 2022, abbreviated to IP 2022. In the texts, the term "Pharmacopoeia" or "IP" without qualification means the Indian Pharmacopoeia 2022 and any amendments and thereto.

Official and Official Articles. The word 'official' wherever used in this Pharmacopoeia or with reference thereto, is synonymous with 'pharmacopoeial', with 'IP' and with 'compendial'. The designation IP in conjunction with the official title on the label of an article is an indication that the article purports to comply with IP standards.

The following terms are used where the articles for which monographs are provided are to be distinguished.

An official substance is a single drug or a drug entity or a pharmaceutical aid for which the monograph title includes no indication of the nature of a dosage form.

An official preparation is a drug product (dosage form) and is the finished or partially finished preparation or product of one or more official substances formulated for use on the patient.

An article is an item for which a monograph is provided, whether an official substance or an official preparation.

Official Standards. The requirements stated in the monographs apply to articles that are intended for medicinal use but not necessarily to articles that may be sold under the same name for other purposes.

An article is not of Pharmacopoeial quality unless it complies with all the requirements stated in the monograph. This does not imply that performance of all the tests in a monograph is necessarily a prerequisite for a manufacturer in assessing compliance with the Pharmacopoeia before release of a product.

Pharmacopoeial requirements for articles used in veterinary medicine are established on the same basis as those used in human medicine. It should be noted that no requirement in the pharmacopoeia can be taken in isolation. A valid interpretation of any particular requirement depends upon it being read in context of the monograph as a whole, the specified method of analysis, the relevant General Notices and where appropriate the General Monographs.

Where a preparation that is the subject of a monograph in the Indian Pharmacopoeia is supplied for use in veterinary medicine, the standards of Indian Pharmacopoeia apply unless otherwise justified and authorized.

The active pharmaceutical ingredients (drug substances), excipients (pharmaceutical aids), pharmaceutical preparations (dosage forms) and other articles described in the monographs are intended for human and veterinary use (unless explicitly restricted to one of these uses). It may be noted, however, that in the event of doubt of interpretation in any text of Veterinary monographs of IP, Indian Pharmacopoeia Commission (IPC) should be consulted.

The requirements given in the monographs are not framed to provide against all possible impurities, contaminants or adulterants; they provide appropriate limitation of potential impurities only.

A preparation must comply with the requirements specified, throughout its shelf-life assigned to it by the manufacturer. For opened or broached containers, the maximum period of validity for use will be as may be stated in the individual monograph. Nevertheless, the responsibility for assigning the period of validity shall be with the manufacturer.

Added Substances. An official substance, as distinguished from an official preparation, contains no added substances except when specifically permitted in the individual monograph. Unless otherwise specified in the individual monograph, or elsewhere in the General Notices, suitable substances may be added to an official preparation to enhance its stability, preserve its properties, usefulness or elegance, or to facilitate its preparation. Such auxiliary substances shall be harmless in the amounts used, shall not exceed the minimum quantity required to provide their intended effect, shall not impair the therapeutic efficacy or the bioavailability or safety of the preparation and shall not interfere with any of the tests and assays prescribed for determining compliance with the official

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standards. Particular care should be taken to ensure that such substances are free from harmful organisms. The freedom to the manufacturers to add auxiliary substances imposes on them the responsibility of satisfying the licensing authorities on the purpose of the addition and the innocuity of such substances. No substance shall be added to conceal any defect or damage or deficiency in the substance or formulation.

Alternative Methods. The tests and assays described are the official methods upon which the standards of the Pharmacopoeia are based. Alternative methods of analysis may be used for control purposes, provided that the methods used are shown to give results of equivalent accuracy and enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. Automated procedures utilising the same basic chemistry as the test procedures given in the monograph may also be used to determine compliance. Such alternative or automated procedures must be validated and are subject to approval by the authority competent to authorised manufacturer of substance or product.

In the event of doubt or dispute, the methods of analysis of the Pharmacopoeia are alone authoritative and only the result obtained by the procedure given in this Pharmacopoeia is conclusive.

Meanings of Terms

Alcohol. The term "alcohol" without qualification means ethanol (95 per cent). Other dilutions of ethanol are indicated by the term "ethanol" or "alcohol" followed by a statement of the percentage by volume of ethanol (C_2H_6O) required.

Desiccator. A tightly-closed container of suitable size and design that maintains an atmosphere of low moisture content by means of silica gel or phosphorus pentoxide or other suitable desiccant.

Drying and ignition to constant weight. Two consecutive weighings after the drying or igniting operations do not differ by more than 0.5 mg, the second weighing following an additional period of drying or of ignition respectively appropriate to the nature and quantity of the residue.

Ethanol. The term "ethanol" without qualification means anhydrous ethanol or absolute alcohol.

Filtration. Unless otherwise stated, filtration is the passing of a liquid through a suitable filter paper or equivalent device until the filtrate is clear.

Freshly prepared. Made not more than 24 hours before it is used.

Label. Any printed packing material, including package inserts that provide information on the article.

Negligible. A quantity not exceeding 0.50 mg.

Solution. Where the name of the solvent is not stated, "solution" implies a solution in water. The water used complies with the requirements of the monograph on Purified Water.

Temperature. The symbol ° used without qualification indicates the use of the Celsius thermometric scale.

Water. If the term is used without qualification it means Purified Water of the Pharmacopoeia. The term 'distilled water' indicates Purified Water prepared by distillation.

Water-bath. A bath of boiling water unless water at another temperature is indicated. Other methods of heating may be used provided the required temperature is approximately maintained but not exceeded.

Provisions Applicable To Monographs and Test Methods

Expression of Contents. Where the content of a substance is defined, the expression "per cent" is used according to circumstances with one of two meanings:

— per cent w/w (percentage, weight in weight) expressing the number of grams of substance in 100 grams of final product,

— per cent v/v (percentage, volume in volume) expressing the number of millilitres of substance in 100 millilitres of final product.

The expression "parts per million" refers to the weight in weight, unless otherwise stated.

Where the content of a substance is expressed in terms of the chemical formula for that substance an upper limit exceeding 100 per cent may be stated. Such an upper limit applies to the result of the assay calculated in terms of the equivalent content of the specified chemical formula. For example, the statement 'contains not less than 99.0 per cent and not more than 101.0 per cent of $C_7H_6O_2$ implies that the result of the assay is not less than 99.0 per cent and not more than 101.0 per cent, calculated in terms of the equivalent content of $C_7H_6O_2$.

Where the result of an assay or test is required to be calculated with reference to the dried, anhydrous, ignited substance, or the substance free from solvent, the determination of loss on drying, water content, loss on ignition, content of the specified solvent, respectively is carried out by the method prescribed in the relevant test in the monograph.

Expression of Concentrations. The following expressions in addition to the ones given under Expression of Content are also used:

— per cent w/v (percentage, weight in volume) expressing the number of grams of substance in 100 millilitres of product,

— per cent v/w (percentage, volume in weight) expressing the number of millilitres of substance in 100 grams of product.

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Usually, the strength of solutions of solids in liquids is expressed as percentage weight in volume, of liquids in liquids as percentage volume in volume, of solids in semi-solid bases (e.g. creams) and of gases in liquids as percentage weight in weight.

When the concentration of a solution is expressed as parts of dissolved substance in parts of solution, it means parts by weight (g) of a solid in parts by volume (ml) of the final solution; as parts by weight (g) of a gas in parts by weight (g) of the final solution.

When the concentration of a solution is expressed in molarity designated by the symbol M preceded by a number, it denotes the number of moles of the stated solute contained in sufficient Purified Water (unless otherwise stated) to produce 1 litre of solution.

Abbreviated Statements. Incomplete sentences are employed in parts of the monographs for directness and brevity (for example, Iodine Value. Not more than ……; Relative Density. …….to……..) Where the tests are abbreviated, it is to be understood that the test method referred to in brackets provides the method to be followed and that the values specified are the applicable limits.

Weights and Measures. The metric system of weights and measures is employed in the Pharmacopoeia. All measures are required to be graduated at 25° and all measurements in tests and assays, unless otherwise stated, are to be made at that temperature. Graduated glass apparatus used in analytical operations shall comply with the requirements stated in Chapter 2.1.6.

Monographs

General Monographs

General monographs on dosage forms include requirements of general application and apply to all preparations within the scope of the Introduction section of the general monograph, except where a preamble limits the application. The requirements are not necessarily comprehensive for a given specific preparation; additional requirements may sometimes be given in the individual monograph for it.

Production. Statements given under the heading Production relate to particular aspects of the manufacturing process and are not necessarily comprehensive. However, they are mandatory instructions to manufacturers. They may relate, for example, to source materials, to the manufacturing process and its validation and control, to any in-process testing that is to be carried out by the manufacturer on the final product either on selected batches or on each batch prior to release. All this cannot be verified on a sample of the final product by an independent analyst. It is for the licensing authority to verify that the instructions have been followed.

The absence of a section on Production does not imply that attention to features such as those given above is not required. An article described in a monograph of the Pharmacopoeia is to be manufactured in accordance with the principles of good manufacturing practice and in accordance with the requirements of the Drugs and Cosmetics Rules, 1945. The general principles applicable to the manufacture and quality assurance of drugs and preparations meant for human use apply equally to veterinary products as well.

Manufacture of Drug Products. The opening definitive statement in certain monographs for drug products is given in terms of the active ingredient(s) only. Any ingredient(s) other than those included in the statement, must comply with the general notice on Excipients and the product must conform to the Pharmacopoeial requirements.

Official preparations are prepared only from ingredients that comply with the requirements of the pharmacopoeial monographs for those individual ingredients for which monographs are provided.

Excipients. Any substance added in preparing an official preparation shall be innocuous, shall have no adverse influence in the therapeutic efficacy of the active ingredients and shall not interfere with the tests and assays of the Pharmacopoeia. Care should be taken to ensure that such substances are free from harmful organisms.

Individual Monographs

Drug products that are the subject of an individual monograph are also required to comply with the tests given in the general monographs.

Titles. The main title for a drug substance is the International Non-proprietary Name (INN) approved by the World Health Organization. Subsidiary names and synonyms have also been given in some cases; where included, they have the same significance as the main title.

The main titles of drug products are the ones commonly recognised in practice. Synonyms drawn from the full non-proprietary name of the active ingredient or ingredients have also been given. Where, however, a product contains one or the other of different salts of an active molecule, the main title is based on the full name of the active ingredient. For example, Chloroquine Phosphate Tablets and Chloroquine Sulphate Tablets.

Chemical Formulae. When the chemical structure of an official substance is known or generally accepted, the graphic and molecular formulae are normally given at the beginning of the monograph for information. This information refers to the chemically pure substance and is not to be regarded as an indication of the purity of the official material. Elsewhere, in statement of purity and strength and in descriptions of

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processes of assay, it will be evident from the context that the formulae denote the chemically pure substances.

Where the absolute stereochemical configuration is specified, the International Union of Pure and Applied Chemistry (IUPAC) *R/S* and *E/Z* systems of designation have been used. If the substance is an enantiomer of unknown absolute stereochemistry, the sign of the optical rotation, as determined in the solvent and under the conditions specified in the monograph, has been attached to the systematic name. An indication of sign of rotation has also been given where this is incorporated in a trivial name that appears on an IUPAC preferred list.

Atomic and Molecular Weights. The atomic weight or molecular weight is shown, as and when appropriate at the top right hand corner of the monograph. The atomic and molecular weights and graphic formulae do not constitute analytical standards for the substances described.

Definition. The opening statement of a monograph is one that constitutes an official definition of the substance, preparation or other article that is the subject of the monograph. In certain monographs for pharmaceutical preparations the statement is given in terms of the principal ingredient(s).

In monographs on vegetable drugs, the definition indicates whether the subject of the monograph is, for example, the whole drug or the drug in powdered form.

Certain pharmaceutical substances and other articles are defined by reference to a particular method of manufacture. A statement that a substance or article is prepared or obtained by a certain method constitutes part of the official definition and implies that other methods are not permitted. A statement that a substance may be prepared or obtained by a certain method, however, indicates that this is one possible method and does not imply that other methods are not permissible.

Statement of content. The limits of content stated are those determined by the method described under Assay.

Category. The statement of category is provided for general information only and is indicative of the medical or pharmaceutical basis for recognition in the Pharmacopoeia. It generally represents an application of the best known pharmacological action of the article or of its active ingredient. The statement under the heading 'Category' are also subject to regulations under the D&C Act 1940 and rules theirunder. In the case of pharmaceutical aids it may indicate the more common usage of the article. The statement is not intended to limit in any way the choice or use of the article nor to indicate that it has no other activity or use.

Usual strength. The statement on the usual strength(s) of a preparation given in the individual monograph indicates the strength(s) usually marketed for information of the pharmacist and the medical practitioner. It does not imply that a strength other than the one(s) mentioned in the individual monograph meeting all the prescribed requirements cannot be manufactured and marketed with the approval of the appropriate authority.

Description. The statements under the heading Description are not to be interpreted in a strict sense and are not to be regarded as official requirements.

Solubility. Statements on solubility are given in Chapter 2.4.26 and are intended as information on the approximate solubility at a temperature between 15° and 30°, unless otherwise stated, and are not to be considered as official requirements. However, a test for solubility stated in a monograph constitutes part of the standards for the substance that is the subject of that monograph.

Residual solvents. The requirements, guidance and information on residual solvents for pharmaceutical use are given in the chapter entitled Residual Solvents (5.4).

All IP articles are subject to relevant control of residual solvents, even when no test is specified in the individual monograph. If solvents are used during production, they must be of suitable quality. In addition, the toxicity and residual level of each solvent shall be taken into consideration and the solvents limited according to the principles defined and the requirements specified in Chapter 5.4. Residual Solvent, using the general methods presented therein or other suitable methods.

Test Methods

References to general methods of testing are indicated by test method numbers in brackets immediately after the heading of the test or at the end of the text.

Identification. The tests given under the heading Identification are not necessarily sufficient to establish absolute proof of identity. They provide a means of verifying that the identity of the material under examination is in accordance with the label on the container.

In certain monographs alternative series of identification tests are given; compliance with either one or the other set of tests is adequate to verify the identity of the article.

When tests for infrared absorption are applied to material extracted from formulated preparations, strict concordance with the specified reference spectrum may not always be possible, but nevertheless a close resemblance between the spectrum of the extracted material and the specified reference spectrum should be achieved.

Tests and Assays

The tests and assays are the official methods upon which the standards of the Pharmacopoeia depend. The requirements are not framed to take into account all possible impurities. It is

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not to be presumed, for example, that an impurity that is not detectable by means of the prescribed tests is tolerated. Material found to contain such an impurity is not of pharmacopoeial quality if the nature or amount of the impurity found is incompatible with good pharmaceutical practice.

Pharmacopoeial methods and limits should be used merely as compliance requirements and not as requirements to guarantee total quality assurance. Tests and assays are prescribed for the minimum sample available on which the attributes of the article should be measured. Assurance of quality must be ensured by the manufacturer by the use of statistically valid sampling and testing programmes.

Tests. Unless otherwise stated, the assays and tests are carried out at a temperature between 20° and 30°.

Where it is directed that an analytical operation is to be carried out 'in subdued light', precautions should be taken to avoid exposure to direct sunlight or other strong light. Where a procedure is directed to be performed 'protected from light' precautions should be taken to exclude actinic light by the use of low-actinic glassware, working in a dark room or similar procedures.

For preparations other than those of fixed strength, the quantity to be taken for a test or an assay is usually expressed in terms of the active ingredient. This means that the quantity of the active ingredient expected to be present and the quantity of the preparation to be taken are calculated from the strength stated on the label.

Other Tests. In the monographs on dosage forms and certain preparations, under the sub-heading 'Other tests' it is stated that the article complies with the tests stated under the general monograph of the relevant dosage form or preparation. Details of such tests are provided in the general monographs.

Limits. The limits given are based on data obtained in normal analytical practice. They take into account normal analytical errors, of acceptable variations in manufacture and of deterioration to an extent that is acceptable. No further tolerances are to be applied to the limits for determining whether or not the article under examination complies with the requirements of the monograph.

Quantities. Unless otherwise stated, the quantities to be taken for assays, limit tests and other tests are of the substance under examination.

In tests with numerical limits and assays, the quantity stated to be taken for testing is approximate. The amount actually used, which may deviate by not more than 10 per cent from that stated, is accurately weighed or measured and the result of analysis is calculated from this exact quantity. In tests where the limit is not numerical but usually depends upon comparison with the behaviour of a reference in the same conditions, the stated quantity is taken for testing. Reagents are used in the prescribed amounts.

Quantities are weighed or measured with an accuracy commensurate with the indicated degree of precision. For weighings, the precision is plus or minus 5 units after the last figure stated. For example, 0.25 g is to be interpreted as 0.245 g to 0.255 g. For the measurement of volumes, if the figure after the decimal point is a zero or ends in a zero, e.g. 10.0 ml or 0.50 ml, the volume is measured using a pipette, a volumetric flask or a burette, as appropriate; in other cases, a graduated measuring cylinder or a graduated pipette may be used. Volumes stated in microlitres are measured using a micropipette or microsyringe.

The term 'transfer' is used generally to indicate a quantitative operation.

Apparatus. Measuring and weighing devices and other apparatus are described in the chapter entitled 'Apparatus for Tests and Assays'. A specification for a definite size or type of container or apparatus in a test or assay is given merely as a recommendation.

Unless otherwise stated, comparative tests are carried out using identical tubes of colourless, transparent, neutral glass with a flat base, commonly known as Nessler cylinders.

Reagents and Solutions. The reagents required for the tests and assays of the Pharmacopoeia are defined in the various chapters showing their nature, degree of purity and the strengths of the solutions to be made from them. The requirements set out are not intended to imply that the materials are suitable for use in the test concerned; reagents not covered by monographs in the pharmacopoeia shall not be claimed to be of IP quality.

The term 'analytical reagent grade of commerce' implies that the chemical is of a high degree of purity wherein the limits of various impurities are known. Where it is directed to use a 'general laboratory reagent grade of commerce' it is intended that a chemically pure grade material, not necessarily required to be tested for limiting or absence of certain impurities, is to be used.

Indicators. Where the use of an indicator solution is mentioned in an assay or test, approximately 0.1 ml of the solution shall be added, unless otherwise directed.

Reference Substances. Certain monographs require the use of a chemical reference substance or a biological reference preparation or a reference spectrum These are authentic specimens chosen and verified on the basis of their suitability for intended use as prescribed in the Pharmacopoeia and are not necessarily suitable in other circumstances.

IP Reference Substances, abbreviated to IPRS are issued by the Indian Pharmacopoeia Commission (IPC). They are the official standards to be used in cases of arbitration.

Biological Reference Substances, also abbreviated to IPRS and Standard Preparations of antibiotics are issued by

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agencies authorised by the IPC. They are standardized against the International Standards and Reference Preparations established by the World Health Organization (WHO). The potency of these preparations is expressed in International Units.

Reference spectra are published by the IPC and they are accompanied by information concerning the conditions used for sample preparation and recording of the spectra.

Test Animals. The animal experiments are carried out in accordance with the provisions of 'The Prevention of Cruelty to Animals Act, 1960' and 'CPCSEA Guidelines' so as to prevent the infliction of unnecessary pain, suffering and prevention of cruelty to animals. Unless otherwise directed, animals used in a test or an assay shall be healthy and are drawn from a uniform stock, and have not previously been treated with any material that will interfere with the test or the assay.

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

When rounding is required, consider only one digit in the decimal place to the right of the last place in the limit expression. If this digit is 4 or smaller, it is eliminated and the preceding digit is left unchanged. If this digit is 5 to 9, it is eliminated and the preceding digit is increased by 1.

Table 1 – Illustration of Rounding Numerical values for comparison with Requirements

Pharmacopoeial Requirement	Unrounded Value (per cent)	Rounded Result (per cent)	Conforms
Assay limit	97.96	98.0	Yes
≥ 98.0 per cent	97.92	97.9	No
	97.95	98.0	Yes
Assay limit	101.55	101.6	No
≤ 101.5 per cent	101.46	101.5	Yes
	101.45	101.5	Yes
Limit test	0.025	0.03	No
≤ 0.02 per cent	0.015	0.02	Yes
	0.027	0.03	No
Limit test ≤ 3 ppm	3.5 ppm	4 ppm	No
	3.4 ppm	3 ppm	Yes
	2.5 ppm	3 ppm	Yes

Storage. Statements under the side-heading 'Storage' constitute non-mandatory advice. The articles of the Pharmacopoeia are to be stored under conditions that prevent contamination and, as far as possible, deterioration. Precautions that should be taken in relation to the effects of the atmosphere, moisture, heat and light are indicated, where appropriate, in the individual monograph.

Specific directions are given in some monographs with respect to the temperatures at which Pharmacopoeial articles should be stored, where it is considered that usage at a lower or higher temperature may produce undesirable results. The storage conditions are defined by the following terms:

— Store in a dry, well-ventilated place at a temperature not exceeding 30°
— Store in a refrigerator (2° to 8°). Do not freeze
— Store in a freezer (-2° to -18°)
— Store in a deep freezer (Below -18°)

Storage conditions not related to temperature are indicated in the following terms:

— Store protected from light
— Store protected from light and moisture

Where no specific storage directions or limitations are given in the monograph or in the D&C rules 1945 or by the manufacturer, it is to be understood that the storage conditions include protection from moisture, direct sunlight, freezing and excessive heat (any temperature above 40°).

Storage Containers. The requirements, guidance and information on containers for pharmaceutical use are given in the chapter entitled Containers (6.2).

In general, an article should be packed in a well-closed container i.e. one that protects the contents from contamination by extraneous solids, liquids, moisture or vapours and from loss of the article under normal conditions of handling and storage and preserves the properties of the drug. Containers, unless otherwise specified, or of the nature such as capsule shall, foils of strips etc, shall allow examination of the contents inside. Closures used shall also of suitable properties and quality to protect the drug from any contamination and shall not be the source of contamination by themselves. Notices as may be needed in respect of Radiopharmaceuticals may also be incorporated.

Where, additionally, loss or deterioration of the article from effervescence, deliquescence or evaporation under normal conditions of storage is likely, the container must be capable of being tightly closed, and re-closed after use.

In certain cases, special requirements of pack have been indicated in some monographs under Storage, using expressions that have been defined in chapter 6.2.

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Labelling. The labelling of drugs and pharmaceuticals is governed by the Drugs and Cosmetics Rules, 1945. The statements that are given in the monographs under the side-heading 'Labelling' are not comprehensive. Only those that are necessary to demonstrate compliance or otherwise with the monograph have been given and they are mandatory. For example, in the monograph on Betamethasone Sodium Tablets the labelling statement is "The label states the strength in terms of the equivalent amount of betamethasone".

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DRUG SUBSTANCES, DOSAGE FORMS AND PHARMACEUTICAL AIDS

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N

Nabumetone 3013
Nabumetone Tablets 3014
Nadifloxacin 3015
Nadifloxacin Cream 3016
Nadifloxacin Gel 3016
Nalidixic Acid 3017
Nalidixic Acid Tablets 3018
Nalorphine Hydrochloride 3019
Nalorphine Injection 3020
Naloxone Hydrochloride 3020
Naloxone Injection 3022
Naltrexone Hydrochloride 3023
Naltrexone Tablets 3024
Nandrolone Decanoate 3025
Nandrolone Decanoate Injection 3026
Nandrolone Phenylpropionate 3026
Nandrolone Phenylpropionate Injection 3027
Naphazoline Nitrate 3028
Naproxcinod 3029
Naproxen 3030
Naproxen Oral Suspension 3031
Naproxen Suppositories 3032
Naproxen Prolonged-release Tablets 3033
Naproxen Tablets 3033
Natamycin 3034
Natamycin Ophthalmic Suspension 3035
Nebivolol Hydrochloride 3035
Nebivolol Tablets 3036
Nelfinavir Mesylate 3037
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Nelfinavir Tablets 3039
Neomycin Sulphate 3040
Neomycin Eye Drops 3041
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Neostigmine Bromide 3043
Neostigmine Tablets 3044
Neostigmine Methylsulphate 3044
Neostigmine Injection 3045
Neotame 3046
Netilmicin Sulphate 3047
Netilmicin Injection 3048
Nevirapine 3049
Nevirapine Oral Suspension 3050
Nevirapine Tablets 3051
Nicardipine Hydrochloride 3052
Niclosamide 3053
Niclosamide Tablets 3054
Nicorandil 3055
Nicorandil Prolonged-release Tablets 3056
Nicorandil Tablets 3058
Nicoumalone 3058
Nicoumalone Tablets 3059
Nifedipine 3060
Nifedipine Capsules 3061
Nifedipine Prolonged-release Capsules 3063
Nifedipine Prolonged-release Tablets 3064
Nifedipine Tablets 3065
Nikethamide 3067
Nikethamide Injection 3067
Nimodipine 3068
Nimodipine Tablets 3069
Nitrazepam 3070
Nitrazepam Tablets 3071

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Nitrofurantoin 3072
Nitrofurantoin Tablets 3074
Nitrofurazone 3075
Nitroglycerin Injection 3076
Nitrous Oxide 3076
Noradrenaline Bitartrate 3078
Noradrenaline Bitartrate Injection 3079
Norethisterone 3079
Norethisterone Tablets 3080
Norethisterone and Ethinyl Estradiol Tablets 3081
Norfloxacin 3083
Norfloxacin Eye Drops 3084
Norfloxacin Tablets 3084
Norgestimate 3085
Norgestimate and Ethinyl Oestradiol Tablets 3086
Norgestrel 3087
Norgestrel and Ethinyloestradiol Tablets 3088
Nortriptyline Hydrochloride 3089
Nortriptyline Tablets 3090
Noscapine 3091
Noscapine Linctus 3092
Novobiocin Sodium 3093
Nystatin 3094
Nystatin Ointment 3095
Nystatin Pessaries 3095
Nystatin Tablets 3096

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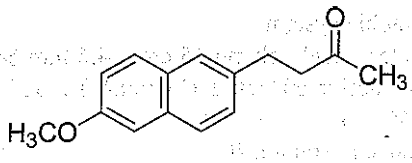
Nabumetone



$C_{15}H_{16}O_2$ Mol. Wt. 228.3

Nabumetone is 4-(6-methoxynaphthalen-2-yl)butan-2-one.

Nabumetone contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{15}H_{16}O_2$, calculated on the anhydrous basis.

Category. Cyclo-oxygenase inhibitor; analgesic; anti-inflammatory.

Description. A white or almost white, crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nabumetone IPRS* or with the reference spectrum of nabumetone.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution (a). Dissolve 50 mg of the substance under examination in *acetonitrile* and dilute to 10.0 ml with *acetonitrile*.

Test solution (b). Dilute 1.0 ml of test solution (a) to 25.0 ml with *acetonitrile*. Dilute 1.0 ml of the solution to 5.0 ml with *acetonitrile*.

Reference solution (a). A 0.004 per cent w/v solution of *nabumetone IPRS* in *acetonitrile*.

Reference solution (b). Dilute 0.5 ml of test solution (a) to 100.0 ml with *acetonitrile*.

Reference solution (c). A 0.0015 per cent w/v solution of *nabumetone impurity F IPRS (6,6'-dimethoxy-2,2'-binaphthalenyl IPRS)* in *acetonitrile*.

Reference solution (d). A 0.004 per cent w/v solution of *nabumetone impurity D IPRS ((E)-4-(6-methoxynaphthalen-2-yl)but-3-en-2-one IPRS)* in *acetonitrile*. To 5.0 ml of the solution, add 5.0 ml of test solution (b).

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with base deactivated octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 12 volumes of *tetrahydrofuran*, 28 volumes of *acetonitrile* and 60 volumes of a 0.1 per cent v/v solution of *glacial acetic acid* in *carbon dioxide-free water*,

  B. a mixture of 24 volumes of *tetrahydrofuran*, 56 volumes of *acetonitrile* and 20 volumes of a 0.1 per cent v/v solution of *glacial acetic acid* in *carbon dioxide-free water*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
12	100	0
28	0	100
33	0	100
35	100	0

The retention time of the nabumetone peak is about 11 minutes.

Inject reference solution (d). The test is not valid unless the resolution between the peaks due to nabumetone and nabumetone impurity D is not less than 1.5.

Inject reference solution (b), (c) and test solution (a). In the chromatogram obtained with test solution (a), the area of any peak corresponding to nabumetone impurity F is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent), the area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks other than nabumetone impurity F is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). 2.0 g complies with limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.2 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modifications.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject reference solution (a) and test solution (b).

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Calculate the content of $C_{15}H_{16}O_2$.

Storage. Store protected from light.

Nabumetone Tablets

Nabumetone Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of nabumetone, $C_{15}H_{16}O_2$.

Usual strengths. 500 mg; 750mg.

Identification

Disperse a quantity of the powdered tablets containing about 0.1 g of nabumetone in 25 ml of *acetone*, with the aid of ultrasound for about 30 minutes and filter. Evaporate the filtrate to dryness on a water-bath and dry the residue in an oven at 60° for about half an hour. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nabumetone IPRS* or with the reference spectrum of nabumetone.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of 2 per cent w/v solution of *sodium lauryl sulphate*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the medium, if necessary, at the maxima at about 270 nm and minima at about 296 nm (2.4.7). Calculate the content of $C_{15}H_{16}O_2$ in the medium from the difference of absorbance obtained at 270 nm and 296 nm by using a solution of known concentration of *nabumetone IPRS* in the same medium.

Q. Not less than 75 per cent of the stated amount of $C_{15}H_{16}O_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 0.25 g of Nabumetone with *acetontrile* and dilute to 50.0 ml with *acetonitrile*, filter.

Reference solution (a). A 0.0025 per cent w/v solution of *nabumetone IPRS* in *acetonitrile*.

Reference solution (b). A 0.0015 per cent w/v solution of *nabumetone impurity F IPRS (6,6'-dimethoxy-2,2'-binaphthalenyl IPRS)* in *acetonitrile*.

Reference solution (c). A solution containing 0.002 per cent w/v, each of, *nabumetone IPRS* and *nabumetone impurity D IPRS ((E)-4-(6-methoxynaphthalen-2-yl)but-3-en-2-one IPRS)* in *acetonitrile*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with base deactivated octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 12 volumes of *tetrahydrofuran*, 28 volumes of *acetonitrile* and 60 volumes of a 0.1 per cent v/v solution of *glacial acetic acid* in *carbon dioxide-free water*,
  B. a mixture of 24 volumes of *tetrahydrofuran*, 56 volumes of *acetonitrile* and 20 volumes of a 0.1 per cent v/v solution of *glacial acetic acid* in *carbon dioxide-free water*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
12	100	0
28	0	100
33	0	100
34	100	0

The retention time of the nabumetone peak is about 11 minutes.

Inject reference solution (c). The test is not valid unless the resolution between the two principal peaks is not less than 1.5.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to nabumetone impurity F is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent) and the sum of the areas of all the secondary peaks other than nabumetone impurity F is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 0.5 g of Nabumetone in 40 ml of *acetonitrile*, with the aid of ultrasound for 15 minutes, allow to cool and dilute to 50.0 ml with *acetonitrile*, filter. Dilute 5.0 ml of the filtrate to 100.0 ml with the mobile phase. Further dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

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Reference solution. Dilute 5.0 ml of a 0.1 per cent w/v solution of *nabumetone IPRS* in *acetonitrile* to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with base deactivated octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 18 volumes of *tetrahydrofuran*, 40 volumes of a 0.1 per cent v/v solution of *glacial acetic acid* in *carbon dioxide-free water* and 42 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

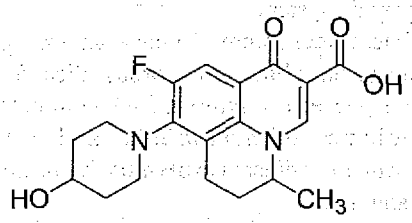
The retention time of the nabumetone peak is about 4 minutes.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{16}O_2$ in the tablets.

Nadifloxacin



$C_{19}H_{21}FN_2O_4$ Mol.Wt. 360.4

Nadifloxacin is 9-Fluoro-8-(4-hydroxy-piperidin-1-yl)-5-methyl-1-oxo-6,7-dihydro-1*H*,5*H*-pyrido[3,2,1-ij]quinoline-2-carboxylic acid.

Nadifloxacin contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{19}H_{21}FN_2O_4$, calculated on the anhydrous basis.

Category. Antibacterial.

Description. An off–white to pale yellow crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nadifloxacin IPRS* or with the reference spectrum of nadifloxacin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 30 volumes of a buffer solution prepared by dissolving 1.927 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 3.6 with *orthophosphoric acid* and 70 volumes of *acetonitrile*.

Test solution. Dissolve 25 mg of the substance under examination in 15 ml of the solvent mixture and dilute to 25.0 ml with the solvent mixture.

Reference solution. A 0.001 per cent w/v solution of *nadifloxacin IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS 3V),
- mobile phase: a mixture of equal volumes of a buffer solution prepared by dissolving 1.927 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 3.6 with *orthophosphoric acid* and *acetonitrile*.
- flow rate: 1 ml per minute,
- spectrophotometer set at 235 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not more than 3.0 per cent, determined on 0.25 g.

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modifications.

Test solution. Dissolve 25 mg of the substance under examination in about 70 ml of the solvent mixture with the aid of ultrasound and dilute to 100.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.0025 per cent w/v solution of *nadifloxacin IPRS* in the solvent mixture.

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Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{21}FN_2O_4$

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Nadifloxacin Cream

Nadifloxacin Cream contains Nadifloxacin in a suitable cream base.

Nadifloxacin Cream contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nadifloxacin, $C_{19}H_{21}FN_2O_4$.

Usual strength. 1 per cent w/w.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.5 to 6.0.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *water* and *acetonitrile.*

Test solution. Disperse a quantity of the cream containing 10 mg of Nadifloxacin in 70 ml of the solvent mixture, heat on a water-bath at 60° for 20 minutes with shaking to obtain a uniform dispersion. Mix with the aid of ultrasound for 15 minutes with intermittent swirling, dilute to 100.0 ml with the solvent mixture and filter, discarding first few ml of filtrate.

Reference solution. A 0.00005 per cent w/v solution of *nadifloxacin IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of *water*, 35 volumes of *acetonitrile* and 1 volume of *triethylamine* adjusted to pH 4.0 with *orthophosphoric acid,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0, and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent).

Other tests. Comply with the tests stated under Creams.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *water* and *acetonitrile.*

Test solution. Disperse a quantity of the cream containing 10 mg of Nadifloxacin in 70 ml of the solvent mixture, heat on a water bath at 60° for 20 minutes with shaking to obtain a uniform dispersion. Mix with the aid of ultrasound for 15 minutes with intermittent swirling, dilute to 100.0 ml with solvent mixture and filter, discarding the first few ml of the filtrate. Dilute 5.0 ml of the solution to 50.0 ml with solvent mixture.

Reference solution. A 0.001 per cent w/v solution of *nadifloxacin IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of *water*, 35 volumes of *acetonitrile* and 1 volume of *triethylamine* adjusted to pH 4.0 with *orthophosphoric acid* and filter.,
- flow rate: 1 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{21}FN_2O_4$ in the cream.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Nadifloxacin Gel

Nadifloxacin Gel contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of Nadifloxacin, $C_{19}H_{21}FN_2O_4$.

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Usual strength. 1 per cent w/w.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 5.0 to 6.0.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. a mixture of 50 volumes of *water* and 50 volumes of *acetonitrile*.

Test solution. Disperse a quantity of the gel containing about 10 mg of Nadifloxacin in 70 ml of the solvent mixture, heat on a water-bath at 60° for 10 minutes with shaking to obtain a uniform dispersion. Mix with the aid of ultrasound for 5 minutes with intermittent swirling and dilute to 100.0 ml with the solvent mixture and filter, discarding the first few ml of the filtrate.

Reference solution. A 0.00005 per cent w/v solution of *nadifloxacin IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of *water*, 35 volumes of *acetonitrile* and 1 volume of *triethylamine* adjusted to pH 4.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0, and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent).

Other tests. Comply with the tests stated under Gels.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. A mixture of equal volumes of *acetonitrile* and *water*.

Test solution. Disperse a quantity of the gel containing 10 mg of Nadifloxacin in 70 ml of the solvent mixture, heat on a water-bath at 60° for 10 minutes with shaking to obtain a uniform dispersion. Mix with the aid of ultrasound for 5 minutes with intermittent swirling dilute to 100.0 ml with the solvent mixture and filter, discarding first few ml of the filtrate. Dilute 5.0 ml of the solution to 50.0 ml with solvent mixture.

Reference solution. A 0.001 per cent w/v solution of *nadifloxacin IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of *water*, 35 volumes of *acetonitrile* and 1 volume of *triethylamine* adjusted to pH 4.0 with *orthophosphoric acid* and filter.
- flow rate: 1 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 10 µl.

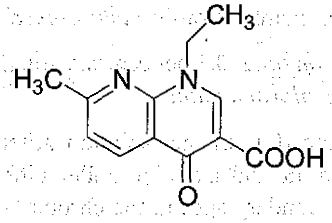
Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution and the test solution.

Calculate the content of $C_{19}H_{21}FN_2O_4$ in the gel.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Nalidixic Acid



$C_{12}H_{12}N_2O_3$ Mol. Wt. 232.2

Nalidixic Acid is 1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid.

Nalidixic Acid contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{12}H_{12}N_2O_3$, calculated on the dried basis.

Category. Antibacterial.

Description. A white to slightly yellow, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

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A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nalidixic acid IPRS* or with the reference spectrum of nalidixic acid.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.0005 per cent w/v solution in *0.1 M sodium hydroxide* shows absorption maxima at about 258 nm and 334 nm; ratio of the absorbance at about 258 nm to that at about 334 nm, 2.2 to 2.4.

C. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (c).

D. Dissolve 0.1 g in 2 ml of *hydrochloric acid* and add 0.5 ml of 10 per cent w/v solution of *2-naphthol* in *ethanol (95 per cent)*; an orange-red colour develops.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Mobile phase. A mixture of 70 volumes of *ethanol (95 per cent)*, 20 volumes of *dichloromethane* and 10 volumes of *5 M ammonia*.

Test solution (a). Dissolve 0.2 g of the substance under examination in 10 ml of *dichloromethane*.

Test solution (b). A 0.1 per cent w/v solution of the substance under examination in *dichloromethane*.

Reference solution (a). A 0.002 per cent w/v solution of the substance under examination in *dichloromethane*.

Reference solution (b). A 0.0008 per cent w/v solution of the substance under examination in *dichloromethane*.

Reference solution (c). A 0.1 per cent w/v solution of *nalidixic acid IPRS* in *dichloromethane*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18) Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh 0.15 g, dissolve in 10 ml of *dichloromethane*, add 30 ml of *2-propanol* and 10 ml of *carbon dioxide-free water* and titrate with *0.1 M ethanolic sodium hydroxide*, determining the end-point potentiometrically (2.4.25) and using a glass electrode as the indicator electrode and a silver-silver chloride reference electrode with a sleeve diaphragm or a capillary tip filled with a saturated solution of *lithium chloride* in *ethanol*. Throughout the titration keep the temperature of the solution between 15° and 20° and pass a current of nitrogen through the solution.

1 ml of *0.1 M ethanolic sodium hydroxide* is equivalent to 0.02322 g of $C_{12}H_{12}N_2O_3$.

Storage. Store protected from light and moisture.

Nalidixic Acid Tablets

Nalidixic Acid Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of nalidixic acid, $C_{12}H_{12}N_2O_3$.

Usual strengths. 250 mg; 500 mg.

Identification

To a quantity of the powdered tablets containing 1 g of Nalidixic Acid add 50 ml of *chloroform*, shake for 15 minutes, filter and evaporate the filtrate to dryness. The residue, after drying at 105°, complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nalidixic acid IPRS* or with the reference spectrum of nalidixic acid.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.0005 per cent w/v solution in *0.1 M sodium hydroxide* shows absorption maxima at about 258 nm and 334 nm; ratio of the absorbance at about 258 nm to that at about 334 nm, 2.2 to 2.4.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium: 900 ml of methanolic phosphate buffer pH 8.6 prepared by mixing 230 ml of *0.2 M sodium hydroxide*, 250 ml of *0.2M potassium dihydrogen orthophosphate* and 200 ml of *methanol* and dilute to 1000 ml with *water*, adjusted to pH 8.6 using *1M sodium hydroxide*.

Speed and time. 60 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the medium, if necessary, at the maximum at about 334 nm. (2.4.7). Calculate the content of $C_{12}H_{12}N_2O_3$ in the medium taking 494 as the specific absorbance at 334 nm.

Q. Not less than 70 per cent of the stated amount of $C_{12}H_{12}N_2O_3$.

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Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Mobile phase. A mixture of 70 volumes of *ethanol (95 per cent)*, 20 volumes of *dichloromethane* and 10 volumes of *5 M ammonia*.

Test solution. Shake a quantity of the powdered tablets containing 0.1 g of Nalidixic Acid with 50 ml of *chloroform* for 15 minutes, filter, evaporate the filtrate to dryness and dissolve the residue in 5 ml of *chloroform*.

Reference solution. Dilute 1 volume of the test solution to 200 volumes with *chloroform*.

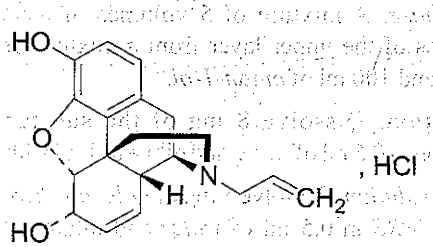
Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 0.1 g of Nalidixic Acid, add 150 ml of *1 M sodium hydroxide*, shake for 3 minutes, dilute to 200.0 ml with *1 M sodium hydroxide*, mix and allow to stand for 15 minutes. Dilute 2.0 ml of the solution to 100.0 ml with water and measure the absorbance of the resulting solution at the maximum at about 334 nm (2.4.7), using *0.01 M sodium hydroxide* as the blank. Calculate the content of $C_{12}H_{12}N_2O_3$ taking 494 as the specific absorbance at 334 nm.

Storage. Store protected from light and moisture.

Nalorphine Hydrochloride



$C_{19}H_{21}NO_3,HCl$ Mol. Wt. 347.8

Nalorphine Hydrochloride is 17-allyl-7,8-didehydro-4,5α-epoxymorphinan-3,6α-diol hydrochloride.

Nalorphine Hydrochloride contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{19}H_{21}NO_3,HCl$, calculated on the dried basis.

Category. Narcotic antagonist.

Description. A white or almost white, crystalline powder. It slowly darkens on exposure to air and light.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests C and D may be omitted if tests A, B and E are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nalorphine hydrochloride IPRS* or with the reference spectrum of nalorphine hydrochloride.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.01 per cent w/v solution in *0.1 M sodium hydroxide* shows an absorption maximum only at about 298 nm; absorbance at about 298 nm, about 0.6.

C. To 10 ml of a 2 per cent w/v solution add 0.05 ml of *dilute ammonia solution*; a white precipitate soluble in sodium hydroxide solution is produced.

D. Dissolve 2 mg in 2 ml of *water*, add 0.15 ml of *potassium ferricyanide solution* containing, in each ml, 0.05 ml of *ferric chloride solution*; a deep bluish green colour is produced immediately.

E. It gives reaction (A) of chlorides (2.3.1).

Tests

Melting range (2.4.21). 260° to 263°.

Acidity. Dissolve 0.2 g in 10 ml of freshly boiled and cooled *water* and titrate with *0.02 M sodium hydroxide* using *methyl red solution* as indicator; not more than 0.2 ml of *0.02 M sodium hydroxide* is required to change the colour of the solution.

Specific optical rotation (2.4.22). – 125° to – 122°, determined in a 2.0 per cent w/v solution.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 100° at a pressure not exceeding 0.7 kPa for 2 hours.

Assay. Dissolve 25 mg in sufficient *water* to produce 250 ml. Measure the absorbance of the resulting solution at the maximum at about 285 nm (2.4.7). Calculate the content of $C_{19}H_{21}NO_3,HCl$ from the absorbance obtained by repeating the operation with *nalorphine hydrochloride IPRS* in place of the substance under examination.

Storage. Store protected from light and moisture.

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Nalorphine Injection

Nalorphine Hydrochloride Injection

Nalorphine Injection is a sterile solution of Nalorphine Hydrochloride in Water for Injections containing suitable buffering agents.

Nalorphine Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nalorphine hydrochloride, $C_{19}H_{21}NO_3,HCl$.

Usual strength. 10 mg per ml.

Identification

A. To a volume containing 50 mg of Nalorphine Hydrochloride add *dilute ammonia solution* until the solution is alkaline and extract with 25 ml of a mixture of 1 volume of *ethanol (95 per cent)* and 3 volumes of *chloroform* and evaporate the extract to dryness. Dry the residue at a pressure not exceeding 2 kPa. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nalorphine hydrochloride IPRS* treated in the same manner or with the reference spectrum of nalorphine.

B. To a volume containing 0.1 g of Nalorphine Hydrochloride add 0.05 ml of *dilute ammonia solution*; a white precipitate soluble in *sodium hydroxide solution* is produced.

C. It gives reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 6.0 to 7.5.

Bacterial endotoxins (2.2.3). Not more than 11.6 Endotoxin Units per mg of nalorphine hydrochloride.

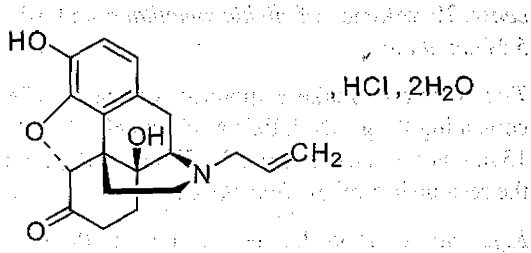
Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Transfer a measured volume containing about 10 mg of Nalorphine Hydrochloride to a separating funnel, add 1 ml of *dilute hydrochloric acid* and dilute to 10 ml with *water*. Extract with five successive quantities, each of 5 ml, of *chloroform*, allowing the layers to separate before drawing off each chloroform extract and discard the chloroform extracts. Transfer the aqueous layer to a 100-ml volumetric flask with the aid of small quantities of *water* and dilute to volume with *water*. Measure the absorbance of the resulting solution at the maximum at about 285 nm (2.4.7). Calculate the content of $C_{19}H_{21}NO_3,HCl$ from the absorbance obtained by repeating the operation with *nalorphine hydrochloride IPRS*.

Storage. Store protected from light.

Naloxone Hydrochloride

Naloxone Hydrochloride Dihydrate



$C_{19}H_{21}NO_4,HCl,2H_2O$ Mol. Wt. 399.9

Naloxone Hydrochloride is 17-allyl-4,5-α-epoxy-3,14-dihydroxymorphinan-3-one hydrochloride dihydrate.

Naloxone Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{19}H_{22}ClNO_4$, calculated on the anhydrous basis.

Category. Antidote for opioids poisoning.

Description. A white to almost white, hygroscopic, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Test B may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6).Compare the spectrum with that obtained with *naloxone hydrochloride dihydrate IPRS* or with the reference spectrum of naloxone hydrochloride.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*

Mobile phase. A mixture of 5 volumes of *methanol* and 95 volumes of the upper layer from a mixture of 60 ml of *ammonia* and 100 ml of *butan-1-ol*.

Test solution. Dissolve 8 mg of the substance under examination in 0.5 ml of *water* and dilute to 1 ml with *methanol*.

Reference solution. Dissolve 8 mg of *naloxone hydrochloride dihydrate IPRS* in 0.5 ml of *water* and dilute to 1 ml with *methanol*.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 8 cm. Dry the plate in air and spray with a freshly prepared 0.5 per cent w/v solution of *potassium ferricyanide* in *ferric chloride solution* and examine in daylight. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

C. It gives reaction (A) of chlorides (2.3.1).

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Tests

Appearance of solution. A 2.0 per cent w/v solution in *carbon dioxide-free water* (Solution A) is clear (2.4.1) and colourless (2.4.1).

Acidity or alkalinity. To 10 ml of solution A add 0.05 ml of *methyl red solution*. Not more than 0.2 ml of *0.02 M sodium hydroxide* or *0.02 M hydrochloric acid* is required to change the colour of the indicator.

Specific optical rotation (2.4.22). – 181° to – 170°, determined in solution A.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.125 g of the substance under examination in 25 ml of *0.1 M hydrochloric acid*.

Reference solution (a). Dissolve 5 mg of *naloxone for peak identification IPRS* (containing naloxone impurity A (4,5α -epoxy-3,14-dihydroxymorphinan-6-one) (noroxymorphone), naloxone impurity B (4,5α-epoxy-14-hydroxy-17-(prop-2-enyl)-3-(prop-2-enyloxy)morphinan-6-one) (3-*O*-allylnaloxone), naloxone impurity C (4,5α -epoxy-3,10α,14-trihydroxy-17-(prop-2-enyl)morphinan-6-one) (10α -hydroxynaloxone), naloxone impurity D (7,8-didehydro-4,5α -epoxy-3,14-dihydroxy-17-(prop-2-enyl)morphinan-6-one) (7,8-didehydronaloxone), naloxone impurity E (4,5 α:4',5' α-diepoxy-3,3',14,14'-tetrahydroxy-17,17'-bis(prop-2-enyl)-2,2'-bimorphinanyl-6,6'-dione) (2,2'-bisnaloxone) and naloxone impurity F (4,5α-epoxy-3,10β,14-trihydroxy-17- (prop-2-enyl)morphinan-6-one) (10α-hydroxynaloxone) in 1 ml of *0.1 M hydrochloric acid*.

Reference solution (b). Dilute 1.0 ml of the test solution to 20 ml with *0.1 M hydrochloric acid*. Dilute 1.0 ml of the solution to 25.0 ml with *0.1 M hydrochloric acid*.

Chromatographic system

- a stainless steel column 12.5 cm x 4 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 2 volumes of *acetonitrile*, 40volumes of *tetrahydrofuran* and 94 volumes of the solution prepared by dissolving 1.1 g of *sodium octanesulphonate* in 1000 ml of *water*, adjusted to pH 2.0 with 50 per cent v/v solution of *orthophosphoric acid*,

  B. a mixture of 4 volumes of *tetrahydrofuran*, 17 volumes of *acetonitrile* and 79 volumes of the solution prepared by dissolving 1.1 g of *sodium octanesulphonate* in 1000 ml of *water*, adjusted to pH 2.0 with a 50 per cent v/v solution of *orthophosphoric acid*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
40	0	100
50	0	100
52	100	0

Inject reference solution (a). Adjust the sensitivity of the system so that the peak-to-valley ratio is minimum 2.0, where H_p is height above the baseline of the peak due to impurity D and H_v is the height above the baseline of the lowest point of the curve separating this peak from the peak due to naloxone.

The relative retention time with reference to naloxone, for naloxone impurity C, naloxone impurity A, naloxone impurity F, naloxone impurity D, naloxone impurity E and naloxone impurity B is about 0.6 minute, 0.8 minute, 0.9 minute, 1.1 minutes, 3.0 minutes and 3.2 minutes respectively.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of each secondary peak corresponding to naloxone impurities A, B, C, E, F multiplied with correction factor 0.5 is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent). Multiply the area of the peak corresponding to naloxone impurity E with correction factor 0.5. The area of secondary peak corresponding to naloxone impurity D is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent), the area of any other impurities is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of the areas of all other secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent). Ignore any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with the reference solution (b) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.2 per cent, determined on 0.5 g.

Water (2.3.43), 7.5 per cent to 11.0 per cent, determined on 0.2 g.

Assay. Weigh 0.3 g, dissolve in 50 ml of *ethanol (95 per cent)* and add 5.0 ml of *0.01 M hydrochloric acid*. Titrate with *0.1 M ethanolic sodium hydroxide*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M ethanolic sodium hydroxide* is equivalent to 0.03638 g of $C_{19}H_{22}ClNO_4$.

Storage. Store protected from light.

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Naloxone Injection

Naloxone Injection is a sterile solution of Naloxone Hydrochloride in Water for Injections.

Naloxone Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of naloxone hydrochloride, $C_{19}H_{21}NO_4,HCl$.

Usual strength. 400 µg per ml.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 5 volumes of *methanol* and 95 volumes of the upper layer from a mixture of 60 ml of *M ammonia* and 100 ml of *butan-1-ol*.

Test solution. Add 1 ml of *ammonia buffer pH 10.0* to a volume of the injection containing the equivalent of 2 mg of anhydrous naloxone hydrochloride, extract with three 20 ml quantities of a mixture of 1volume of *propan-2-ol* and 3 volumes of *chloroform*, dry the combined extracts over *anhydrous sodium sulphate*, filter, evaporate the filtrate to dryness and dissolve the residue in 1 ml of *methanol*. Dilute 1 ml of the solution to 20 ml with *methanol*.

Reference solution. A 0.01 per cent w/v solution of *naloxone hydrochloride IPRS* in *methanol*.

Apply to the plate 20 µl of each solution. Allow the mobile phase to rise 10 cm, protecting the plate from light. After development, dry the plate in a current of air, spray with a freshly prepared 0.5 per cent w/v solution of *potassium hexacyanoferrate (III)* in *iron(III) chloride solution* and examine in daylight. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 3.0 to 4.5.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 5 volumes of *methanol* and 95 volumes of the upper layer from a mixture of 60 ml of *2 M ammonia* and 100 ml of *butan-1-ol*.

Test solution. Transfer a volume of the injection containing about 2 mg of Naloxone Hydrochloride in 1 ml of *ammonia buffer pH 10.0*, extract with three 20 ml quantities of a mixture of 1 volume of *propan-2-ol* and 3 volumes of *chloroform*, dry the combined extracts over *anhydrous sodium sulphate*, filter, evaporate the filtrate to dryness and dissolve the residue in 1 ml of *methanol*.

Reference solution. Dilute 0.5 ml of the test solution to 100.0 ml with *methanol*.

Apply to the plate 20 µl of each solution. Allow the mobile phase to rise 10 cm, protecting the plate from light. After development, dry the plate in a current of air, spray with a freshly prepared 0.5 per cent w/v solution of *potassium hexacyanoferrate(III)* in *iron(III) chloride solution* and examine in daylight. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent). Ignore any spot remaining on the line of application.

Bacterial endotoxins (2.2.3). Not more than 70 Endotoxin Units per ml of the injection, diluted if necessary, with *water BET* to give a solution containing 0.04 per cent w/v of anhydrous Naloxone Hydrochloride.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 0.1 volume of *orthophosphoric acid*, 45 volumes of *methanol* and 55 volumes of *water*.

Test solution. Dilute the injection equivalent to 0.001 per cent w/v of Naloxone Hydrochloride with the solvent mixture.

Reference solution (a). A 0.001 per cent w/v solution of *naloxone hydrochloride IPRS* in the solvent mixture.

Reference solution (b). A 0.001 per cent w/v solution of *naloxone hydrochloride IPRS* and 0.0005 per cent w/v solution of *noroxymorphone* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with end-capped octadecylsilane bonded to porous silica (5 to 10 µm) (Such as Zorbax C18),
- mobile phase: a solution containing 0.068 per cent w/v of *sodium octanesulphonate* and 0.1 per cent w/v of *sodium chloride* in the solvent mixture,
- flow rate: 1 ml per minute,
- spectrophotometer set at 229 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to naloxone and noroxymorphone in the chromatogram obtained with reference solution (b) is not less than 1.3.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{19}H_{21}NO_4,HCl$ in the injection.

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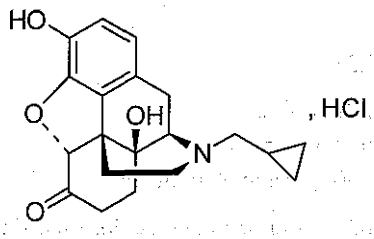
Storage. Store protected from light.

Labelling. The label states the quantity of active ingredient in terms of the equivalent amount of anhydrous naloxone hydrochloride.

When naloxone is prescribed for neonatal use, Neonatal Naloxone Injection (containing the equivalent of 20 micrograms per ml of anhydrous naloxone hydrochloride) shall be dispensed.

Naltrexone Hydrochloride



$C_{20}H_{24}ClNO_4$ Mol. Wt. 377.9

Naltrexone Hydrochloride is 17-(cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxymorphinan-6-one hydrochloride.

Naltrexone Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{20}H_{24}ClNO_4$, calculated on the anhydrous and ethanol free basis.

Category. Antidote for opioids poisoning.

Description. A white or almost white powder, very hygroscopic.

Identification

A. Dissolve 20 mg of the substance under examination in 5 ml of *water* and make alkaline with *dilute ammonia*. Shake with 10 ml of *dichloromethane*, separate the organic layer and evaporate the solvent. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *naltrexone hydrochloride IPRS* treated in the same manner or with the reference spectrum of naltrexone.

B. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 2.0 per cent w/v solution in *carbon-dioxide free water* is clear (2.4.1) and not more intensely coloured than reference solution YS5 or BS5 (2.4.1).

Acidity or alkalinity. To 10 ml of 2.0 per cent w/v solution in *carbon-dioxide free water*, add 0.05 ml of *methyl red solution*. Not more than 0.2 ml of *0.02 M sodium hydroxide* or *0.02 M hydrochloric acid* is required to change the colour of the indicator.

Specific optical rotation (2.4.22). – 195° to – 187°, determined in a 2.0 per cent w/v solution in *water*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in 10.0 ml of *0.1 M hydrochloric acid*.

Reference solution (a). Dissolve 5 mg of *17-but-3-enyl-4,5α-epoxy-3,14-dihydroxymorphinan-6-one IPRS (naltrexone impurity C IPRS)* in 2.5 ml of *0.1 M hydrochloric acid*.

Reference solution (b). Dilute 1.0 ml of the test solution and 1.0 ml of reference solution (a) to 100.0 ml with *0.1 M hydrochloric acid*. Dilute 1.0 ml of the solution to 10.0 ml with *0.1 M hydrochloric acid*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. a 0.11 per cent w/v solution of *sodium octanesulphonate*, adjusted to pH 2.3 with *orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
45	55	45
47	90	10
55	90	10

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to naltrexone and naltrexone impurity C is not less than 2.0. The relative retention time with reference to naltrexone for 17-formyl-4,5α-epoxy-3,14-dihydroxymorphinan-6-one (naltrexone impurity A) is about 0.4, for 4,5α-epoxy-3,14-dihydroxymorphinan-6-one (noroxymorphone) (naltrexone impurity B) is about 0.7, For 17-(cyclopropylmethyl)-4,5α-epoxy-3,10α,14-trihydroxymorphinan-6-one (naltrexone impurity F) is about 0.8, for 17-(cyclopropylmethyl)-4,5α-epoxy-3,10β,14-trihydroxymorphinan-6-one (naltrexone impurity G) is about 0.9, for 17-but-3-enyl-4,5α-epoxy-3,14-dihydroxymorphinan-6-one

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(naltrexone impurity C) is about 1.05, for 17-butyl-4,5α-epoxy-3,14-dihydroxymorphinan-6-one (naltrexone impurity H) is about 1.1, for 17-(cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxymorphinan-6,10-dione (naltrexone impurity I) is about 1.2, for 7-(cyclopropylmethyl)-4,5α-epoxy-14-hydroxy-3-methoxymorphinan-6-one (naltrexone impurity J) is about 1.3, for 17,17′-bis(cyclopropylmethyl)-4,5α:4′,5′-α-diepoxy-3,3′,14,14′-tetrahydroxy-2,2′-bimorphinanyl-6,6′-dione (pseudonaltrexone) (naltrexone impurity D) is about 1.4, for 3-(cyclopropylmethoxy)-17-(cyclopropylmethyl)-4,5α-epoxy-14-hydroxymorphinan-6-one (naltrexone impurity E) is about 1.7.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution the area any peak corresponding to naltrexone impurity C, D, E, F and G, each of, is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any peak corresponding to naltrexone impurity A, B, H, I and J, each of, is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of the areas of all secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Ethanol (2.3.45). Not more than 3.0 per cent, determined by Method I using the following solutions.

Test solution. Dissolve 0.25 g of the substance under examination in 10 ml of *water.*

Reference solution. Dilute 0.75 g of *anhydrous ethanol* to 1000 ml with *water.*

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 10.0 per cent, determined on 0.2 g.

Assay. Weigh 0.2 g of the substance under examination, dissolve in 60 ml of *ethanol (95 per cent)* and add 1.0 ml of *0.1 M hydrochloric acid.* Titrate with *0.1 M sodium hydroxide,* determining the end-point potentiometrically (2.4.25). The curve shows 3 points of inflexion. Read the volume added between the first 2 points of inflexion. Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.03779 g of $C_{20}H_{24}ClNO_4$.

Storage. Store protected from light and moisture.

Naltrexone Tablets

Naltrexone Hydrochloride Tablets

Naltrexone Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of naltrexone hydrochloride, $C_{20}H_{23}NO_4.HCl$.

Usual strength. 50 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water,*
Speed and time. 50 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with *water.*

Reference solution. A solution of *naltrexone hydrochloride IPRS* in *water* to obtain the same concentration as given in the test solution.

Chromatographic system
- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: a mixture of 600 volumes of 0.05 M buffer solution prepared by dissolving 7 g of *monobasic sodium phosphate* in 1000 ml of *water*, add 1.1 g of *sodium 1-octane sulphonate monohydrate* and 400 ml of *methanol*, adjusted to pH 6.7 with *dilute sodium hydroxide*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 100 µl.

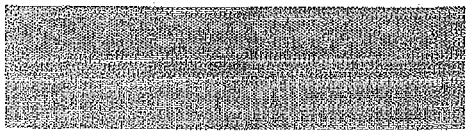
Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{23}NO_4.HCl$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{20}H_{23}NO_4.HCl$.

Other tests. Comply with the tests stated under Tablets.



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Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powdered tablets containing about 250 mg of Naltrexone with 80 ml of *0.1 M orthophosphoric acid*, sonicate for 30 minutes and dilute to 100.0 ml with the same solvent, filter.

Reference solution (a). Dissolve 22.5 mg of *naltrexone IPRS* in 1.5 ml of *methanol* and 0.6 ml of *0.1 M hydrochloric acid.* Dilute to 10.0 ml with *0.1 M orthophosphoric acid.*

Reference solution (b). Dissolve about 3 mg of *N-(3-butenyl)-noroxymorphone hydrochloride IPRS (naltrexone impurity A IPRS)* in 3.0 ml of *methanol* and dilute to 10 ml with *0.1 M orthophosphoric acid.* To 0.5 ml of the solution, add 5.0 ml of reference solution (a) and dilute to 10.0 ml with *0.1 M orthophosphoric acid.*

Chromatographic system

- a stainless steel column 15 cm x 3.9 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: A. dissolve 1.08 g of *sodium 1-octanesulphonate* and 23.8 g of *sodium acetate* in 800 ml of *water*. Add 1.0 ml of *triethylamine* and 200 ml of *methanol*, adjusted to pH 6.5 with *glacial acetic acid*, B. dissolve 1.08 g *sodium 1-octanesulphonate* and 23.8 g *sodium acetate* in 400 ml of *water*. Add 1.0 ml of *triethylamine* and 600 ml of *methanol*, adjusted to pH 6.5 with *glacial acetic acid*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

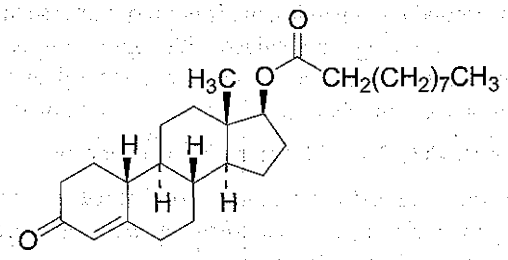
Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
1	100	0
35	0	100
50	100	0

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to naltrexone and naltrexone impurity A is not less than 2.0, the tailing factor is not more than 1.4, and the relative standard deviation for replicate injections is not more than 2.0 per cent. The relative retention time with reference to naltrexone for noroxymorphone is about 0.55, for 10-hydroxynaltrexone is about 0.7, for naltrexone impurity A is about 1.26, for 2,2α-bisnaltrexone is about 1.80 and for 10-ketonaltrexone is about 1.99.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{20}H_{23}NO_4.HCl$ in the Tablets.

Nandrolone Decanoate



$C_{28}H_{44}O_3$ Mol. Wt. 428.7

Nandrolone Decanoate is 3-oxo-4-estren-17β-yl decanoate.

Nandrolone Decanoate contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{28}H_{44}O_3$, calculated on the dried basis.

Category. Anabolic steroid.

Description. A white to creamy-off white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nandrolone decanoate IPRS* or with the reference spectrum of nandrolone decanoate.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *ethanol (95 per cent)* shows an absorption maximum at about 239 nm is about 0.41.

C. Dissolve 25 mg in 1 ml of *methanol*, add 2 ml of *semicarbazide acetate solution*, heat under a reflux condenser for 30 minutes and cool; the precipitate, after recrystallisation from *ethanol (95 per cent)*, melts at about 175° (2.4.21).

Tests

Specific optical rotation (2.4.22). +32.0° to +36.0°, determined in a 2.0 per cent w/v solution in *dioxan*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *heptane* and 30 volumes of *acetone*.

Test solution. Dissolve 0.1 g of the substance under examination in 10 ml of *chloroform*.

Reference solution (a). A 0.005 per cent w/v solution of the substance under examination in *chloroform*.

Reference solution (b). A 0.01 per cent w/v solution of *nandrolone IPRS* in *chloroform*.

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Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. In the chromatogram obtained with the test solution any spot corresponding to nandrolone is not more intense than the spot in the chromatogram obtained with reference solution (b) and any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (a).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa for 4 hours.

Assay. Weigh 10 mg and dissolve in sufficient *ethanol (95 per cent)* to produce 100.0 ml. Dilute 5.0 ml of the solution to 50.0 ml with *ethanol (95 per cent)* and measure the absorbance of the resulting solution at the maximum at about 239 nm (2.4.7). Calculate the content of $C_{28}H_{44}O_3$ taking 407 as the specific absorbance at 239 nm.

Storage. Store protected from light and moisture, at a temperature between 2° to 8°.

Nandrolone Decanoate Injection

Nandrolone Decanoate Injection is a sterile solution of Nandrolone Decanoate in Ethyl Oleate or other suitable ester, in a suitable fixed oil or in any mixture of these.

Nandrolone Decanoate Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nandrolone decanoate, $C_{28}H_{44}O_3$.

Usual strength. 25 mg per ml.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *heptane* and 30 volumes of *acetone*.

Test solution. Dilute a suitable volume of the injection with *chloroform* to give a solution containing 0.5 per cent w/v solution of Nandrolone Decanoate.

Reference solution. A 0.5 per cent w/v solution of *nandrolone decanoate IPRS* in *chloroform*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air until the odour of solvent is no longer detectable, spray with a 10 per cent v/v solution of *sulphuric acid* in *ethanol (95 per cent)*, heat at 105° for 30 minutes and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution. Ignore any subsidiary spots due to the vehicle.

Tests

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of the injection with *ethanol* to obtain a solution containing 0.02 per cent w/v of Nandrolone Decanoate.

Reference solution. Dissolve a quantity of *nandrolone decanoate IPRS* in *ethanol* to obtain a solution containing 0.02 per cent w/v of nandrolone decanoate.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: a mixture of 90 volumes of *methanol* and 10 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 10000 theoretical plates, the tailing factor is not more than 1.5 and the relative standard deviation of replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

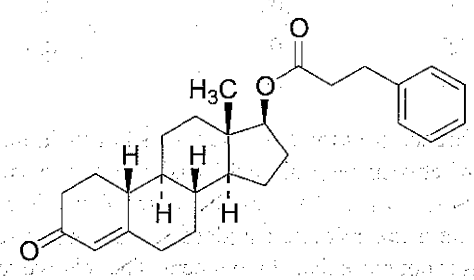
Calculate the content of $C_{28}H_{44}O_3$.

Storage. Store protected from light.

Labelling. The label states that the preparation is for intramuscular injection only.

Nandrolone Phenylpropionate

Nandrolone Phenpropionate



$C_{27}H_{34}O_3$ Mol.Wt. 406.6

Nandrolone Phenylpropionate is 3-oxo-4-estren-17β-yl 3-phenylpropionate.

Nandrolone Phenylpropionate contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{27}H_{34}O_3$, calculated on the dried basis.

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Category. Anabolic steroid.

Description. A white to creamy-white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nandrolone phenylpropionate IPRS* or with the reference spectrum of nandrolone phenylpropionate.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *ethanol (95 per cent)* shows an absorption maximum at about 240 nm is about 0.43.

C. Dissolve 25 mg in 1 ml of *methanol*, add 2 ml of *semicarbazide acetate solution*, heat under a reflux condenser for 30 minutes and cool; the precipitate, after recrystallisation from *ethanol (95 per cent)* melts at about 182° (2.4.21).

Tests

Specific optical rotation (2.4.22). +48.0° to +51.0°, determined in a 1.0 per cent w/v solution in *dioxan*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *heptane* and 30 volumes of *acetone*.

Test solution. Dissolve 0.1 g of the substance under examination in 10 ml of *chloroform*.

Reference solution (a). A 0.005 per cent w/v solution of the substance under examination in *chloroform*.

Reference solution (b). A 0.01 per cent w/v solution of *nandrolone IPRS* in *chloroform*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. In the chromatogram obtained with the test solution any spot corresponding to nandrolone is not more intense than the spot in the chromatogram obtained with reference solution (b) and any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (a).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa for 4 hours.

Assay. Weigh 10 mg, dissolve in sufficient *ethanol* to produce 100.0 ml, dilute 5.0 ml to 50.0 ml with *ethanol* and measure the absorbance of the resulting solution at the maximum at about 240 nm (2.4.7). Calculate the content of $C_{27}H_{34}O_3$ taking 430 as the specific absorbance at 240 nm.

Storage. Store protected from light.

Nandrolone Phenylpropionate Injection

Nandrolone Phenylpropionate Injection is a sterile solution of Nandrolone Phenylpropionate in Ethyl Oleate or other suitable ester, in a suitable fixed oil or in a mixture of these.

Nandrolone Phenylpropionate Injection contains not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of nandrolone phenylpropionate, $C_{27}H_{34}O_3$.

Usual strengths. 25 mg per ml; 50 mg per ml.

Identification

Dissolve a volume of the injection containing 50 mg of Nandrolone Phenylpropionate in 8 ml of *light petroleum (40° to 60°)* and extract with three 8-ml quantities of a mixture of 7 volumes of *glacial acetic acid* and 3 volumes of *water*. Wash the combined extracts with 10 ml of *light petroleum (40° to 60°)*, dilute with *water* until the solution becomes turbid, allow to stand for 2 hours in ice and filter. The precipitate, after washing with *water* and drying over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa, complies with the following test.

Determine by thin-layer chromatography (2.4.17), using a *silica gel GF254* precoated plate the surface of which has been modified by chemically-bonded octadecylsilyl groups.

Mobile phase. A mixture of 20 volumes of *water*, 40 volumes of *acetonitrile* and 60 volumes of *propan-2-ol*.

Test solution. A 0.5 per cent w/v solution of the dried precipitate in *chloroform*.

Reference solution (a). A 0.5 per cent w/v solution of *nandrolone phenylpropionate IPRS* in *chloroform*.

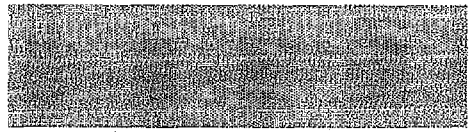
Reference solution (b). A mixture of equal volumes of the test solution and reference solution (a).

Apply to the plate 5 µl of each solution. After development, dry the plate in air until the solvent has evaporated and heat it at 100° for 10 minutes. Allow to cool and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single spot.

Tests

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. To a measured volume containing 0.1 g of Nandrolone Phenylpropionate add sufficient *chloroform* to produce 100.0 ml. Dilute 3.0 ml of the solution to 50.0 ml with *chloroform*.



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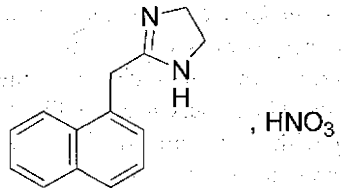
To 5.0 ml of the resulting solution add 10 ml of *isoniazid solution* and sufficient *methanol* to produce 20.0 ml. Allow to stand for 45 minutes and measure the absorbance of the solution at the maximum at about 380 nm (2.4.7), using as blank 5 ml of *chloroform* treated in the same manner. Calculate the content of $C_{27}H_{34}O_3$ from the absorbance obtained from a 0.006 per cent w/v solution of *nandrolone phenylpropionate IPRS* treated in the same manner.

Storage. Store protected from light.

Labelling. The label states that the preparation is for intramuscular injection only.

Naphazoline Nitrate



, HNO_3

$C_{14}H_{14}N_2,HNO_3$ Mol. Wt. 273.3

Naphazoline Nitrate is 2-(1-napthylmethyl)-2-imidazoline nitrate.

Naphazoline Nitrate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_4H_{14}N_2,HNO_3$ calculated on the dried basis.

Category. Sympathomimetic.

Description. A white or almost white crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *naphazoline nitrate IPRS* or with the reference spectrum of naphazoline nitrate.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution in *0.01 M hydrochloric acid* shows absorption maxima at about 270 nm, 280 nm, 287 nm and 291 nm; absorbances at these maxima are about 0.43, 0.50, 0.35 and 0.34 respectively.

C. To 1 ml of 0.05 per cent w/v in *methanol,* add 0.5 ml of a freshly prepared 5 per cent w/v solution of *sodium nitroprusside* and 0.5 ml of a 2 per cent w/v solution of *sodium hydroxide*, allow to stand for 10 minutes and add 1 ml of a 8 per cent w/v solution of *sodium bicarbonate;* a violet colour is produced.

D. Dissolve about 10 mg in 5 ml of *water,* add 0.2 g of *magnesium oxide,* shake mechanically for 30 minutes, add 10 ml of *chloroform* and shake vigorously. Allow to stand, separate the chloroform layer, filter and evaporate the aqueous layer to dryness. The residue gives reaction (A) for nitrates (2.3.1).

Tests

Appearance of solution. A 1.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1) and colourless (2.4.1).

pH (2.4.24). 5.0 to 6.5, determined in a 1.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 100.0 ml of the mobile phase.

Reference solution (a). Dissolve 5 mg of *1-naphthylacetic acid* in the mobile phase, add 5 ml of the test solution and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *naphazoline impurity A IPRS* in 100 ml of the mobile phase. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 2.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (4 µm),
- mobile phase: dissolve 1.1 g of *sodium octanesulphonate* in a mixture of 5 volumes of *glacial acetic acid,* 300 volumes of *acetonitrile* and 700 volumes of *water,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to naphazoline and naphazoline impurity B is not less than 5.0. The relative retention time with reference to naphazoline for naphthylacetylethylenediamine (naphazoline impurity A) is about 0.76, for 1-naphthylacetic acid (naphazoline impurity B) is about 1.27, for 1-naphthylacetonitrile (naphazoline impurity C) is about 2.8, for ß-naphazoline (naphazoline impurity D) is about 1.28.

Inject reference solution (b), (c) and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of the peak corresponding to naphazoline impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any other secondary peak is not more than 0.5 times the area

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of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent). Ignore any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Chlorides (2.3.12). To 0.75 g, add 30 ml of *water* and 1 ml of *nitric acid*, heat at 50° for 10 minutes. This solution complies with the limit test for chlorides (330 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

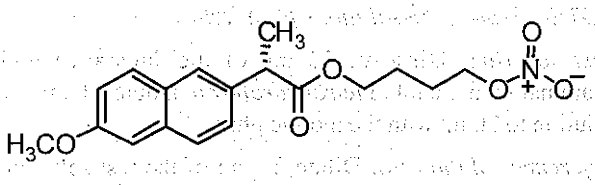
Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 0.2 g in 30 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02733 g of $C_{14}H_{14}N_2,HNO_3$.

Storage. Store protected from light.

Naproxcinod



$C_{18}H_{21}NO_6$ Mol Wt. 347.4

Naproxcinod is 4-Nitrooxybutyl (2*S*)-2-(6-methoxy-2-naphthyl)propanoate.

Naproxcinod contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{18}H_{21}NO_6$, calculated on the anhydrous basis.

Category. Analgesic.

Description. A light yellow to yellow liquid.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *naproxcinod IPRS* or with the reference spectrum of naproxcinod.

Tests

Specific optical rotation (2.4.22). +32° to +36°, determined on 1.0 per cent w/v solution in *methanol*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in mobile phase A and dilute to 100.0 ml with mobile phase A.

Reference solution. A 0.005 per cent w/v solution of *naproxcinod IPRS* and *naproxcinod impurity A IPRS [2(S)-(-methoxy-napthalen-2-yl) propionic acid 4-(chloro) butyl ester]* in mobile phase A.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: A. a mixture of 60 volumes of a buffer solution prepared by mixing 1.0 ml of *orthophosphoric acid* in 1000 ml of *water* and 40 volumes of *acetonitrile*,
  B. a mixture of 85 volumes of *acetonitrile* and 15 volumes of buffer solution,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	60	40
35	30	70
47	30	70
47.1	60	40
52	60	40

The relative retention time for naproxcinod impurity A with respect to naproxcinod is 1.15.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 5000 theoretical plates and the tailing factor is not more than 2.0.

Inject the test solution. In the chromatogram obtained with the test solution the area of any peak corresponding to naproxcinod impurity A is not more than 0.25 per cent, the area of any other secondary peak is not more than 0.5 per cent and sum of the area of all the secondary peaks is not more than 1.0 per cent, calculated by area normalization.

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 0.25 g.

Assay. Determine by liquid chromatography (2.4.14).

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Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of mobile phase A. Dilute 5.0 ml of the solution to 100.0 ml with mobile phase A.

Reference solution. A 0.005 per cent w/v solution of *naproxcinod IPRS* in mobile phase A.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: A. a mixture of 60 volumes of buffer solution prepared by mixing 1.0 ml of *orthophosphoric acid* in 1000 ml of *water* and 40 volumes of *acetonitrile*, B. a mixture of 85 volumes of *acetonitrile* and 15 volumes of the buffer solution,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	30	70
10	30	70
11	0	100
15	0	100
15.1	30	70
20	30	70

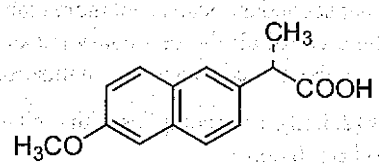
Inject the reference solution. The test is not valid unless the column efficiency is not less than 5000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{21}NO_6$

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Naproxen



$C_{14}H_{14}O_3$ Mol. Wt. 230.3

Naproxen is (2*S*)-2-(6-methoxynaphthalen-2-yl)propionic acid.

Naproxen contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{14}H_{14}O_3$, calculated on the dried basis.

Category. Nonsteroidal antiinflammatory.

Description. A white or almost white crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *naproxen IPRS* or with the reference spectrum of naproxen.

B. When examined in the range 230 nm to 350 nm (2.4.7), a 0.004 per cent w/v solution in *methanol* shows absorption maxima at about 262 nm, 271 nm, 316 nm and 331 nm. The specific absorbance at the maxima are 216 to 238, 219 to 241, 61 to 69 and 79 to 87 respectively.

C. Melting range (2.4.21). 154° to 158°.

Tests

Appearance of solution. A 5.0 per cent w/v solution in *methanol* is clear (2.4.1) and not more intensely coloured than reference solution BYS6 (2.4.1).

Specific optical rotation (2.4.22). + 59° to + 62°, determined in a 2.0 per cent w/v solution in *ethanol (95 per cent)*.

Enantiomeric purity. Determine by liquid chromatography (2.4.14).

NOTE—Protect the solutions from light.

Test solution. Dissolve 25 mg of the substance under examination in 50 ml of *tetrahydrofuran*. Dilute 2.0 ml of the solution to 20 ml with the mobile phase.

Reference solution (a). Dilute 2.5 ml of the test solution to 100 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *racemic naproxen IPRS* in 10 ml of *tetrahydrofuran*, dilute to 100 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with silica gel π-acceptor/π-donor for chiral separations (5 µm),
- column temperature: 25°,
- mobile phase: a mixture of 5 volumes of *glacial acetic acid*, 50 volumes of *acetonitrile*, 100 volumes of *2-propanol* and 845 volumes of *hexane*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 263 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to (2*R*)-2-(6-methoxynaphthalen-2-yl) propanoic acid (naproxen impurity G) ((*R*)-enantiomer) and naproxen is not less than 3.0.

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Inject reference solution (a) and the test solution. Run the chromatogram 1.5 times the retention time of the principal peak. The area of the peak corresponding to naproxen impurity G is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Protect the solutions from light.

Test solution. Dissolve 12 mg of the substance under examination in 20.0 ml of the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 50 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 6 mg of *2-bromo-6-methoxynaphthalene IPRS (Naproxen impurity N IPRS)*, 6 mg of *1-(6-methoxynaphthalen-2-yl)ethanone IPRS (Naproxen impurity L IPRS)* and 6 mg of *(1RS)-1-(6-methoxynaphthalen-2-yl)ethanol IPRS (Naproxen impurity K IPRS)* in 10 ml of *acetonitrile*. To 1.0 ml of the solution, add 1.0 ml of the test solution and dilute to 50 ml with the mobile phase. Dilute 1.0 ml of the solution to 20 ml with the mobile phase.

Chromatographic system

- a stainless steel column 10 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 50°,
- mobile phase: a mixture of 42 volumes of *acetonitrile* and 58 volumes of a 0.136 per cent w/v solution of *potassium dihydrogen phosphate*, adjusted to pH 2.0 with *orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to naproxen impurity K and naproxen is not less than 2.2. The relative retention time with reference to naproxen for naproxen impurity K is about 0.9, for naproxen impurity L is about 1.4 and naproxen impurity N is about 5.3.

Inject reference solution (a), (b) and the test solution . Run the chromatogram 1.5 times the retention time of naproxen impurity N. The area of the peak corresponding to naproxen impurity L is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent); the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all impurities is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 0.2 g in a mixture of 25 ml of *water* and 75 ml of *methanol*. Titrate with *0.1 M sodium hydroxide* using 1 ml of *phenolphthalein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.02303 g of $C_{14}H_{14}O_3$.

Storage. Store protected from light.

Naproxen Oral Suspension

Naproxen Oral Suspension is an aqueous suspension of Naproxen in a suitable flavoured vehicle.

Naproxen Oral Suspension contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of naproxen, $C_{14}H_{14}O_3$.

Usual strength. 25 mg per ml.

Identification

Evaporate 50 ml of solution A obtained in the Assay, to dryness using a rotary evaporator. The residue complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6).Compare the spectrum with that obtained with *naproxen IPRS* or with the reference spectrum of naproxen.

B. When examined in the range 230 nm to 350 nm (2.4.7), a 0.004 per cent w/v solution in *methanol* exhibits maxima at 262 nm, 271 nm, 316 nm and 331 nm.

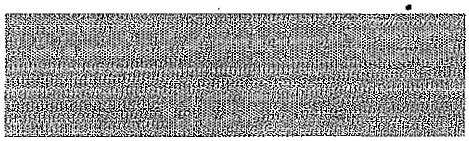
Tests

pH (2.4.24). 2.1 to 4.0.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 3 volumes of *glacial acetic acid*, 9 volumes of *tetrahydrofuran* and 90 volumes of *toluene*.

Test solution. Evaporate solution A obtained in the Assay to dryness on a rotary evaporator and dissolve the residue in sufficient *methanol* to produce a solution containing 5.0 per cent w/v of naproxen.



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Reference solution. Dilute 1.0 ml of the test solution to 200.0 ml with *methanol.*

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Other tests. Comply with the tests stated under Oral Liquids.

Assay. To a quantity of the oral suspension containing 0.5 g of Naproxen add 20 ml of *3.5 M hydrochloric acid*, mix, extract with three 50 ml quantities of *chloroform*, filter each extract through *anhydrous sodium sulphate*, combine the filtrates and add sufficient *chloroform* to produce 200.0 ml (solution A). Dilute 5.0 ml of solution A add sufficient *methanol* to produce 250.0 ml and measure the absorbance of the resulting solution at the maximum at about 331 nm (2.4.7).

Calculate the content of $C_{14}H_{14}O_3$ taking 81 as the specific absorbance at 331 nm.

Naproxen Suppositories

Naproxen Suppositories contain Naproxen in a suitable suppository base.

Naproxen Suppositories contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of naproxen, $C_{14}H_{14}O_3$.

Usual strength. 500 mg.

Identification

Dissolve a quantity of the suppositories containing 0.5 g of Naproxen in 50 ml of *2,2,4-trimethylpentane* and extract with four 25 ml quantities of *methanol (80 per cent)*. To the combined extracts add 100 ml of a 2 per cent w/v solution of *sodium chloride*, extract with four 25 ml quantities of *chloroform*, dry the combined extracts over *anhydrous sodium sulphate*, filter and add sufficient *chloroform* to produce 200 ml (solution A). Evaporate 100 ml of solution A to dryness using a rotary evaporator. The residue complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *naproxen IPRS* or with the reference spectrum of naproxen.

B. When examined in the range 230 nm to 350 nm (2.4.7), a 0.004 per cent w/v solution in *methanol* exhibits maxima at 262 nm, 271 nm, 316 nm and 331 nm.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 3 volumes of *glacial acetic acid*, 9 volumes of *tetrahydrofuran* and 90 volumes of *toluene.*

Test solution. Weigh 20 suppositories and cut into small pieces. Dissolve a quantity of the suppositories containing 0.5 g of Naproxen in 50 ml of *2,2,4-trimethylpentane* and extract with four 25-ml quantities of *methanol (80 per cent).* Combine the extracts, add 100 ml of a 2 per cent w/v solution of *sodium chloride*, extract with four 25-ml quantities of *chloroform* filtering each extract through a layer of *anhydrous sodium sulphate* on an absorbent cotton plug moistened with *chloroform*. Evaporate the combined filtrates to dryness using a rotary evaporator with the aid of gentle heat. Shake the residue with 10 ml of *methanol*, centrifuge and use the supernatant liquid.

Reference solution. Dilute1.0 ml of the test solution to 200.0 ml with *methanol.*

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Other tests. Comply with the tests stated under Suppositories.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Disperse ten suppositories in 500 ml of *methanol* on a water-bath for 40 minutes with the aid of ultrasound and by swirling the flask. Cool at 5° for 1 hour, centrifuge and use the clear, supernatant liquid; if the solution is still cloudy filter through glass-fibre paper (such as Whatman GF/C). To 5 ml of the filtrate add sufficient of the mobile phase to produce a solution containing 0.01 per cent w/v of naproxen.

Reference solution (a). A 0.01 per cent w/v solution of *naproxen IPRS* in the mobile phase.

Reference solution (b). A 0.01 per cent w/v each of *naproxen IPRS* and *2-naphthylacetic acid* in the mobile phase.

Chromatographic system

- a stainless steel column 20 cm x 4 mm, packed with end-capped octadecylsilane bonded to porous silica (5 µm) (Such as Nucleosil C18),
- mobile phase: a mixture of 1 volume of a 0.52 per cent w/v solution of *sodium acetate*, adjusted to pH 5.8 with *glacial acetic acid* and 1 volume of *methanol,*
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to naproxen and 2-naphthyl-

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acetic acid in the chromatogram obtained with the reference solution (b) is more than 3.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{14}H_{14}O_3$ in the suppositories.

Storage. Store protected from light.

Naproxen Prolonged-release Tablets

Naproxen Sustained-release Tablets; Naproxen Extended-release Tablets

Naproxen Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Naproxen Prolonged-release Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of naproxen, $C_{14}H_{14}O_3$.

Usual strength. 375 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution (b).

Tests

Dissolution (2.5.2). Complies with the test stated under tablets.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture A: 90 volumes of *acetonitrile* and 10 volumes of *water*.

Solvent mixture B. Equal volumes of *acetonitrile* and *water*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 250 mg of Naproxen in 70 ml of solvent mixture B, sonicate for 15 minutes and dilute to 100.0 ml with solvent mixture B and filter. Dilute 2.0 ml of the filtrate to 50 ml with the mobile phase.

Reference solution (a). A 0.05 per cent w/v solution of *naproxen IPRS* in solvent mixture A.

Reference solution (b). Dilute 10 ml of reference solution (a) to 50 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 55 volumes of 1.0 per cent v/v solution of *acetic acid* and 45 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 50 µl.

Inject reference solution (b). The test is not valid unless the tailing factor of the principal peak is not more than 1.5 per cent and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (b) and the test solution.

Calculate the content of $C_{14}H_{14}O_3$ in the tablet.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Naproxen Tablets

Naproxen Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of naproxen, $C_{14}H_{14}O_3$.

Usual strengths. 250 mg; 500 mg.

Identification

A. Extract a quantity of the powdered tablets containing 20 mg of Naproxen with 100 ml of *methanol* and filter. Evaporate and dry the residue at 105°. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *naproxen IPRS* or with the reference spectrum of naproxen.

B. When examined in the range 230 nm to 350 nm (2.4.7), a 0.002 per cent w/v solution in *methanol* exhibits maxima at 262 nm, 271 nm, 316 nm and 331 nm.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of a phosphate buffer prepared by dissolving 2.62 g of *sodium dihydrogen orthophospate monohydrate* and 11.5 g of *anhydrous disodium hydrogen orthophosphate* in sufficient *water* to produce 1000 ml, adjusted to pH 7.4 with *0.1 M sodium hydroxide* or *0.1 M hydrochloric acid*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the dissolution medium if necessary, at the maximum at about 332 nm (2.4.7). Calculate the content of $C_{14}H_{14}O_3$, in the medium from the absorbance obtained from a solution of known concentration of *naproxen IPRS* in the same medium.

Q. Not less than 70 per cent of the stated amount of $C_{14}H_{14}O_3$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 3 volumes of *glacial acetic acid*, 9 volumes of *tetrahydrofuran* and 90 volumes of *toluene*.

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Test solution. Shake a quantity of the powdered tablets containing about 0.5 g of Naproxen with 10 ml of *methanol* for 15 minutes, centrifuge and use the supernatant liquid.

Reference solution. Dilute 1.0 ml of the test solution to 200.0 ml with *methanol.*

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

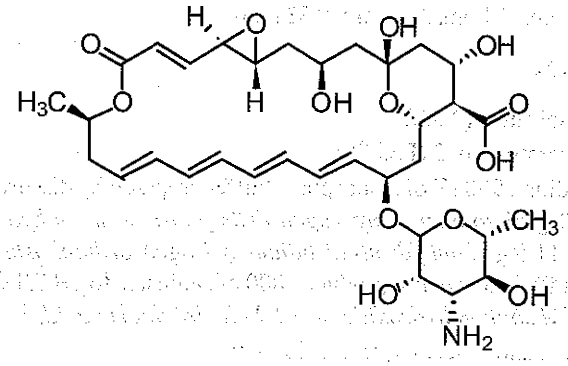
Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 50 mg of Naproxen with 70 ml of *methanol* for 30 minutes, add sufficient *methanol* to produce 100.0 ml and filter. Dilute 10.0 ml of the filtrate to 50.0 ml with *methanol* and measure the absorbance at the maximum at about 331 nm (2.4.7) using *methanol* as blank.

Calculate the content of $C_{14}H_{14}O_3$ in the tablets, from the absorbance obtained from a solution containing 0.01 per cent w/v solution of *naproxen IPRS* in *methonol.*

Storage. Store protected from light.

Natamycin



$C_{33}H_{47}NO_{13}$ Mol. Wt. 665.7

Category. Antibacterial.

Natamycin is stereoisomer of (1*R*,3*S*,5*R*,7*R*,8*E*,12*R*,14*E*,16*E*,18*E*,20*E*,22*R*,24*S*,25*R*,26*S*)-22-[(3-Amino-3,6-dideoxy-*D*-mannopyranosyl)oxy]-1,3,26-trihydroxy-12-methyl-10-oxo-6,11,28-trioxatricyclo[22.3.1.05,7]octacosa8,14,16,18,20-pentaene-25-carboxylic acid.

Natamycin contains not less than 90.0 per cent and not more than 102.0 per cent of $C_{33}H_{47}NO_{13}$, calculated on the anhydrous basis.

Description. A white to yellow, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *natamycin IPRS* or with the reference spectrum of natamycin.

B. To 50 mg, add 5 ml of *water* and 100 ml of 0.1 per cent v/v solution of *glacial acetic acid* in *methanol* and dilute with 0.1 per cent v/v solution of *glacial acetic acid* in *methanol* to obtain a solution containing 0.0005 per cent w/v of Natamycin. When examined in the range 230 nm to 380 nm (2.4.7), the solution exhibits maximum and minimum absorption at the same wavelengths with that obtained with *natamycin IPRS.*

NOTE — Carry out the procedure protected from light.

Tests

pH (2.4.24). 5.0 to 7.5, determined in a 1.0 per cent w/v solution.

Water (2.3.43). 6.0 to 9.0 per cent.

Assay. Determined by liquid chromatography (2.4.14).

NOTE—Carry out the following procedure protected from direct light.

Test solution. Dissolve 20 mg of the substance under examination in 5.0 ml of *tetrahydrofuran* with aid of ultrasound for 10 minutes, add 60 ml of *methanol*, add 25 ml of *water*, and mix. Dilute to 100.0 ml with *water* and filter.

Reference solution (a). Weigh a quantity of 20 mg of *Natamycin IPRS,* add 5.0 ml of *tetrahydrofuran* and dissolve with aid of ultra sound for 10 minutes, add 60 ml of *methanol*, add 25 ml of *water*, and mix. Dilute to 100.0 ml with *water* and filter.

Reference solution (b). Dissolve 20 mg of *Natamycin* in 1.0 per cent of *0.1 M hydrochloric acid* in *methanol* and allow to stand for 2 hours.

NOTE—Use this solution within 1 hour.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica or ceramic microparticles (10µm).
- mobile phase: a mixture of 760 volumes of a buffer solution containing 3.0 g of *ammonium acetate* and 1.0 g of *ammonium chloride* in *water*, 240 volumes of *acetonitrile* and 5 volumes of *tetrahydrofuran*, mix and filter,
- flow rate: 3 ml per minute,
- spectrophotometer set at 303 nm,
- injection volume: 20 µl.

Inject reference solution (a) and (b). The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not less than 0.8 and not more than 1.3 and

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the relative standard deviation for replicate injection is not more than 1.0 per cent in the chromatogram obtained with reference solution (a). The resolution between the peaks due to natamycin and its methyl ester is not less than 2.5 in the chromatogram obtained with reference solution (b) and the relative retention times are about 0.7 for natamycin and 1.0 for its methyl ester.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{33}H_{47}NO_{13}$.

Storage. Store protected from light and moisture.

Natamycin Ophthalmic Suspension

Natamycin Ophthalmic Suspension is a sterile aqueous solution of Natamycin in a suitable aqueous vehicle.

Natamycin Ophthalmic Suspension contains not less than 90.0 per cent and not more than 125.0 per cent of the labeled amount of $C_{33}H_{47}NO_{13}$. It contains one or more suitable preservatives.

Usual strengths. 5.0 per cent w/v.

Identification

Take a volume of ophthalmic suspension equivalent to 50 mg, add 5 ml *water* and add 100 ml of *glacial acetic acid* in *methanol* (1:1000). Dilute to 200 ml with *glacial acetic acid* and *methanol* solution. Dilute 2.0 ml of the solution to 100.0 ml with same solution. Compare the UV absorption spectrum (2.4.7) of the solution so obtained exhibits maxima and minima at the same wavelengths with that obtained with *Natamycin IPRS.*

Tests

pH (2.4.24). 5.0 to 7.5.

Sterility (2.2.11). Complies with the test for sterility.

Other tests. Comply with the tests stated under Eye Drops.

Assay. Determined by liquid chromatography (2.4.14).

NOTE—Carry out the following procedure protected from direct light.

Test solution. Measure a volume of Ophthalmic Suspension containing 50 mg of Natamycin, add 12.5 ml of *tetrahydrofuran,* and dissolve with the aid of ultrasound for 10 minutes, allow to cool to room temperature. Add 150 ml of *methanol,* add 60 ml of *water,* mix and dilute with *water* to produce 250.0 ml, mix and filter.

Reference solution (a). Weigh a quantity of 20 mg of *Natamycin IPRS,* add 5.0 ml *of tetrahydrofuran* and dissolve with the aid of ultrasound for 10 minutes, add 60 ml of *methanol* and add 25 ml of *water,* and mix. Dilute to 100.0 ml with *water* and filter.

Reference solution (b). Dissolve 20 mg of *Natamycin* in 1.0 per cent of *0.1 M hydrochloric acid* in *methanol* and allow to stand for 2 hours.

NOTE—Use this solution within 1 hour.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica or ceramic microparticles (10 µm).
- mobile phase: a mixture of 760 volumes of a buffer solution containing 3.0 g of *ammonium acetate* and 1.0 g of *ammonium chloride* in *water,* 240 volumes of *acetonitrile* and 5 volumes of *tetrahydrofuran,* mix and filter,
- flow rate: 3 ml per minute,
- spectrophotometer set at 303 nm,
- injection volume: 20 µl.

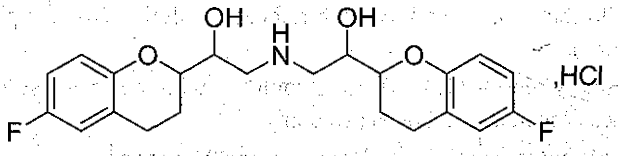
Inject reference solution (a) and (b). The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not less than 0.8 and not more than 1.3 and the relative standard deviation for replicate injection is not more than 1.0 per cent in the chromatogram obtained with reference solution (a). The resolution between the peaks due to natamycin and its methyl ester is not less than 2.5 in the chromatogram obtained with reference solution (b) and the relative retention times are about 0.7 for natamycin and 1.0 for its methyl ester.

Inject reference solution (a) and the test solution.

Calculate the quantity in mg, of $C_{33}H_{47}NO_{13}$ in Ophthalmic Suspension.

Storage. Store protected from light and moisture in container. The container should be sterile, tamper evident and sealed so as to exclude microorganism.

Nebivolol Hydrochloride



$C_{22}H_{25}F_2NO_4$, HCl Mol. Wt. 441.9

Nebivolol Hydrochloride is (1*RS*,1'*RS*)-1, 1'-[(2*RS*,2'*SR*)-bis (6-flurochroman-2-yl)]-2,2'-iminodiethanol hydrochloride.

Nebivolol Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{22}H_{25}F_2NO_4$,HCl, calculated on the anhydrous basis.

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Category. Antihypertensive.

Description. A white to off-white powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nebivolol hydrochloride IPRS* or with the reference spectrum of nebivolol hydrochloride.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 30 mg of the substance under examination in 5 ml of *acetonitrile* and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 30 mg of *nebivolol hydrochloride IPRS* in 5 ml of *acetonitrile* and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with porous silica with bonded phenyl groups (5 µm),
- mobile phase: a mixture of 28 volumes of *acetonitrile*, 72 volumes of a buffer solution prepared by dissolving 3.4 g of *tetrabutylammonium hydrogen sulphate* in 1000 ml of *water* and 0.3 volume of *diethylamine*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than the area of the peak in the chromatogram obtained with reference solution (b) (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 0.3 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 35 mg of the substance under examination in 5 ml of *acetonitrile* and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution. Dissolve 35 mg of *nebivolol hydrochloride IPRS* in 5 ml of *acetonitrile* and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Use the chromatographic system described under Related substances.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution the test solution.

Calculate the content of $C_{22}H_{25}F_2NO_4$, HCl.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Nebivolol Tablets

Nebivolol Hydrochloride Tablets

Nebivolol Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nebivolol, $C_{22}H_{25}F_2NO_4$.

Usual strength. 5 mg

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 500 ml *0.1 M hydrochloric acid,*

Speed and time. 75 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14) as described under Assay using the following solutions and a flow rate of 1.5 ml per minute.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *nebivolol hydrochloride IPRS* in *methanol* and dilute with dissolution medium to obtain a solution having a known concentration similar to the test solution.

Q. Not less than 70 per cent of the stated amount of $C_{22}H_{25}F_2NO_4$.

Uniformity of content. Complies with the test stated under Tablets.

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Determine by liquid chromatography (2.4.14), using the chromatographic conditions and the reference solution described under Assay.

Test solution. Disperse one tablet in the mobile phase, dilute to obtain a solution containing 0.005 per cent w/v of nebivolol in the mobile phase and filter.

Calculate the content of $C_{22}H_{25}F_2NO_4$ in the tablets.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 5 mg of nebivolol, disperse in 100.0 ml of the mobile phase and filter.

Reference solution. A solution containing *nebivolol hydrochloride IPRS* equivalent to 0.005 per cent w/v of nebivolol, in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 45 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adding 2 ml of *triethylamine* and adjusted to pH 3.0 with 10 per cent v/v *orthophosphoric acid*, 25 volumes of *acetonitrile* and 30 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 281 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

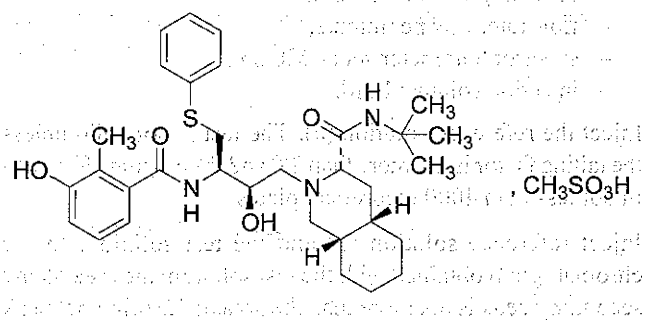
Inject the reference solution the test solution.

Calculate the content of $C_{22}H_{25}F_2NO_4$ in the tablets.

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of the equivalent amount of nebivolol.

Nelfinavir Mesylate



$C_{32}H_{45}N_3O_4S,CH_4O_3S$ Mol. Wt. 663.9

Nelfinavir Mesylate is (3*S*,4a*S*,8a*S*)-*N*-(*tert*-butyldecahydro-2-[(2*R*,3*R*)-3-(3-hydroxy-*o*-toluamido)-hydroxy-4-phenylthio) butyl]isoquinoline-3-carboxamide methyl sulphonate.

Nelfinavir Mesylate contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{32}H_{45}N_3O_4S,CH_4O_3S$, calculated on the anhydrous basis.

Category. Antiretroviral.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nelfinavir mesylate IPRS* or with the reference spectrum of nelfinavir mesylate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). – 120° to – 105°, determined in a 1.0 per cent w/v solution in *methanol*.

Related substances. Determine by liquid chromatography (2.4.14), using the chromatographic system as described under Assay.

Test solution. A 0.1 per cent w/v solution of the substance under examination in the mobile phase.

Reference solution (a). A 0.001 per cent w/v solution of *nelfinavir mesylate IPRS* in the mobile phase.

Reference solution (b). A 0.01 per cent w/v solution of *methanesulphonic acid* in the mobile phase.

Inject reference solution (a). The test is not valid unless the column efficiency determined from the nelfinavir peak is not less than 4000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (a), (b) and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak other than the principal peak is not greater than half of the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of the areas of all such peaks is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak due to methanesulphonic acid corresponding to the retention time of the principal peak in the chromatogram obtained with reference solution (b).

Methanesulphonic acid. 13.5 per cent to 15.5 per cent w/w, calculated on the anhydrous basis, determined by the following

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method. Weigh 0.6 g, dissolve in 50 ml of *dimethylformamide* and titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.00961 g of CH_3SO_3H.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 3.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. A 0.01 per cent w/v solution of the substance under examination in the mobile phase.

Reference solution. A 0.01 per cent w/v solution of *nelfinavir mesylate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a filtered and degassed mixture of 45 volumes of *acetonitrile*, 20 volumes of *methanol* and 35 volumes of a buffer prepared by dissolving 4.0 g of *sodium dihydrogen phosphate* in 1000 ml of *water*, to which 1 ml of *dimethylamine* solution and 1 g of *sodium octanesulphonate* are added and mixed to dissolve,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency determined from the nelfinavir peak is not less than 5000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{32}H_{45}N_3O_4S,CH_4O_3S$.

Storage. Store protected from light.

Nelfinavir Mesylate Oral Powder

Nelfinavir Mesylate Oral Powder contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nelfinavir, $C_{32}H_{45}N_3O_4S$.

Usual strength. 50 mg per g.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 75 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate and, if necessary, dilute with the dissolution medium.

Reference solution. A 0.065 per cent w/v solution of *nelfinavir mesylate IPRS* in *methanol*. Dilute 10.0 ml of the solution to 100.0 ml with the dissolution medium.

Use the chromatographic system as described under Assay.

Inject the reference solution the test solution.

Q. Not less than 75 per cent of the stated amount of $C_{32}H_{45}N_3O_4S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the oral powder containing 50 mg of Nelfinavir Mesylate, disperse in 10 ml of *methanol*, dilute to 50.0 ml with the mobile phase and filter.

Reference solution (a). Dissolve 10 mg of *nelfinavir mesylate IPRS* in 2 ml of *methanol* and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: a mixture of 28 volumes of a buffer solution prepared by dissolving 4.88 g of *anhydrous sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 3.4 with *orthophosphoric acid* and filter, 27 volumes of *acetonitrile*, 20 volumes of *methanol* and 25 volumes of *water*. Adjusted to pH 4.8 with *0.1 M sodium hydroxide* or *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

Inject the reference solution (a). The test is not valid unless the tailing factor is not more than 2.0 and the column efficiency in not less than 4000 theoretical plates.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b)

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(1.0 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

Water (2.3.43). Not more than 12.0 per cent, determined on 0.5 g.

Other tests. Comply with the tests stated under Oral Powder.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 30 volumes of *water* and 70 volumes of *methanol*.

Test solution. Disperse a quantity of the powder containing 50 mg of Nelfinavir Mesylate in 50 ml of *0.1 M hydrochloric acid* and dilute to 250.0 ml with the solvent mixture.

Reference solution. Dissolve 10 mg of *nelfinavir mesylate IPRS* in 10 ml of *0.1 M hydrochloric acid* and dilute to 50.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 35 volumes of a buffer solution prepared by dissolving 4 g of *sodium dihydrogen phosphate dihydrate* and 1g of *1-octane sulphonic acid sodium salt* into 1000 ml of *water*, adding 1ml of *dimethylamine* and filtering, 45 volumes *acetonitrile* and 20 volumes of *methanol*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency in not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution the test solution.

Calculate the content of $C_{32}H_{45}N_3O_4S$ in the oral powder.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of nelfinavir.

Nelfinavir Tablets

Nelfinavir Mesylate Tablets

Nelfinavir Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nelfinavir, $C_{32}H_{45}N_3O_4S$.

Usual strengths. 250 mg; 625 mg.

Identification

A. Shake a quantity of the powdered tablets containing about 0.1 g of Nelfinavir Mesylate with 80 ml of *methanol* for 10 minutes, add sufficient *methanol* to produce 100 ml, mix and filter. Dilute 5 ml of the filtrate to 100 ml with *methanol*.

When examined in the range 200 nm to 300 nm the resulting solution shows an absorption maximum only at about 254 nm (2.4.7).

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.01 M hydrochloric acid*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter promptly through a membrane filter disc with an average pore diameter not greater than 1.0 µm. Reject the first few ml of the filtrate and dilute a suitable volume of the filtrate with the same solvent. Measure the absorbance of the resulting solution at the maximum at about 250 nm (2.4.7). Calculate the content of $C_{32}H_{45}N_3O_4S$ from the absorbance of a solution of known concentration of *nelfinavir mesylate IPRS*.

Q. Not less than 75 per cent of the stated amount of $C_{32}H_{45}N_3O_4S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the powdered tablets containing about 100 mg of Nelfinavir Mesylate, add about 20 ml of *methanol*, mix with the aid of ultrasound for 10 minutes and dilute to 100 ml with the mobile phase.

Reference solution. Weigh 10 mg of *nelfinavir mesylate IPRS*, add about 10 ml of *methanol*, shake for 10 minutes and dilute to 50 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica particles (5 µm),
- mobile phase: a mixture of 35 volumes of a buffer solution prepared by dissolving 4.0 g of *sodium dihydrogen phosphate* in 1000 ml of *water*, add 1 ml of *dimethylamine solution* and 1 g of *sodium octanesulphonate* and mixing

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to dissolve, 45 volumes of *acetonitrile* and 20 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency determined from the nelfinavir mesylate peak is not less than 4000 theoretical plates and the tailing factor is not more than 2.0.

Inject the diluent (10 ml of *methanol* diluted to 50 ml with the mobile phase) and the test solution and continue the chromatography for 4 times the retention time of the principal peak. Examine the diluent chromatogram for any extraneous peaks and ignore the corresponding peaks observed in the chromatogram obtained with the test solution.

Any secondary peak observed in the chromatogram obtained with the test solution should not be more than 1.0 per cent and the sum of the areas of all the secondary peaks should not be more than 2.0 per cent when calculated by area normalisation. Inhibit integration of peak due to methanesulphonic acid.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the powdered tablets containing about 200 mg of Nelfinavir Mesylate, add about 20 ml of *methanol*, mix with the aid of ultrasound for 10 minutes and dilute to 100.0 ml with the mobile phase. Filter through a membrane filter disc with an average pore diameter not greater than 1.0 µm, rejecting the first few ml of the filtrate. Further dilute 5.0 ml of the filtrate to 100.0 ml with the mobile phase.

Reference solution. Weigh 50 mg of *nelfinavir mesylate IPRS*, add about 10 ml of *methanol*, mix with the aid of ultrasound to dissolve and dilute to 50.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Use the chromatographic system as described under Related substances.

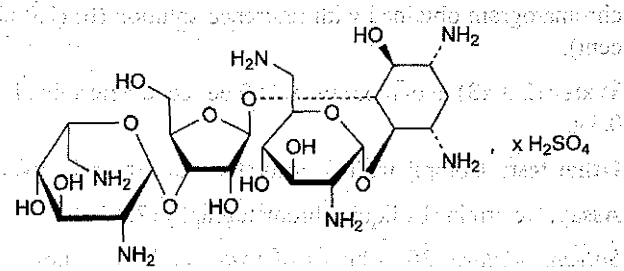
Inject the reference solution. The test is not valid unless the column efficiency determined from the nelfinavir mesylate peak is not less than 5000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{32}H_{45}N_3O_4S$ in the tablets.

Storage. Store protected from light.

Neomycin Sulphate



$C_{23}H_{46}N_6O_{13},xH_2SO_4$ Mol. Wt. 615 (base)

Neomycin Sulphate is a mixture of the sulphates of substances obtained by the growth of certain selected strains of *Streptomyces fradiae*.

Neomycin Sulphate has a potency of not less than 680 Units per mg, calculated on the dried basis.

Category. Antibacterial.

Description. A white or yellowish-white powder; hygroscopic.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H*.

Mobile phase. A freshly prepared 3.85 per cent w/v solution of *ammonium acetate*.

Test solution. Dissolve 0.2 g of the substance under examination in 10 ml of *water*.

Reference solution. A 2.0 per cent w/v solution of *neomycin sulphate IPRS* in *water*.

Apply to the plate 1 µl of each solution. After development, dry the plate in air for 10 minutes, heat at 100° for 1 hour and spray with a 0.1 per cent w/v solution of *ninhydrin* in *1-butanol* saturated with *water*. Heat again at 100° for 5 minutes. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. Dissolve about 10 mg in 5 ml of *water*, add 0.1 ml of *pyridine* and 2 ml of a 0.1 per cent w/v solution of *ninhydrin* and heat on a water-bath at a temperature of about 70° for 10 minutes; a deep violet colour is produced.

C. A 5 per cent w/v solution gives the reactions of sulphates (2.3.1).

Tests

pH (2.4.24). 5.0 to 7.5, determined in a 1.0 per cent w/v solution.

Specific optical rotation (2.4.22). + 53.5° to + 59.0°, determined in a 10.0 per cent w/v solution.

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Neamine. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H.*

Mobile phase. A mixture of 30 volumes of *methanol*, 20 volumes of *strong ammonia solution* and 10 volumes of *dichloromethane.*

Test solution. Dissolve 0.25 g of the substance under examination in 10.0 ml of *water.*

Reference solution. A 0.05 per cent w/v solution of *neamine IPRS* in *water.*

Apply to the plate as 5-mm bands 5 µl of each solution. Dry the bands; allow the mobile phase to rise at least 8 cm. Dry the plate in a current of warm air, heat at 110° for 10 minutes, spray the plate with *ninhydrin and stannous chloride reagent* and heat at 110° for 15 minutes. Spray the plate again with the same reagent and heat at 110° for 15 minutes. Any band corresponding to neamine in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Neomycin C. Determine by thin-layer chromatography (2.4.17), coating the plate with silica gel of a suitable grade.

Mobile phase. A mixture of 80 volumes of a 20 per cent w/v solution of *sodium chloride* and 20 volumes of *methanol.*

Test solution. Dissolve 40 mg of the substance under examination in *water* and dilute to 5.0 ml with the same solvent.

Reference solution (a). Dissolve 30 mg of *framycetin sulphate IPRS* in *water* and dilute to 25.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 25.0 ml with *water.*

Reference solution (c). Dissolve 40 mg of *neomycin sulphate IPRS* in *water* and dilute to 5.0 ml with the same solvent.

Apply to the plate as 5 mm bands 5 µl of each solution. Dry the bands; allow the mobile phase to rise at least 12 cm. Dry the plate at 100° to 105° for 10 minutes. Spray the plate with *ethanolic ninhydrin solution* and heat at 100° to 105° for 10 minutes. In the chromatogram obtained with the test solution the principal band corresponds to the principal band in the chromatogram obtained with reference solution (c) and the band due to neomycin C with R_f value slightly less than that of the principal band is not more intense than the band obtained with reference solution (a) (15 per cent) but is more intense than the band in the chromatogram obtained with reference solution (b) (3 per cent). The test is not valid unless in the chromatogram obtained with reference solution (c) a band appears with R_f value slightly less than that of the principal band.

Sulphated ash (2.3.18). Not more than 1.0 per cent.

Loss on drying (2.4.19). Not more than 8.0 per cent, determined on 0.5 g by drying in an oven at 60° over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa for 3 hours.

Assay. Determine by the microbiological assay of antibiotics (2.2.10).

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of Units of neomycin per mg.

Neomycin Eye Drops

Neomycin Sulphate Eye Drops

Neomycin Sulphate Eye Drops are a sterile solution of Neomycin Sulphate in Purified Water.

Neomycin Sulphate Eye Drops contain not less than 90.0 per cent and not more than 115.0 per cent w/v of the stated amount of neomycin sulphate.

Usual strength. 0.35 per cent w/v of neomycin.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel.*

Mobile phase. A mixture of 60 volumes of *methanol*, 40 volumes of *strong ammonia solution* and 20 volumes of *chloroform.*

Test solution. Dilute if necessary a volume of the eye drops to produce a solution containing 0.5 per cent w/v of Neomycin Sulphate in *water.*

Reference solution (a). A 0.5 per cent w/v solution of *neomycin sulphate IPRS* in *water.*

Reference solution (b). A mixture of equal volumes of the eye drops and reference solution (a).

Apply to the plate 1 µl of each solution. After development, dry the plate in air, spray with 1 per cent w/v solution of *ninhydrin* in *1-butanol* and heat at 105° for 2 minutes. The principal red spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a) and the principal red spot in the chromatogram obtained with reference solution (b) appears as a single spot.

Tests

Neamine. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H.*

Mobile phase. A mixture of 30 volumes of *methanol*, 20 volumes of *strong ammonia solution* and 10 volumes of *dichloromethane.*

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Test solution. A volume of the eye drops containing 5 µg (3.5 Units).

Reference solution. The same volume of water containing 0.1 µg of *neamine IPRS*.

Apply to the plate each solution. After development, dry the plate in a current of warm air, heat at 110° for 10 minutes, spray the plate with *ninhydrin and stannous chloride reagent* and heat at 110° for 15 minutes. Spray the plate again with the same reagent and heat at 110° for 15 minutes. Any spot corresponding to neamine in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Neomycin C. Determine by liquid chromatography (2.4.14).

Test solution. Dilute the eye drops with *0.02 M borax* to contain 1 mg (700 Units) per ml. To 0.5 ml of the diluted solution add 1.5 ml of a freshly prepared 2 per cent w/v solution of *1-fluoro-2,4-dinitrobenzene* in *methanol*, dilute to 25 ml with the mobile phase, allow to stand and use the clear lower layer.

Reference solution. Add 1.5 ml of the 1-fluoro-2,4-dinitrobenzene solution to 0.5 ml of 0.1 per cent w/v solution of *neomycin sulphate IPRS* in *0.02 M borax*, heat in a water-bath at 60° for 1 hour and cool; dilute the solution to 25 ml with the mobile phase, allow to stand and use the clear lower layer.

Chromatographic system

- a stainless steel column 20 cm x 4.6 mm, packed with porous silica particles (5 µm) (Such as Nucleosil 100-5),
- mobile phase: a mixture of 97 ml of *tetrahydrofuran*, 1.0 ml of *water* and 0.5 ml of *glacial acetic acid* diluted with sufficient of 2.0 per cent v/v solution of *ethanol* in *ethanol-free chloroform* to produce 250 ml,
- flow rate: 1.6 ml per minute,
- spectrophotometer set at 350 nm,
- injection volume: 10 µl.

If necessary the tetrahydrofuran and water content of the mobile phase may be adjusted so that the chromatogram obtained with the reference solution shows resolution similar to that in the specimen chromatogram supplied with framycetin sulphate. The mobile phase should be passed through the column for several hours before the solutions are injected. Continue the chromatography for 1.4 times the retention time of the peak due to neomycin B.

The column efficiency, determined using the peak due to Neomycin B in the chromatogram obtained with the test solution, should be not less than 13,000 theoretical plates.

In the chromatogram obtained with the test solution the area of the peak corresponding to neomycin C is not less than 3.0 per cent and not more than 15.0 per cent of sum of the areas of the peaks corresponding to Neomycin B and Neomycin C.

Other tests. Comply with the tests stated under Eye Drops.

Assay. Measure a quantity containing 5 mg of Neomycin Sulphate and dilute to 50.0 ml with sterile *phosphate buffer pH 8.0* and mix. Dilute 10.0 ml of the solution to 100.0 ml with the same solvent.

Determine by the microbiological assay of antibiotics (2.2.10).

Storage. Store protected from light.

Labelling. The strength is stated in terms of number of Units per ml.

Neomycin Eye Ointment

Neomycin Sulphate Eye Ointment

Neomycin Sulphate Eye Ointment is a sterile preparation containing Neomycin Sulphate in a suitable base.

Neomycin Sulphate Eye Ointment contains not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of neomycin sulphate.

Usual strength. 0.35 per cent w/v of neomycin.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel*.

Mobile phase. A mixture of 60 volumes of *methanol*, 40 volumes of *strong ammonia solution* and 20 volumes of *chloroform*.

Test solution. Disperse a quantity of the eye ointment containing 20 mg of Neomycin Sulphate in 20 ml of *chloroform*, extract with 5 ml of *water* and use the aqueous extract.

Reference solution (a). A 0.4 per cent w/v solution of *neomycin sulphate IPRS* in *water*.

Reference solution (b). A mixture of equal volumes of test solution and reference solution (a).

Apply to the plate 1 µl of each solution. After development, dry the plate in air, spray with a 1 per cent w/v solution of *ninhydrin* in *1-butanol* and heat at 105° for 2 minutes. The principal red spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a) and the principal red spot in the chromatogram obtained with reference solution (b) appears as a single spot.

Tests

Neamine. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H*.

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Mobile phase. A mixture of 30 volumes of *methanol*, 20 volumes of *strong ammonia solution* and 10 volumes of *dichloromethane*.

Test solution. Disperse a quantity of the eye ointment containing 20 mg of Neomycin Sulphate in 20 ml of *chloroform*, shake gently with 8 ml of *water*, allow the layers to separate and use the aqueous layer.

Reference solution. A 0.005 per cent w/v solution of *neamine IPRS* in *water*.

Apply to the plate 2 µl of each solution. After development, dry the plate in a current of warm air, heat at 110° for 10 minutes, spray with *ninhydrin and stannous chloride reagent* and heat at 110° for 15 minutes. Spray the plate again with the same reagent and heat at 110° for 15 minutes. Any spot corresponding to neamine in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Neomycin C. Determine by liquid chromatography (2.4.17)

Test solution. Disperse a quantity of the eye ointment containing 5 mg of Neomycin Sulphate in 20 ml of *light petroleum (120° to 160°)*, add 5 ml of *0.02 M borax*, shake, separate the aqueous layer and centrifuge. To 0.5 ml of the separated aqueous layer add 1.5 ml of a freshly prepared 2 per cent w/v solution of *1-fluoro-2,4-dinitrobenzene* in *methanol*, heat on a water-bath at 60° for 1 hour and cool. Dilute the resulting solution to 25 ml with the mobile phase, allow to stand and use the clear lower layer.

Reference solution. Add 1.5 ml of the *1-fluoro-2,4-dinitrobenzene solution* to 0.5 ml of a 0.1 per cent w/v solution of *neomycin sulphate IPRS* in *0.02 M borax* and proceed as for the test solution.

Chromatographic system

- a stainless steel column 20 cm x 4.6 mm, packed with porous silica particles (5 µm),
- mobile phase: 97 ml of *tetrahydrofuran*, 1.0 ml of *water* and 0.5 ml of *glacial acetic acid* with sufficient of 2.0 per cent v/v solution of *ethanol* in *ethanol-free chloroform* to produce 250 ml,
- flow rate: 1.6 ml per minute,
- spectrophotometer set at 350 nm,
- injection volume: 10 µl.

If necessary the tetrahydrofuran and water content of the mobile phase may be adjusted so that the chromatogram obtained with reference solution shows resolution similar to that in the specimen chromatogram supplied with framycetin sulphate. The mobile phase should be passed through the column for several hours before the solutions are injected. Continue the chromatography for 1.4 times the retention time of the peak due to neomycin B.

The column efficiency, determined using the peak due to Neomycin B in the chromatogram obtained with the test solution, should be not less than 13,000 theoretical plates.

In the chromatogram obtained with the test solution the area of the peak corresponding to neomycin C is not less than 3.0 per cent and not more than 15.0 per cent of the sum of the areas of the peaks corresponding to Neomycin B and Neomycin C.

Other tests. Comply with the tests stated under Eye Ointments.

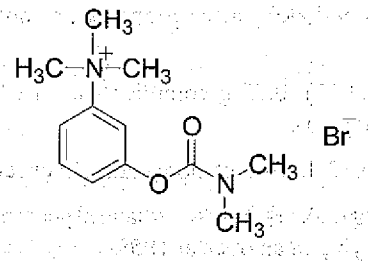
Assay. Weigh a quantity containing 5 mg of Neomycin Sulphate, dissolve in 25 ml of *chloroform*, extract with four quantities, each of 20 ml, of sterile *phosphate buffer pH 8.0*, combine the extracts and add sufficient of the buffer solution to produce 100.0 ml.

Carry out the microbiological assay of antibiotics (2.2.10).

Storage. Store protected from light.

Labelling. The strength is stated in terms of number of Units per g.

Neostigmine Bromide



$C_{12}H_{19}BrN_2O_2$ Mol. Wt. 303.2

Neostigmine Bromide is 3-(dimethylcarbamoyloxy) trimethylanilinium bromide.

Neostigmine Bromide contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{12}H_{19}BrN_2O_2$, calculated on the dried basis.

Category. Anticholinesterase.

Description. Colourless crystals or a white, crystalline powder; hygroscopic.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests B, C and D may be omitted if tests A and E are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *neostigmine*

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bromide IPRS or with the reference spectrum of neostigmine bromide.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.02 per cent w/v solution in *0.5 M sulphuric acid* shows absorption maxima at about 260 nm and 266 nm.

C. Warm about 50 mg with 1 ml of *dilute sodium hydroxide solution*; an odour of dimethylamine develops slowly.

D. Warm about 50 mg with 0.4 g of *potassium hydroxide* and 2 ml of *ethanol (95 per cent)* on a water-bath for 3 minutes, replacing the evaporated *ethanol*. Cool, add 2 ml of *dilute diazobenzenesulphonic acid solution*; an orange-red colour is produced.

E. It gives the reactions of bromides (2.3.1).

Tests

Appearance of solution. A 0.5 per cent w/v solution is clear (2.4.1), and colourless (2.4.1).

Acidity. Dissolve 0.2 g in 20 ml of *carbon dioxide-free water* and titrate to pH 7.0 with *0.02 M sodium hydroxide* (carbonate-free); not more than 0.1 ml is required.

3-Hydroxytrimethylanilinium bromide. Dissolve 50 mg in a mixture of 1 ml of *sodium carbonate solution* and 9 ml of *water*. Absorbance of the resulting solution at about 294 nm, measured immediately after preparation, not more than 0.25 (2.4.7).

Sulphates (2.3.17). 0.75 g complies with the limit test for sulphates (200 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.5 g in 20 ml of *anhydrous glacial acetic acid*, add 5 ml of *acetic anhydride*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03032 g of $C_{12}H_{19}BrN_2O_2$.

Storage. Store protected from light and moisture.

Neostigmine Tablets

Neostigmine Bromide Tablets

Neostigmine Bromide Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of neostigmine bromide, $C_{12}H_{19}BrN_2O_2$.

Usual strength. 15 mg.

Identification

Triturate a quantity of the powdered tablets containing about 0.3 g of Neostigmine Bromide with three quantities, each of 5 ml of *ether* and discard the ether. Macerate the residue with several quantities, each of 10 ml of *ethanol (95 per cent)*, filtering after each maceration. Evaporate the combined filtrates on a water-bath and dry the residue at 105° for 1 hour. The residue melts at about 167°, with decomposition. The residue complies with the following tests.

A. Warm about 50 mg with 0.4 g of *potassium hydroxide* and 2 ml of *ethanol (95 per cent)* on a water-bath for 3 minutes, replacing the evaporated *ethanol*. Cool, add 2 ml of *dilute diazobenzenesulphonic acid solution*; an orange-red colour is produced.

B. It gives the reactions of bromides (2.3.1).

Tests

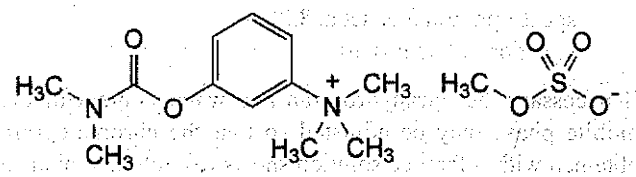
Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 0.15 g of Neostigmine Bromide, transfer to a semi-micro ammonia-distillation apparatus, add 20 ml of a 50 per cent w/v solution of *sodium hydroxide* and 0.5 ml of a 2 per cent w/v solution of *2-octanol* in *liquid paraffin*. Pass a current of steam through the mixture, collect the distillate in 50 ml of *0.01 M sulphuric acid* until the volume is about 200 ml and titrate the excess of acid with *0.02 M sodium hydroxide* using *methyl red solution* as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of sulphuric acid required to neutralise the dimethylamine produced.

1 ml of *0.01 M sulphuric acid* is equivalent to 0.006064 g of $C_{12}H_{19}BrN_2O_2$.

Storage. Store protected from light and moisture.

Neostigmine Methylsulphate



$C_{13}H_{22}N_2O_6S$ Mol. Wt. 334.4

Neostigmine Methylsulphate is 3-(dimethylcarbamoyloxy)-trimethylanilinium methyl sulphate.

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Neostigmine Methylsulphate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{13}H_{22}N_2O_6S$, calculated on the dried basis.

Category. Anticholinesterase.

Description. Colourless crystals or a white, crystalline powder; hygroscopic.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *neostigmine methylsulphate IPRS* or with the reference spectrum of neostigmine methylsulphate.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.05 per cent w/v solution in *0.5 M sulphuric acid* shows absorption maxima, at about 261 nm and 267 nm. The ratio of the absorbance at the maximum at about 267 nm to that at the maximum at 261 nm is 0.84 to 0.87.

C. Dissolve 0.1 g in 5 ml of *distilled water* and add 1 ml of a 6 per cent w/v solution of *barium chloride*; no precipitate is produced. Add 2 ml of *hydrochloric acid* and heat in a water-bath for 10 minutes; a white precipitate is produced.

D. Warm about 50 mg with 0.4 g of *potassium hydroxide* and 2 ml of *ethanol (95 per cent)* on a water-bath for 3 minutes, replacing the evaporated *ethanol*. Cool, add 2 ml of *dilute diazobenzenesulphonic acid solution*; an orange-red colour is produced.

Tests

Appearance of solution. A 5.0 per cent w/v solution in *distilled water* is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. To 4.0 ml of a 5.0 per cent w/v solution in *distilled water* add 6.0 ml of *water* and 0.1 ml of *phenolphthalein solution*; the solution is colourless. Add 0.3 ml of *0.01 M sodium hydroxide*; the solution becomes red. Add 0.4 ml of *0.01 M hydrochloric acid*; the solution becomes colourless. Add 0.1 ml of *methyl red solution*; the solution becomes red or yellowish-red.

3-Hydroxytrimethylanilinium methyl sulphate. Dissolve 50 mg in a mixture of 1 ml of *sodium carbonate solution* and 9 ml of *water*. Absorbance of the resulting solution at about 294 nm, measured immediately after preparation, not more than 0.20 (2.4.7).

Chlorides (2.3.12). 1.0 g complies with the limit test for chlorides (250 ppm).

Sulphates (2.3.17). 0.75 g complies with the limit test for sulphates (200 ppm).

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Dissolve 0.3 g in 150 ml of *water*. Add 100 ml of *2 M sodium hydroxide*, distill and collect the distillate in 50 ml of 4 per cent w/v solution of *boric acid* until a total volume of 250 ml is reached. Titrate the distillate with *0.1 M hydrochloric acid* using 0.25 ml of *methyl red-methylene blue solution* as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of hydrochloric acid required.

1 ml of *0.1 M hydrochloric acid* is equivalent to 0.03344 g of $C_{13}H_{22}N_2O_6S$.

Storage. Store protected from light and moisture.

Neostigmine Injection

Neostigmine Methylsulphate Injection

Neostigmine Injection is a sterile solution of Neostigmine Methylsulphate in Water for Injections.

Neostigmine Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of neostigmine methylsulphate, $C_{13}H_{22}N_2O_6S$.

Usual strengths. 0.5 mg per ml; 2.5 mg per ml.

Identification

A. Dilute, if necessary, a volume of the injection containing 2.5 mg of Neostigmine Methylsulphate to 5 ml with *water*, shake with three quantities, each of 10 ml, of *ether* and discard the ether extracts.

When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows absorption maxima at about 260 nm and 267 nm.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*

Mobile phase. A mixture of 50 volumes of *chloroform*, 35 volumes of *methanol*, 10 volumes of *formic acid* and 5 volumes of *water*.

Test solution. Dilute the injection under examination, if necessary, with *water* to produce a solution containing 0.05 per cent w/v of Neostigmine Methylsulphate.

Reference solution (a). A 0.05 per cent w/v solution of *neostigmine methylsulphate IPRS* in *water*.

Reference solution (b). A mixture of equal volumes of the test solution and reference solution (a).

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Apply to the plate 10 µl of each solution. After development, dry the plate in air, spray with *dilute potassium iodobismuthate solution*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

C. To 1 ml add 0.5 ml of *sodium hydroxide solution* and evaporate to dryness on a water-bath. Heat quickly in an oil-bath to about 250° and maintain at this temperature for about 30 seconds. Cool, dissolve the residue in 1 ml of *water*, cool in ice water and add 1 ml of *diazobenzenesulphonic acid solution*; an orange-red colour is produced.

Tests

pH. (2.4.24) 4.5 to 6.5.

3-Hydroxy trimethylanilinium methyl sulphate. Determine by liquid chromatography (2.4.14).

Test solution. Dilute the injection if necessary, with *water* to contain a 0.05 per cent w/v solution of Neostigmine Methylsulphate.

Reference solution (a). Dilute 1 volume of the test solution to 100 volumes with *water*.

Reference solution (b). Add 0.05 ml of *5 M sodium hydroxide* to 1 ml of the test solution and allow to stand for 5 minutes. Add 0.1 ml of *5 M hydrochloric acid* and use immediately.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane chemically bonded to porous silica particles (5 µm) (Such as Lichrosphere 60 RP-select B),
- mobile phase: *0.0015 M solution of sodium heptanesulphonate* in a mixture of 15 volumes of *acetonitrile* and 85 volumes of *0.05 M potassium dihydrogen orthophosphate* adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 1.1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 10 µl.

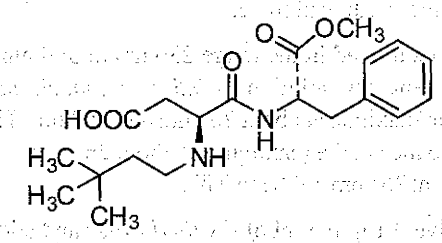
In the chromatogram obtained with reference solution (b) the principal peak has a retention time of about 6.8 minutes (neostigmine methylsulphate) and there is a peak with a relative retention time of about 0.5 (3-hydroxy) trimethylanilinium methylsulphate. In the chromatogram obtained with the test solution, the area of any secondary peak with a retention time corresponding to that of the peak due to (3-hydroxy) trimethylanilinium methylsulphate in the chromatogram obtained with reference solution (b) is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Dilute a measured volume containing about 25 mg of Neostigmine Methylsulphate to 50.0 ml with *water*. Measure the absorbance of the resulting solution at the maximum at about 260 nm (2.4.7). Calculate the content of $C_{13}H_{22}N_2O_6S$ taking 14.35 as the specific absorbance at 260 nm.

Storage. Store protected from light.

Neotame



$C_{20}H_{30}N_2O_5$ Mol.Wt. 378.4

Neotame is *N*-(3,3-dimethylbutyl)-L-α-aspartyl-D-phenylalanine 2-methyl ester.

Neotame contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{20}H_{30}N_2O_5$, calculated on the anhydrous basis.

Category. Non-nutritive sweetener.

Description. A white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *neotame IPRS* or with the reference spectrum of neotame.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). −43.4° to −40°, determined in a 0.5 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of substance under examination in 50.0 ml of the mobile phase.

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Reference solution (a). A 0.002 per cent w/v solution of *neotame IPRS* in the mobile phase.

Reference solution (b). A 0.003 per cent w/v solution of *neotame impurity A IPRS* in the mobile phase.

Use the chromatographic system as described under Assay.

Inject reference solution (b). The test is not valid unless the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject reference solution (a), reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of peak corresponding to *neotame impurity A (N-[3,3-Dimethylbutyl)-L-á-aspartyl]-L-phenylalanine)* is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent) and the sum of the areas of all other secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). Not more than 5.0 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of substance under examination in 25.0 ml of mobile phase. Dilute 5.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution. A 0.1 per cent w/v solution of *neotame IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: dissolve 3 g of *sodium 1-heptane-sulphonate* and 3.8 ml of *triethylamine* in 750 ml of *water*, adjusted to pH 3.5 with *orthophosphoric acid*. Add 250 ml of *acetonitrile*, adjusted to pH 3.7 with *orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

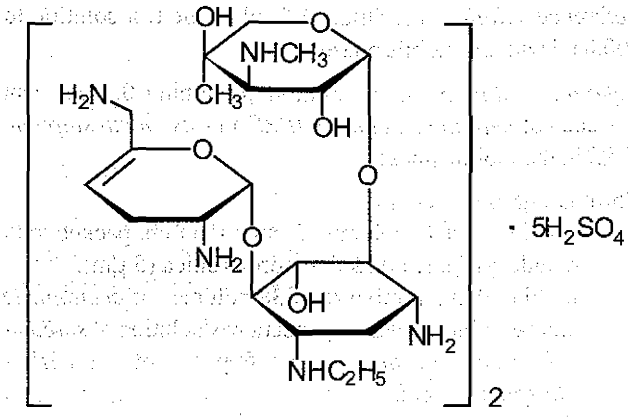
Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution the test solution.

Calculate the content of $C_{20}H_{30}N_2O_5$.

Storage. Store protected from moisture.

Netilmicin Sulphate



$C_{42}H_{82}N_{10}O_{14}, 5H_2SO_4$ Mol. Wt. 1441.6

Category. Aminoglycoside antibacterial.

Netilmicin Sulphate is 4-*O*-[(2*R*,3*R*)-*cis*-3-Amino-6-aminomethyl-3,4-dihydro-2*H*-pyran-2-yl]-2-deoxy-6-*O*-(3-deoxy-4-*C*-methyl-3-methylamino--β-L-arabino-pyranosyl)-3*N*-ethylstreptamine sulphate.

Netilmicin sulphate is obtained by synthesis from sisomicin. Semi-synthetic product derived from a fermentation product.

Netilmicin sulphate has a potency of not less than 595 µg per mg of netilmicin ($C_{21}H_{41}N_5O_7$), calculated on the dried basis.

Description. A white or yellowish-white powder; very hygroscopic.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

B. It gives reaction of (A) of sulphates (2.3.1).

Tests

pH (2.4.24). 3.5 to 5.5, determined in 4.0 per cent w/v solution in *carbon dioxide-free water.*

Appearance of solution. A 4.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1) and when examined at about 400 nm (2.4.7) shows maximum absorbance of 0.08.

Specific optical rotation (2.4.22). + 88.0° to + 96.0°, determined in a 5.0 per cent w/v solution in *carbon dioxide- free water.*

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware.

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Test solution. Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.1 per cent w/v each of *netilmicin sulphate IPRS* and *sisomicin sulphate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase. a mixture of 38 volumes of *acetonitrile* and 62 volumes of 2.02 per cent w/v solution of *sodium-1-heptane sulphonate* in 0.5 per cent v/v *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to netilmicin sulphate and sisomicin sulphate is not less than 1, in the chromatogram obtained with reference solution (b). The column efficiency is not less than 3000 theoretical plates and tailing factor is not more than 2.0 in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (5.0 per cent).

Sulphates. 31.5 to 35.0 per cent calculated on dried basis.

Dissolve 0.12 g in 100 ml of *water* and adjusted to pH 11 with *concentrated ammonia*. Add 30.0 ml of *0.1 M barium chloride* and 0.5 mg of *phthalein purple* and titrate with *0.1M sodium edetate*, adding 50 ml of *ethanol (95 per cent)* when the colour of the solution begins to change and continuing the titration until the violet-blue colour disappears.

1 ml of *0.1 M barium chloride* is equivalent to 0.009606 g of SO_4.

Sulphated ash (2.3.18). Not more than 1.0 per cent, determined on 0.5 g.

Loss on drying (2.4.19). Not more than 15.0 per cent, determined on 0.5 g by drying in an oven at 110° under high vaccum for 3 hours.

Netilmicin sulphate intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxin complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 1.25 Endotoxin Unit per mg of netilmicin sulphate.

Assay. Determine by liquid chromatography (2.4.14) as described under Related substances with the following modifications.

Reference solution (a). A 0.1 per cent w/v solution of *netilmicin sulphate IPRS* in the mobile phase.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to netilmicin sulphate and sisomicin sulphate is not less than 1, in the chromatogram obtained with reference solution (b). The column efficiency is not less than 3000 theoretical plates, tailing factor is not more than 2.0 per cent and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{21}H_{41}N_5O_7$.

Storage. Store protected from light and moisture in container. If the material is sterile, store in a sterile, airtight, tamper-proof container.

Netilmicin Injection

Netilmicin Sulphate Injection

Netilmicin Injection is a sterile solution of Netilmicin Sulphate in Water for Injections.

Netilmicin Injection contains not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of netilmicin, $C_{21}H_{41}N_5O_7$.

Usual strengths. 10 mg per ml; 25 mg per ml; 50 mg per ml; 100 mg per ml.

Identification

In the Assay the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

pH (2.4.24). 3.5 to 6.0.

Bacterial endotoxins (2.2.3). Not more than 1.25 Endotoxin Units per mg of netilmicin.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14)

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Test solution. Dilute a volume of injection with the mobile phase to obtain a solution containing 0.1 per cent w/v of netilmicin.

Reference solution (a). A solution of *netilmicin sulphate IPRS* equivalent to 0.1 per cent w/v of netilmicin in the mobile phase.

Reference solution (b). A solution containing 0.1 per cent w/v each of *netilmicin sulphate IPRS* and *sisomicin sulphate IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase. a mixture of 38 volumes of *acetonitrile* and 62 volumes of 2.02 per cent w/v solution of *sodium-1-heptane sulphonate* in 0.5 per cent v/v *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

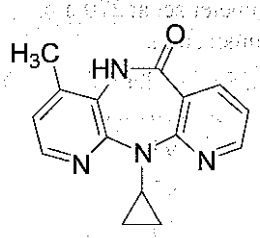
Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to netilmicin sulphate and sisomicin sulphate is not less than 1.0 in the chromatogram obtained with reference solution (b) and the column efficiency is not less than 3000 theoretical plates, tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{21}H_{41}N_5O_7$.

Storage. Store in single dose or multiple dose containers, preferably of Type-1 glass.

Nevirapine



$C_{15}H_{14}N_4O$ Mol. Wt. 266.3
(anhydrous)

$C_{15}H_{14}N_4O.\ ½H_2O$ Mol. Wt. 275.3
(hemihydrate)

Nevirapine is 11-cyclopropyl-5,11-dihydro-4-methy-6*H*-dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one.

Nevirapine is anhydrous or contains one-half molecule of water of hydration. It contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{15}H_{14}N_4O$, calculated on the anhydrous basis.

Category. Antiretroviral.

Description. A white or almost white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nevirapine IPRS* or with the reference spectrum of nevirapine.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in 100 ml of *methanol.*

Reference solution. Dilute 1 ml of the test solution to 100 ml with *methanol.*

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 12.0 g of *sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and filter, 20 volumes of *methanol* and 20 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency for nevirapine peak is not less than 5000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak other than the principal peak is not greater than half of the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all such peaks is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

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Water (2.3.43). Not more than 0.2 per cent for Nevirapine anhydrous and 3.1 per cent to 3.9 per cent for Nevirapine hemihydrates.

Assay: Determine by liquid chromatography (2.4.14).

Test solution. A 0.005 per cent w/v solution of the substance under examination in *methanol.*

Reference solution. A 0.005 per cent w/v solution of *nevirapine IPRS* in *methanol.*

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 12.0 g of *sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and filter, 20 volumes of *methanol* and 20 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency for nevirapine peak is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{14}N_4O$.

Storage. Store protected from moisture.

Labelling. The label states whether the material is anhydrous or hemihydrates.

Nevirapine Oral Suspension

Nevirapine Oral Suspension is a suspension of Nevirapine in a suitable flavoured vehicle.

Nevirapine Oral Suspension contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nevirapine, $C_{15}H_{14}N_4O$.

Usual strength. 50 mg in 5 ml.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 40 volumes of *1-butanol*, 30 volumes of *heptane*, 30 volumes of *acetone* and 10 volumes of *strong ammonia solution.*

Test solution. Dilute the preparation under examination with *methanol* to obtain a solution containing 1 mg of Nevirapine per ml.

Reference solution. A 0.1 per cent w/v solution of *nevirapine IPRS* in a mixture of 75 volumes of *methanol* and 25 volumes of *water.*

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 5.0 to 7.0.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. To a measured volume of the preparation under examination containing about 25 mg of Nevirapine add about 10 ml of *methanol*, mix with the aid of ultrasound for 10 minutes, dilute to 50 ml with *water*, mix and filter.

Reference solution. Weigh 25 mg of *nevirapine IPRS*, add about 10 ml of *methanol*, mix with the aid of ultrasound to dissolve and dilute to 50 ml with *water.*

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilyl silica gel for chromatography (5 µm) (Such as Hypersil C8),
- mobile phase: A. *0.1 M ammonium acetate*,
  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
5	95	5
25	20	80
30	20	80
31	95	5
40	95	5

Inject the reference solution. The test is not valid unless the column efficiency determined from the nevirapine peak is not less than 3000 theoretical plates and the tailing factor is not more than 2.0.

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Inject the diluent (dilute 10 ml of *methanol* to 50 ml with *water*) and the test solution. Examine the diluent chromatogram for any extraneous peaks and ignore the corresponding peaks observed in the chromatogram obtained with the test solution. Ignore any peaks due to preservatives also.

Any secondary peak observed in the chromatogram obtained with the test solution should not be more than 1.0 per cent and the sum of the areas of all the secondary peaks should not be more than 2.0 per cent when calculated by area normalisation.

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the preparation under examination containing 25 mg of Nevirapine, add about 10 ml of *methanol*, mix with the aid of ultrasound for 10 minutes, dilute to 50.0 ml with *water*, mix and filter. Further dilute 10.0 ml of the filtrate to 25.0 ml with *water*.

Reference solution. Weigh 50 mg of *nevirapine IPRS*, add about 20 ml of *methanol*, mix with the aid of ultrasound to dissolve and dilute to 100.0 ml with *water*. Dilute 10.0 ml of the solution to 25.0 ml with *water*.

Use the chromatographic system described under the test for Related substances.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Determine the weight per ml of the oral suspension (2.4.29) and calculate the content of $C_{15}H_{14}N_4O$ weight in volume.

Storage. Store protected from light.

Nevirapine Tablets

Nevirapine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nevirapine, $C_{15}H_{14}N_4O$.

Usual strength. 200 mg.

Identification

A. When examined in the range 220 nm to 400 nm (2.4.7) a 0.001 per cent w/v solution in *methanol*, shows an absorption maximum at about 283 nm.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter through a membrane filter disc with an average pore diameter not greater than 1.0 µm, rejecting the first few ml of the filtrate. Dilute suitably, if necessary. Measure the absorbance of the resulting solution, at the maximum at about 313 nm (2.4.7).

Calculate the content of $C_{15}H_{14}N_4O$ from the absorbance obtained from a solution of known concentration of *nevirapine IPRS* in *0.1 M hydrochloric acid*.

Q. Not less than 70 per cent of the stated amount of $C_{15}H_{14}N_4O$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets with a suitable quantity of the mobile phase to obtain a mixture containing 0.05 per cent w/v of Nevirapine and filter through a membrane filter disc with an average diameter not exceeding 1.0 µm, rejecting the first few ml of the filtrate.

Reference solution. A 0.05 per cent w/v solution of *nevirapine IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 12.0 g of *sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and filter, 20 volumes of *methanol* and 20 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency for nevirapine peak is not less than 7500 theoretical plates and the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2 per cent.

Inject the test solution and continue the chromatography for at least five times the retention time of the principal peak. Determine the amount of related substances by the area normalisation method. Any individual impurity is not more than 1.0 per cent and the sum of all impurities is not more than 2.0 per cent.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

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Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 100 mg of Nevirapine with sufficient of the mobile phase to obtain a mixture containing 0.05 per cent w/v of Nevirapine. Mix and filter through a membrane filter disc with an average pore diameter not greater than 1.0 µm, rejecting the first few ml of the filtrate.

Reference solution. A 0.05 per cent w/v solution of *nevirapine IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 12.0 g of *sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and filter, 20 volumes of *methanol* and 20 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

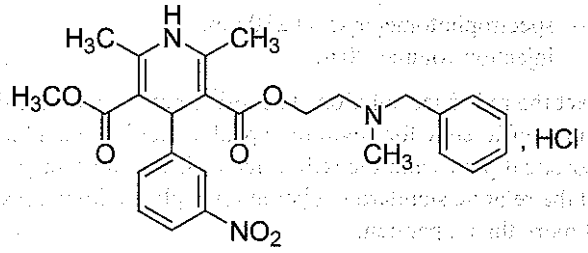
Inject the reference solution. The test is not valid unless the column efficiency for nevirapine peak is not less than 7500 theoretical plates, the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{14}N_4O$ in the tablets.

Storage. Store protected from moisture at a temperature not exceeding 30°.

Nicardipine Hydrochloride



$C_{26}H_{29}N_3O_6,HCl$ Mol.Wt.516.0.

Nicardipine Hydrochloride is 3, 5-pyridinedicarboxylic acid, 1, 4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-,methyl2-[methyl(phenylmethyl)amino]ethyl ester,monohydrochloride.

Nicardipine Hydrochloride contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{26}H_{29}N_3O_6,HCl$ calculated on the dried basis.

Category. Antihypertensive.

Description. A greenish yellow crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nicardipine hydrochloride IPRS* or with the reference spectrum of nicardipine hydrochloride.

B. When examined in the range 230 nm to 400 nm (2.4.7), a 0.05 per cent w/v solution in *ethanol* shows an absorption maximum at 355 nm.

C. A 0.25 per cent w/v solution in *methanol* gives the reaction of chlorides (2.3.1).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use and protected from light.

Test solution. Dissolve 60 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 60 mg of *nicardipine hydrochloride IPRS* in 10 ml of the mobile phase, mix with the aid of ultrasound, add 10.0 ml of a 10 per cent *hydrogen peroxide solution*, allow to stand for 30 minutes and dilute to 100.0 ml with the mobile phase.

NOTE— Nicardipine Hydrochloride degrades to produce nicardipine pyridine analog, nicardipine dimethyl ester analog, and nicardipine bis analog. Use a freshly prepared sample to avoid further degradation for analysis.

Reference solution (b). A 0.0003 per cent w/v solution of *nicardipine hydrochloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane chemically bonded to totally porous silica particles (5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 60 volumes of buffer solution prepared by dissolving 6.8 of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 4.8 with *potassium hydroxide solution* and 40 volumes of a mixture of 82 volumes of *acetonitrile* and 18 volumes of *methanol*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 237 nm,
- injection volume: 50 µl.

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Name	Relative retention time
Nicardipine impurity A[1]	0.67
Nicardipine (Retention time about 10 minutes)	1.0
Nicardipine impurity B[2]	1.2
Nicardipine impurity C[3]	1.3

[1]3-{2-[benzyl(methyl)amino]ethyl} 5-methyl 2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate dihydrochloride,

[2]dimethyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate,

[3]bis{2-[benzyl(methyl)amino]ethyl} 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate dihydrochloride.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to nicardipine and nicardipine impurity A is not less than 1.5 and the resolution between the peaks due to nicardipine impurity C and nicardipine impurity B is not less than 1.5.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak due to nicardipine impurity A is not more than 0. 2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent), the area of any peak due to nicardipine impurity B and nicardipine impurity C is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore any peak with an area less than 0.02 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.01 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1 g by drying in an oven at 105°.

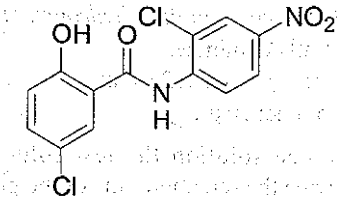
Assay. Dissolve 0.3 g in 50 ml of *glacial acetic acid* and add 10 ml of *mercuric acetate solution* by warming if necessary, allow to cool. Titrate with *0.1 M perchloric acid*, determining the end- point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.0516 g of $C_{26}H_{29}N_3O_6$,HCl.

Storage. Store protected from light.

Niclosamide

Anhydrous Niclosamide



$C_{13}H_8Cl_2N_2O_4$ Mol. Wt. 327.1

Niclosamide is 2′,5-dichloro-4′-nitrosalicylanilide.

Niclosamide contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{13}H_8Cl_2N_2O_4$, calculated on the dried basis.

Category. Anthelmintic.

Description. A yellowish white to yellowish, fine crystals or powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *niclosamide IPRS* or with the reference spectrum of niclosamide.

B. Heat 50 mg with 5 ml of *1 M hydrochloric acid* and 0.1 g of *zinc powder* in a water-bath for 10 minutes, cool and filter. To the filtrate add 1 ml of a 0.5 per cent w/v solution of *sodium nitrite* and allow to stand for 3 minutes. Add 2 ml of a 2 per cent w/v solution of *ammonium sulphamate*, shake, allow to stand for 3 minutes and add 2 ml of a 0.5 per cent w/v solution of *N-(1-naphthyl) ethylenediamine dihydrochloride*; a violet colour is produced.

C. Heat the substance under examination on a copper wire in a non-luminous flame; a green colour is imparted to the flame.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of the *methanol*.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile*. Dilute 1.0 ml of the solution to 20.0 ml with *acetonitrile*.

Chromatographic system

– a stainless steel column 12.5 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),

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- mobile phase: a mixture of 50 volumes of *acetonitrile*, 50 volumes of a solution containing 0.2 per cent w/v of *potassium dihydrogen orthophosphate*, 0.1 per cent w/v of *disodium hydrogen phosphate* and 0.2 per cent w/v of *tetrabutylammonium hydrogen sulphate*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Inject the reference solution the test solution. Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution, the sum of the areas of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with the reference solution. Ignore any peak with an area less than 10 per cent of the area of the principal peak in the chromatogram obtained with the reference solution.

Chlorides (2.3.12). To 2.0 g add a mixture of 40 ml of *water* and 1.2 ml of *5 M acetic acid*, boil for 2 minutes, cool and filter; 10 ml of the filtrate diluted to 15 ml with *water* complies with the limit test for chlorides (500 ppm).

2-Chloro-4-nitroaniline. Not more than 100 ppm, determined by the following method. Boil 0.25 g with 5 ml of *methanol*, cool, add 45 ml of *1 M hydrochloric acid*, heat to boiling, cool, filter and dilute the filtrate to 50.0 ml with *1 M hydrochloric acid*. To 10.0 ml of the solution add 0.5 ml of a 0.5 per cent w/v solution of *sodium nitrite* and allow to stand for 3 minutes. Add 1.0 ml of a 2 per cent w/v solution of *ammonium sulphamate*, shake, allow to stand for 3 minutes and add 1.0 ml of a 0.5 per cent w/v solution of N-*(1-naphthyl) ethylenediamine dihydrochloride*. Any pinkish violet colour produced is not more intense than that obtained in a solution prepared at the same time and in the same manner using 10.0 ml of a solution prepared by diluting 2.0 ml of a 0.00050 per cent w/v solution of *2-chloro-4-nitroaniline* in *methanol* to 20 ml with *1 M hydrochloric acid* and beginning at the words "add 0.5 ml of a 0.5 per cent w/v solution of *sodium nitrite*.....".

5-Chlorosalicylic acid. Not more than 60 ppm, determined by the following method. Boil 1.0 g with 15 ml of *water* for 2 minutes, cool, filter through a membrane filter (pore size 0.45 µm), wash the filter and dilute the combined filtrate and washings to 20 ml with *water* (solution A). Dissolve 30 mg of *5-chlorosalicylic acid* in 20 ml of *methanol* and add sufficient *water* to produce 100.0 ml. Dilute 1.0 ml of the solution to 100.0 ml with *water* (solution B). To 10.0 ml of each of solutions A and B add separately 0.1 ml of *ferric chloride solution*; any violet colour produced in solution A is not more intense than that obtained in solution B.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 4 hours.

Assay. Dissolve 0.3 g in 80 ml of a mixture of equal volumes of *acetone* and *methanol*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M tetrabutylammonium hydroxide* is equivalent to 0.03271 g of $C_{13}H_8Cl_2N_2O_4$.

Storage. Store protected from light and moisture.

Niclosamide Tablets

Niclosamide Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of niclosamide, $C_{13}H_8Cl_2N_2O_4$. The tablets may contain sweetening and flavouring agents.

Usual strength. 500 mg.

Identification

Heat a quantity of the powdered tablets containing 0.5 g of Niclosamide with 25 ml of hot *ethanol (95 per cent)*, filter while hot and evaporate the filtrate to dryness on a water-bath. The residue complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *niclosamide IPRS* or with the reference spectrum of niclosamide.

B. Heat 50 mg with 5 ml of *1 M hydrochloric acid* and 0.1 g of *zinc powder* in a water-bath for 10 minutes, cool and filter. To the filtrate add 1 ml of a 0.5 per cent w/v solution of *sodium nitrite* and allow to stand for 3 minutes. Add 2 ml of a 2 per cent w/v solution of *ammonium sulphamate*, shake, allow to stand for 3 minutes and add 2 ml of a 0.5 per cent w/v solution of N-*(1-naphthyl) ethylenediamine dihydrochloride*; a violet colour is produced.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets containing about 100 mg of anhydrous nicolsamide with 80 ml of *methanol* for 15 minutes and dilute to 100.0 ml with the same solvent.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile* and further dilute 1.0 ml of the solution to 20.0 ml with *acetonitrile*.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of *acetonitrile* and 50 volumes of a solution containing about 0.2 per

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cent w/v of *potassium dihydrogen orthophosphate*, 0.2 per cent w/v of *tetrabutylammonium hydrogen sulphate* and 0.1 per cent w/v of *disodium hydrogen ortho-phosphate*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Inject the reference solution. Adjust the sensitivity so that the height of the principal peak is not less than 20 per cent of full scale of the recorder.

Inject the reference solution the test solution. Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution, the sum of the areas of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with the reference solution. Ignore any peak with an area less than 10 per cent of the area of the principal peak in the chromatogram obtained with the reference solution.

2-Chloro-4-nitroaniline. Not more than 100 ppm.

Boil a quantity of the powdered tablets containing 0.1 g of Niclosamide with 20 ml of *methanol*, cool, and dilute to 50.0 ml with *1 M hydrochloric acid*, filter. To 10.0 ml of the solution add 0.5 ml of a 0.5 per cent w/v solution of *sodium nitrite* and allow to stand for 3 minutes. Add 1.0 ml of a 2 per cent w/v solution of *ammonium sulphamate*, shake, allow to stand for 3 minutes and add 1.0 ml of a 0.5 per cent w/v solution of *N-(1-naphthyl) ethylenediamine dihydrochloride*. Any pinkish violet colour produced is not more intense than that obtained in a solution prepared at the same time and in the same manner using 10.0 ml of a solution prepared by diluting 2.0 ml of a 0.0005 per cent w/v solution of *2-chloro-4-nitroaniline* in *methanol* to 50 ml with *1 M hydrochloric acid* and beginning at the words "add 0.5 ml of a 0.5 per cent w/v solution of *sodium nitrite* .....".

5-Chlorosalicylic acid. Not more than 60 ppm, determined by the following method. Boil a quantity of powdered tablets containing 0.5 g of niclosamide with 15 ml of *water* for 2 minutes, cool, filter through a membrane filter (pore size 0.45 µm), wash the filter and dilute the combined filtrate and washings to 20 ml with *water* (solution A). Dissolve 15 mg of *5-chlorosalicylic acid* in 20 ml of *methanol* and add sufficient *water* to produce 100.0 ml. Dilute 1.0 ml of the solution to 100.0 ml with *water* (solution B). To 10.0 ml of each of solution A and B add separately 0.1 ml of *ferric chloride solution; any* violet colour produced in solution A is not more intense than that obtained in solution B.

Other tests. Comply with the tests stated under Tablets.

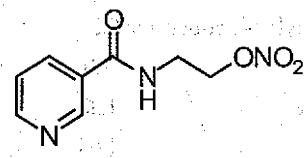
Assay. Weigh and powder 20 tablets. Disperse a quantity of the powdered tablets containing about 0.3 g of Niclosamide dissolved in 60 ml of *dimethylformamide*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M tetrabutylammonium hydroxide* is equivalent to 0.03271 g of $C_{13}H_8Cl_2N_2O_4$.

Storage. Store protected from light and moisture.

Labelling. The label states that the tablets should be chewed thoroughly before swallowing.

Nicorandil



$C_8H_9N_3O_4$ Mol. Wt. 211.2

Nicorandil is 2-(3-Pyridinecarboxamido)ethyl nitrate.

Nicorandil contains not less than 98.0 per cent and not more than 102.0 per cent of $C_8H_9N_3O_4$, calculated on the anhydrous basis.

Category. Selective ATP dependent potassium channel opener.

Description. A white to off white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nicorandil IPRS* or with the reference spectrum of nicorandil.

B. In the Assay, the retention time of the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Disperse 15 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution. A 0.00003 per cent w/v solution of *nicorandil IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),

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- mobile phase: a mixture of 70 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, 22 volumes of *methanol* and 8 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Name	Relative retention time	Relative response factor
Nitrate	0.22	1.77
Nicotinic acid	0.26	1.1
2-amino ethyl nicotinate	0.29	1.15
N-[2-Hydroxy ethyl]-nicotinamide	0.34	1.15
Nicorandil	1.0	–
Methyl nicotinate	1.38	1.53
Oxazoline	1.68	1.01

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with the reference solution (0.6 per cent). Ignore any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphates (2.3.17). To 7.5 ml of 5 per cent w/v solution of the substance under examination in *1 M hydrochloric acid*, add 7.5 ml of *water* and mix. The solution complies with the limit test for sulphates (0.04 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.3 per cent, determined on 0.3 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Disperse 0.1 g of the substance under examination in 70 ml of the mobile phase with the aid of ultrasound and dilute to 100.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 50.0 ml with the mobile phase. Further dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution. A 0.002 per cent w/v solution of *nicorandil IPRS* in the mobile phase.

Use chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_9N_3O_4$.

Storage. Store protected from moisture, at a temperature between 2° to 8°.

Nicorandil Prolonged-release Tablets

Nicorandil Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Nicorandil Prolonged-release Tablets contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nicorandil, $C_8H_9N_3O_4$.

Usual strengths. 10 mg; 20 mg.

Identification

In the Assay, the retention time of the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2). Complies with the test stated under Tablets.

Nitrate. Not more than 2.5 per cent.

Determine by liquid chromatography (2.4.14).

Test solution. Disperse 5 tablets in 180 ml of *methanol* with the aid of ultrasound for 45 minutes and dilute to 250.0 ml with *methanol*, filter. Dilute the filtrate with *methanol* to obtain a solution containing 0.004 per cent w/v of Nicorandil.

Reference solution. Disperse 33 mg of *potassium nitrate* (equivalent to 20 mg of nitrate) with 5 ml of *water* and dilute to 100.0 ml with *methanol*. Further dilute the solution with *methanol* to obtain a solution of 0.0001 per cent w/v of nitrate.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with aminopropylsilane bonded to porous silica (3 µm) (Such as APS-2 Hypersil),
- mobile phase: a mixture of 70 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen*

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orthophosphate in 1000 ml of *water*, 30 volumes of *methanol*, adjusted to pH 5.2 with *potassium hydroxide solution*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of nitrate.

1 g of potassium nitrate is equivalent to 0.61 g of nitrate.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 70 volumes of *water* and 30 volumes of *methanol*.

Test solution. Disperse 5 tablets in 180 ml of *methanol* with the aid of ultrasound for 45 minutes and dilute to 250.0 ml with *methanol*. Dilute the solution with the solvent mixture to obtain a solution containing 0.004 per cent w/v of Nicorandil.

Reference solution. A 0.0002 per cent w/v solution of *nicorandil IPRS* in *methanol*. Further dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 73 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, 25 volumes of *methanol* and 2 volumes of *acetonitrile*, adjusted to pH 5.7 with *potassium hydroxide solution*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Nitrate	0.16	—
Nicotinic acid	0.20	0.88
2-amino ethyl nicotinate	0.25	0.95
N-[2-hydroxy ethyl]-nicotinamide	0.32	0.95
Nicorandil	1.0	—
Methyl nicotinate	1.64	0.64
Oxazoline	2.30	0.92

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak corresponding to nicotinic acid and methyl nicotinate, each of, is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent), the area of any secondary peak corresponding to 2-amino ethyl nicotinate nitrate and oxazoline, each of, is not more than 3 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent), the area of any secondary peak corresponding to N-[2-hydroxy ethyl]-nicotinamide is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of areas of all other secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent) and the sum of areas of all the secondary peaks is not more than 12 times the area of the principal peak in the chromatogram obtained with the reference solution (6.0 per cent). Ignore any peak due to nitrate.

Uniformity of content. Complies with the test stated under Tablets as described under Assay using the following test solution.

Test solution. Disperse 1 tablet in 180 ml of *methanol* with the aid of ultrasound for 45 minutes and dilute to 250.0 ml with *methanol*, filter. Dilute the filtrate to obtain a solution containing 0.004 per cent w/v of Nicorandil.

Calculate the content of $C_8H_9N_3O_4$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 40 mg of Nicorandil in 70 ml of *methanol* with the aid of ultrasound for 45 minutes and dilute to 100.0 ml with *methanol*, filter. Dilute 1.0 ml of the solution to 10.0 ml with *methanol*.

Reference solution. A 0.004 per cent w/v solution of *nicorandil IPRS* in *methanol*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as zorbax Rx C8),
- mobile phase: a mixture of 70 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water* and 30 volumes of *methanol*, adjusted to pH 5.2 with *dilute potassium hydroxide solution*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the

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tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not be more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_9N_3O_4$ in the tablets.

Storage. Store protected from moisture at a temperature between 2° to 8°.

Nicorandil Tablets

Nicorandil Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of Nicorandil, $C_8H_9N_3O_4$.

Usual strengths. 10 mg; 20 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 500 ml of *water*,

Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Test solution. Use the filtrate, dilute if necessary, with *water*.

Reference solution. Dissolve a suitable quantity of *nicorandil IPRS* in *water* and dilute with *water* in such manner to obtain a similar concentration to that of the test solution.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_9N_3O_4$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_8H_9N_3O_4$.

Uniformity of content. Complies with the test stated under Tablets.

Solvent mixture. 80 volumes of *water* and 20 volumes of *methanol*.

Test solution. Disperse one tablet in the solvent mixture and sonicate for about 5 minutes and dilute with the solvent mixture to obtain a solution containing 0.005 per cent w/v of Nicorandil and filter.

Reference solution. Dissolve a suitable quantity of *nicorandil IPRS* in the solvent mixture and dilute with the solvent mixture in such a manner to get similar concentration to that of the test solution.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_9N_3O_4$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of *water* and 20 volumes of *methanol*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 10 mg of Nicorandil and dissolve in 150 ml of the solvent mixture, sonicate for about 5 minutes and dilute to 250.0 ml with the solvent mixture, filter.

Reference solution. A 0.004 per cent w/v solution of *nicorandil IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 80 volumes of buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 7.2 with *1 M potassium hydroxide solution* and 20 volumes of *methanol*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3500 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

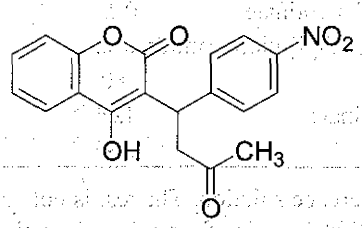
Inject the reference solution and the test solution.

Calculate the content of $C_8H_9N_3O_4$ in tablets.

Storage. Store protected from moisture, at a temperature 2° to 8°.

Nicoumalone

Acenocoumarol



$C_{19}H_{15}NO_6$ Mol. Wt. 353.3

Nicoumalone is (*RS*)-4-hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl]coumarin.

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Nicoumalone contains not less than 98.5 per cent and not more than 100.5 per cent of $C_{19}H_{15}NO_6$, calculated on the dried basis.

Category. Anticoagulant.

Description. A white to brownish-white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nicoumalone IPRS* or with the reference spectrum of nicoumalone.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in a mixture of 9 volumes of *methanol* and 1 volume of *1 M hydrochloric acid* shows absorption maxima at about 283 nm and 306 nm; absorbances at the maxima, about 0.65 and about 0.52, respectively.

C. Heat 50 mg with 2.5 ml of *glacial acetic acid*, 0.5 ml of *hydrochloric acid* and 0.2 g of *zinc powder* on a water-bath for 5 minutes, cool and filter. To the filtrate add 0.05 ml of *sodium nitrite solution* and add the mixture to 10 ml of a 1 per cent w/v solution of *2-naphthol* containing 3 ml of *5 M sodium hydroxide*; a bright red precipitate is produced.

Tests

Appearance of solution. A 2.0 per cent w/v solution in *acetone* is clear (2.4.1).

B. A 2.0 per cent w/v solution in *0.1 M sodium hydroxide* is clear (2.4.1), and yellow.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 50 volumes of *chloroform*, 50 volumes of *cyclohexane* and 20 volumes of *glacial acetic acid*.

Test solution. Dissolve 0.2 g of the substance under examination in 10 ml of *acetone*.

Reference solution. A 0.002 per cent w/v solution of the substance under examination in *acetone*.

Apply to the plate 20 µl of each solution. After development, dry the plate in air and immediately examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.75 g in 50 ml of *acetone* and titrate with *0.1 M sodium hydroxide* using *bromothymol blue solution* as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of sodium hydroxide required.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.03533 g of $C_{19}H_{15}NO_6$.

Storage. Store protected from light.

Nicoumalone Tablets

Acenocoumarol Tablets

Nicoumalone Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nicoumalone, $C_{19}H_{15}NO_6$.

Usual strengths. 1 mg; 2 mg; 3 mg; 4 mg.

Identification

A. Heat a quantity of the powdered tablets containing 50 mg of Nicoumalone with 30 ml of *acetone* under a reflux condenser for 5 minutes, filter and wash the residue with two quantities, each of 10 ml, of *acetone*. Evaporate the combined filtrate and washings to 5 ml, add *water* dropwise until the solution becomes turbid, heat on a water-bath until the solution is clear and allow to stand. Filter, wash the crystals with a mixture of equal volumes of *acetone* and *water* and dry at 100° at a pressure of 2 kPa for 30 minutes.

On the residue determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nicoumalone IPRS* or with the reference spectrum of nicoumalone.

B. When examined in the range 230 nm to 360 nm (2.4.7), the final solution obtained in the Assay shows absorption maxima at about 283 nm and 306 nm.

C. Heat 50 mg of the residue obtained in test A, with 2.5 ml of *glacial acetic acid*, 0.5 ml of *hydrochloric acid* and 0.2 g of *zinc powder* on a water-bath for 5 minutes, cool and filter. To the filtrate add 0.05 ml of *sodium nitrite solution* and add the mixture to 10 ml of a 1 per cent w/v solution of *2-naphthol* containing 3 ml of *5 M sodium hydroxide*; a bright red precipitate is produced.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 50 volumes of *chloroform*, 50 volumes of *cyclohexane* and 20 volumes of *glacial acetic acid*.

Test solution. Shake a quantity of the powdered tablets containing 20 mg of Nicoumalone with 5 ml of *acetone*, centrifuge and use the supernatant liquid.

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Reference solution. Dilute 1.0 volume of the test solution to 200.0 volumes with *acetone*.

Apply to the plate 20 µl of each solution. After development, dry the plate in air and immediately examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Uniformity of content. Complies with the test stated under Tablets.

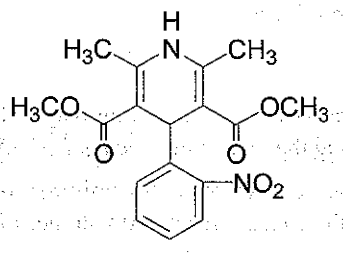
Finely crush one tablet, add 30 ml of *methanol*, stir the mixture for 30 minutes and filter through sintered glass, washing the residue with three quantities, each of 15 ml, of *methanol*. To the combined filtrate and washings add 10 ml of *1 M hydrochloric acid* and sufficient *methanol* to produce 100.0 ml. If necessary, dilute further with a solvent prepared by diluting 1 volume of *1 M hydrochloric acid* to 10 volumes with *methanol* to produce a solution containing about 0.001 per cent w/v solution of Nicoumalone. Measure the absorbance of the resulting solution at the maximum at about 306 nm (2.4.7). Calculate the content of $C_{19}H_{15}NO_6$ taking 521 as the specific absorbance at 306 nm.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 10 mg of Nicoumalone, add 30 ml of *methanol*, stir the mixture for 30 minutes and filter through sintered-glass, washing the residue with three quantities, each of 15 ml, of *methanol*. To the combined filtrate and washings add 10 ml of *1 M hydrochloric acid* and sufficient *methanol* to produce 100.0 ml. Dilute 5.0 ml of the solution to 50.0 ml with a solvent prepared by diluting 1 volume of *1 M hydrochloric acid* to 10 volumes with *methanol* and measure the absorbance of the resulting solution at the maximum at about 306 nm (2.4.7). Calculate the content of $C_{19}H_{15}NO_6$ taking 521 as the specific absorbance at 306 nm.

Storage. Store protected from light and moisture.

Nifedipine



$C_{17}H_{18}N_2O_6$ Mol. Wt. 346.3

Nifedipine is dimethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate.

Nifedipine contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{17}H_{18}N_2O_6$, calculated on the dried basis.

Category. Antianginal (calcium channel blocker).

Description. A yellow, crystalline powder; readily affected by exposure to light.

NOTE — Nifedipine, when exposed to daylight and certain wavelengths of artificial light, readily converts to a nitrosophenyl derivative. Exposure to ultraviolet light leads to the formation of a nitrophenyl derivative. Perform the tests and assay in the dark or under long-wavelength light (greater than 420 nm). Use low-actinic glassware.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nifedipine IPRS* or with the reference spectrum of nifedipine.

B. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to nifedipine in the chromatogram obtained with the reference solution (a).

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *cyclohexane* and 40 volumes of *ethyl acetate*.

Test solution. Dissolve 0.1 g of the substance under examination in 100.0 ml of *methanol*.

Reference solution. A 0.1 per cent w/v solution of *nifedipine IPRS* in *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

D. To 25 mg add 10 ml of a mixture of 5 volumes of *ethanol (95 per cent)*, 3.5 volumes of *water* and 1.5 volumes of *hydrochloric acid* and dissolve with gentle heating. Add 0.5 g of granulated zinc and allow to stand for 5 minutes, swirling occasionally. Filter, add 5 ml of a 1 per cent w/v solution of *sodium nitrite* to the filtrate and allow to stand for 2 minutes. Add 2 ml of a 5 per cent w/v solution of *ammonium sulphamate*, shake vigorously with care and add 2 ml of a 0.5 per cent w/v solution of *N-(1-naphthyl) ethylenediamine dihydrochloride*; an intense red colour develops which persists for more than 5 minutes.

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Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.2 g of the substance under examination in 20 ml of *methanol* and dilute to 50 ml with the mobile phase.

Reference solution (a). Dissolve a weighed quantity of *nifedipine IPRS* in sufficient *methanol* to produce a 1.0 per cent w/v solution and dilute quantitatively with the mobile phase to obtain a 0.4 per cent w/v solution.

Reference solution (b). A 0.04 per cent w/v solution of *dimethyl-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicaboxylate IPRS* (nitrophenylpyridine analogue) in *methanol*.

Reference solution (c). A 0.04 per cent w/v solution of *dimethyl-2,6-dimethyl-4-(2-nitrosophenyl)pyridine-3,5-dicarboxylate IPRS* (nitroso-phenylpyridine analogue) in *methanol*.

Reference solution (d). Mix 1 volume of each of reference solution (b) and (c) and 0.1 volume of the test solution, dilute to 10 volumes with the mobile phase and then dilute 2 volumes of the resulting solution to 10 volumes with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 55 volumes of *water*, 36 volumes of *methanol* and 9 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 235 nm,
- injection volume: 20 µl.

Inject reference solution (d). The peaks appear in the order nitrophenylpyridine analogue, nitrosophenylpyridine analogue and nifedipine (retention time about 15.5 minutes). The test is not valid unless, in the chromatogram obtained with reference solution (d), (a) the resolution factor between the peaks due to the nitrophenylpyridine analogue and the nitrosophenylpyridine analogue is greater than 1.5, (b) the resolution between the peaks due to the nitrosophenylpyridine analogue and nifedipine is greater than 1.5, and (c) the height of the peak due to the nitrophenyl-pyridine analogue is at least 20 per cent of the full-scale deflection.

Inject reference solution (a), (d) and the test solution and record the chromatograms for twice the retention time of nifedipine. In the chromatogram obtained with the test solution no secondary peak other than any peaks corresponding to the nitrophenylpyridine analogue and the nitrosophenylpyridine analogue has an area greater than that of the peak due to nifedipine in the chromatogram obtained with reference solution (d) and the areas of any peaks corresponding to the nitrophenylpyridine analogue and the nitrosophenylpyridine analogue are not greater than the areas of the corresponding peaks in the chromatogram obtained with reference solution (d) (0.2 per cent). The total amount of related substances is not greater than 0.3 per cent. Ignore any peak with an area less than 10 per cent of the area of the peak due to nifedipine in the chromatogram obtained with reference solution (d).

Heavy metals (2.3.13). 2.0 g complies with limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Assay. Weigh 0.13 g, dissolve in a mixture of 25 ml of *2-methyl-2-propanol* and 25 ml of *1 M perchloric acid* and titrate with *0.1 M ceric ammonium sulphate*, using 0.1 ml of *ferroin solution* as indicator until the pink colour is discharged, titrating slowly towards the end-point. Carry out a blank titration.

1 ml of *0.1 M ceric ammonium sulphate* is equivalent to 0.01732 g of $C_{17}H_{18}N_2O_6$.

Storage. Store protected from light.

Nifedipine Capsules

Nifedipine Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nifedipine, $C_{17}H_{18}N_2O_6$.

Usual strengths. 5 mg; 10 mg.

NOTE — Nifedipine, when exposed to daylight and certain wavelengths of artificial light, readily converts to a nitrosophenyl derivative. Exposure to ultraviolet light leads to the formation of a nitrophenyl derivative. Perform the tests and assay in the dark or under long-wavelength light (greater than 420 nm). Use low-actinic glassware.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of equal volumes of *ethyl acetate* and *cyclohexane*.

Test solution. Transfer a quantity of the contents of the capsules containing 30 mg of Nifedipine into a centrifuge tube containing *0.1 M sodium hydroxide*, add 25 ml of *dichloromethane*, stopper the tube and shake gently for 1 hour. Centrifuge for 10 minutes at 2000 to 2500 rpm. Remove the supernatant aqueous layer by aspiration with a syringe and transfer 5 ml of the clarified lower layer to a suitable vial.

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Reference solution (a). A 0.12 per cent w/v solution of *nifedipine IPRS* in *dichloromethane.*

Reference solution (b). A mixture of equal volumes of test solution and reference solution (a).

Apply to the plate 500 µl of each solution as bands 20 mm by 3 mm. After development, dry the plate in air until the odour of the solvent is not detectable and immediately examine under ultraviolet light at 254 nm. The principal band, appearing as a dark blue band, in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution (a). Spray with a solution prepared in the following manner. Dissolve 3 g of *bismuth subnitrate* and 30 g of *potassium iodide* in 10 ml of *3 M hydrochloric acid* and dilute with *water* to 100 ml; dilute 10 ml to 100 ml with *0.3 M hydrochloric acid.* In the chromatogram obtained with test solution the principal band, appearing as a compact light orange band against a yellow background, corresponds to that in the chromatogram obtained with reference solution (a). The band obtained with reference solution (b) appears as a single band under both visualisation procedures.

Tests

Dissolution (2.5.2).

A. Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid,*
Speed and time. 50 rpm and 20 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the dissolution medium, if necessary, at the maximum at about 340 nm (2.4.7). Calculate the content of $C_{17}H_{18}N_2O_6$ in the medium from the absorbance obtained from a solution of known concentration of *nifedipine IPRS* prepared by dissolving weighed quantity of *nifedipine IPRS* completely in *methanol* to prepare the first dilution and further dilute with dissolution medium.

Q. Not less than 70 per cent of the stated amount of $C_{17}H_{18}N_2O_6$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of mixed contents of capsules containing 10 mg nifedipine in 100-ml volumetric flask. Dissolve in minimum quantity of *methanol*, shake, and further dilute to volume with the mobile phase. Mix and filter.

Reference solution (a). A 0.1 per cent w/v solution of *nifedipine IPRS* in methanol. Further dilute suitably with mobile phase to produce a solution containing 0.03 per cent w/v solution of nifedipine.

Reference solution (b). A 0.04 per cent w/v solution of *dimethyl-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate IPRS* (nitrophenypyridine analogue) in *methanol.* Further dilute suitably with mobile phase to produce a solution containing 0.0006 per cent w/v of nitrophenylpyridine analogue.

Reference solution (c). A 0.04 per cent w/v solution of *dimethyl-2,6-dimethyl-4-(2-nitrosophenyl)pyridine-3,5 dicarboxylate IPRS* (nitrosophenylpyridine analogue) in *methanol.* Further dilute suitably with mobile phase to produce a solution containing 0.00015 per cent w/v of nitrosophenylpyridine analogue.

Reference solution (d). Mix equal volume of *reference solution (b),(c)* and *mobile phase.*

Reference solution (e). Mix equal volume of *reference solution (a), (b) and (c).*

Chromatographic system.

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A mixture of 50 volumes of *water*, 25 volumes of *methanol* and 25 volumes of *acetonitrile,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 25 µl.

Inject reference solution (e). The peaks appear in the order of nitrophenylpyridine analogue, nitrosophenylpyridine analogue and nifedipine. The test is not valid unless, in the chromatogram obtained with reference solution (e), the resolution between the peaks due to nitrophenylpyridine analogue and nitrosophenylpyridine analogue is not less than 1.5, and the resolution between the peaks due to nitrosophenylpyridine analogue and nifedipine is not less than 1.0. The test is not valid unless the relative standard deviation for replicate injections is not more than 10.0 per cent.

Inject the reference solution (d) and the test solution. In the chromatogram obtained with the test solution, area of any peak corresponding to nitrophenylpyridine analogue is not more than the corresponding peak obtained with reference solution (d) (2.0 per cent) and area of any peak corresponding to nitrosophenylpyridine analogue is not more than the corresponding peak obtained with reference solution (d) (0.5 per cent).

Uniformity of content. Complies with the test stated under Capsules.

Transfer the contents of a capsule with *methanol* and dilute to obtain a solution containing 0.005 per cent w/v of Nifedipine. Complete the Assay beginning at the words "Measure the absorbance...." and calculate the content of $C_{17}H_{18}N_2O_6$ in the capsule.

Other tests. Comply with the tests stated under Capsules.

Assay. Transfer the contents of capsules containing about 50 mg of Nifedipine quantitatively to a 200-ml volumetric flask

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with the aid of small quantities of *methanol*. Dilute to volume with *methanol* and mix. To 20.0 ml add sufficient *methanol* to produce 100.0 ml and mix. Measure the absorbance of the resulting solution at the maximum at about 350 nm (2.4.7). Calculate the content of $C_{17}H_{18}N_2O_6$ in the capsules from the absorbance obtained by repeating the operation with a 0.005 per cent w/v solution of *nifedipine IPRS* in *methanol*.

Storage. Store protected from light.

Nifedipine Prolonged-release Capsules

Nifedipine Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Nifedipine Prolonged-release Capsules contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of nifedipine, $C_{17}H_{18}N_2O_6$.

Usual strengths. 10 mg; 20 mg.

NOTE — Carry out all the following procedures in the dark or under long-wavelength light (greater than 420 nm). Prepare solutions immediately before use and protect them from light.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 40 volumes of *ethyl acetate* and 60 volumes of *cyclohexane*.

Solvent mixture. Equal volumes of *dichloromethane* and *methanol*.

Test solution. Shake a quantity of the contents of the capsules containing 20 mg of Nifedipine with solvent mixture and dilute to 100 ml with the solvent mixture, filter through a Whatman GF/C filter.

Reference solution (a). A 0.02 per cent w/v solution of *nifedipine IPRS* in the solvent mixture.

Reference solution (b). A mixture of equal volumes of test solution and reference solution (a).

Apply to the plate 20 µl of each solution.Allow the mobile phase to rise 15 cm.After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution (b).

B. In the Assay, the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with reference solution (a).

Tests

Dissolution. Complies with the tests stated under Capsules.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered contents of the capsules containing 50 mg of Nifedipine in 15 ml of *methanol* and dilute to 25 ml with the *methanol*, filter. Dilute 1 volume of resulting solution with 1 volume of the mobile phase.

Reference solution (a). Dilute 1.0 ml of test solution to 100.0 ml with mobile phase and further dilute 1.0 ml of the resulting solution to 5.0 ml with mobile phase.

*Reference solution (b).*A 0.0005 per cent w/v solution of *dimethyl-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicaboxylate IPRS* in the mobile phase.

Reference solution (c). A 0.0005per cent w/v solution of *dimethyl-2,6-dimethyl-4-(2-nitrosophenyl)pyridine-3,5-dicarboxylate IPRS* in the mobile phase.

Reference solution (d). Dilute 1 ml of the test solution to 100 ml with mobile phase and further dilute 1 volume of the resulting solution with 1 volume of reference solution (b) and 1 volume of reference solution (c).

Reference solution (e). Dilute 1 volume of reference solution (a) to 4 volume with mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Lichrosorb RP18),
- mobile phase: a mixture of 55 volumes of *water*, 36 volumes of *methanol* and 9 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 235 nm,
- injection volume: 20 µl.

Inject reference solution (d). The test is not valid unless, the resolution between the peaks due to dimethyl-2,6-dimethyl-4-(2- nitrophenyl)pyridine-3,5-dicarboxylate and dimethyl-2,6-dimethyl-4-(2- nitrosophenyl)pyridine-3,5-dicarboxylate is not less than 1.5 and the resolution between the peaks due to dimethyl-2,6-dimethyl-4-(2- nitrosophenyl) pyridine-3,5-dicarboxylate and nifedipine is not less than 1.5.

Inject reference solution (a), (b), (c), (e) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to *dimethyl-2,6-dimethyl-4-(2-nitrophenyl)pyridine- 3,5-dicarboxylate* is not more than the

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area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),the area of any peak corresponding to *dimethyl-2,6-dimethyl-4-(2- nitrosophenyl) pyridine-3,5-dicarboxylate* is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent), the area of any other secondary peak is not morethan the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent)and the sum of the areas of any other secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.05 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of mixed content of 20 capsules containing about 0.05 g of nifedipine in 15 ml of *methanol* and dilute to 50 ml with the *methanol* and filter. Dilute 1 volume of resulting solution to 5 volume of the mobile phase.

Reference solution (a).A 0.02 per cent w/v solution of *nifedipine IPRS* in the mobile phase.

Reference solution (b).A solution containg 0.0003 per cent w/v of *nifedipine IPRS* and 0.002 per cent w/v each of*dimethyl-2,6-dimethyl-4-(2- nitrophenyl)pyridine-3,5-dicarboxylate IPRS* and *dimethyl-2,6-dimethyl-4-(2-nitrosophenyl) pyridine-3,5-dicarboxylate IPRS* in the mobile phase.

Use chromatographic system as described under Related substances.

Inject reference solution (b). The test is not valid unless, the resolution between the peaks due to dimethyl-2,6-dimethyl-4-(2- nitrophenyl)pyridine-3,5-dicarboxylate and dimethyl-2,6-dimethyl-4-(2- nitrosophenyl)pyridine-3,5-dicarboxylate is not less than 1.5 and the resolution between the peaks due to dimethyl-2,6-dimethyl-4-(2- nitrosophenyl)pyridine-3,5-dicarboxylate and nifedipine is not less then 1.5.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{17}H_{18}N_2O_6$ in the capsule.

Nifedipine Prolonged-release Tablets

Nifedipine Sustained-release Tablets; Nifedipine Extended-release Tablets

Nifedipine Prolonged-release Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nifedipine, $C_{17}H_{18}N_2O_6$.

NOTE — Nifedipine, when exposed to daylight and certain wavelengths of artificial light, readily converts to a nitrosophenyl derivative. Exposure to ultraviolet light leads to the formation of a nitrophenyl derivative. Perform the tests and the assay in the dark or under long-wavelength light (greater than 420 nm). Use low-actinic glassware.

Usual strengths. 10 mg; 20 mg.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of equal volumes of *ethyl acetate* and *cyclohexane*.

Test solution. Transfer a quantity of the powdered tablets containing 30 mg of Nifedipine into a centrifuge tube containing *0.1 M sodium hydroxide*, add 25 ml of *dichloromethane*, stopper the tube and shake gently for 1 hour. Centrifuge for 10 minutes at 2000 rpm to 2500 rpm. Remove the supernatant aqueous layer by aspiration with a syringe and use 5 ml of the clarified lower layer.

Reference solution. A 0.12 per cent w/v solution of *nifedipine IPRS* in *dichloromethane*.

Apply to the plate 500 µl of each solution as bands 20 mm by 3 mm. After development, dry the plate in air until the odour of the solvent is not detectable and immediately examine under ultraviolet light at 254 nm. The principal band, appearing as a dark blue band, in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution. Spray with a solution prepared in the following manner. Dissolve 3 g of *bismuth subnitrate* and 30 g of *potassium iodide* in 10 ml of *3 M hydrochloric acid* and dilute to 100 ml with *water*; dilute 10 ml of the solution to 100 ml with *0.3 M hydrochloric acid*. In the chromatogram obtained with the test solution the principal band, appearing as a compact light orange band against a yellow background, corresponds to that in the chromatogram obtained with reference solution.

Tests

Dissolution (2.5.2).

A. Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 150 rpm and 120 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the dissolution medium, if necessary, at the maximum at about 340 nm (2.4.7).

Calculate the content of $C_{17}H_{18}N_2O_6$ in the medium from the absorbance obtained from a solution of known concentration

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of *nifedipine IPRS* prepared by dissolving weighed quantity of *nifedipine IPRS* completely in *methanol* and diluting further with the dissolution medium.

Not less than 25 per cent and not more than 45 per cent of the stated amount of $C_{17}H_{18}N_2O_6$.

B. Apparatus No. 2 (Paddle),

Medium. 900 ml of phosphate buffer pH 6.8 prepared by dissolving 30 g of *sodium lauryl sulphate*, 24.8 g of *disodium hydrogen phosphate anhydrous*, 2.85 g of *citric acid monohydrate* and 0.75 ml of *orthophosphoric acid* in sufficient *water* to produce 6000 ml. Adjusted to pH 6.8, if necessary,

Replace *0.1 M hydrochloric acid* with phosphate buffer pH 6.8 and run the apparatus at 150 rpm for 6 hours. Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the dissolution medium, if necessary, at the maximum at about 340 nm (2.4.7).

Calculate the content of $C_{17}H_{18}N_2O_6$ in the medium from the absorbance obtained from a solution of known concentration of *nifedipine IPRS* prepared by dissolving weighed quantity of *nifedipine IPRS* completely in *methanol* and diluting further with the dissolution medium.

Q. Not less than 60 per cent cumulative of both the medium of the stated amount of $C_{17}H_{18}N_2O_6$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of tablet powder containing 10 mg of nifedipine in 100-ml volumetric flask. Dissolve in minimum quantity of *methanol*, dilute to volume with the mobile phase, mix and filter.

Reference solution (a). A 0.1 per cent w/v solution of *nifedipine IPRS* in methanol. Further dilute suitably with mobile phase to produce a solution containing 0.03 per cent w/v solution of nifedipine.

Reference solution (b). A 0.04 per cent w/v solution of *dimethyl-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate IPRS* (nitrophenypyridine analogue) in *methanol*. Further dilute suitably with mobile phase to produce a solution containing 0.0006 per cent w/v of nitrophenylpyridine analogue.

Reference solution (c). A 0.04 per cent w/v solution of *dimethyl-2,6-dimethyl-4-(2-nitrosophenyl)pyridine-3,5 dicarboxylate IPRS* (nitrosophenylpyridine analogue) in *methanol*. Further dilute suitably with mobile phase to produce a solution containing 0.00015 per cent w/v of nitrosophenylpyridine analogue.

Reference solution (d). Mix equal volume of *reference solution (b),(c)* and *mobile phase.*

Reference solution (e): Mix equal volume of *reference solution (a), (b) and (c).*

Chromatographic system.

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A mixture of 50 volumes of *water*, 25 volumes of *methanol* and 25 volumes of *acetonitrile,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 25 µl.

Inject reference solution (e). The peaks appear in the order of nitrophenylpyridine analogue, nitrosophenylpyridine analogue and nifedipine. The test is not valid unless, in the chromatogram obtained with reference solution (e), the resolution between the peaks due to nitrophenylpyridine analogue and nitrosophenylpyridine analogue is not less than than 1.5, and the resolution between the peaks due to nitrosophenylpyridine analogue and nifedipine is not less than 1.0. The test is not valid unless the relative standard deviation for replicate injections is not more than 10.0 per cent.

Inject the reference solution (d) and the test solution. In the chromatogram obtained with the test solution, area of any peak corresponding to nitrophenylpyridine analogue is not more than the corresponding peak obtained with reference solution (d) (2.0 per cent), and area of any peak corresponding to nitrosophenylpyridine analogue is not more than the corresponding peak obtained with reference solution (d) (0.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 25 mg of Nifedipine, disperse in *methanol*, shake and dilute to 100.0 ml with *methanol*, filter. Dilute 20.0 ml of the filtrate to 100.0 ml with *methanol*. Measure the absorbance of the resulting solution at the maximum at about 350 nm (2.4.7). Calculate the content of $C_{17}H_{18}N_2O_6$ from the absorbance obtained with a 0.005 per cent w/v solution of *nifedipine IPRS* in *methanol*.

Storage. Store protected from light and moisture.

Nifedipine Tablets

Nifedipine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nifedipine, $C_{17}H_{18}N_2O_6$.

Usual strengths. 5 mg; 10 mg.

NOTE — Nifedipine, when exposed to daylight and certain wavelengths of artificial light, readily converts to a nitrosophenyl derivative. Exposure to ultraviolet light leads to the formation of a nitrophenyl derivative. Perform the tests and assay in the dark or under long-wavelength light (greater than 420 nm). Use low-actinic glassware.

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Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of equal volumes of *ethyl acetate* and *cyclohexane*.

Test solution. Transfer a quantity of the powdered tablets containing 30 mg of Nifedipine to a centrifuge tube containing 20 ml of *0.1 M sodium hydroxide*, add 25 ml of *dichloromethane*, stopper the tube and shake gently for 1 hour. Centrifuge for 10 minutes at 2000 to 2500 rpm. Remove the supernatant aqueous layer by aspiration with a syringe and transfer 5.0 ml of the clarified lower layer to a suitable vial.

Reference solution (a). A 0.12 per cent w/v solution of *nifedipine IPRS* in *dichloromethane*.

Reference solution (b). A mixture of equal volumes of the test solution and reference solution (a).

Apply to the plate 500 µl of each solution as bands 20 mm by 3 mm. After development, dry the plate in air until the solvent is not detectable and immediately examine under ultraviolet light at 254 nm. The principal band, appearing as a dark blue band, in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). Spray with a solution prepared in the following manner. Dissolve 3 g of *bismuth subnitrate* and 30 g of *potassium iodide* in 10 ml of *3 M hydrochloric acid* and dilute with *water* to 100 ml; dilute 10 ml to 100 ml with *0.3 M hydrochloric acid*. In the chromatogram obtained with the test solution the principal band, appearing as a compact light orange band against a yellow background, corresponds to that in the chromatogram obtained with reference solution (a). The band obtained with reference solution (b) appears as a single band under both visualisation procedures.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of tablet powder containing 10 mg of nifedipine in 100-ml volumetric flask. Dissolve in minimum quantity of *methanol*, shake, and further dilute to volume with the mobile phase. Mix and filter.

Reference solution (a). A 0.1 per cent w/v solution of *nifedipine IPRS* in *methanol*. Dilute suitably with mobile phase to produce a solution containing 0.03 per cent w/v solution of nifedipine.

Reference solution (b). A 0.04 per cent w/v solution of *dimethyl-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate IPRS* (nitrophenypyridine analogue) in *methanol*. Further dilute suitably with mobile phase to produce a solution containing 0.0006 per cent w/v of nitrophenylpyridine analogue.

Reference solution (c). A 0.04 per cent w/v solution of *dimethyl-2,6-dimethyl-4-(2-nitrosophenyl)pyridine-3,5 dicarboxylate IPRS* (nitrosophenylpyridine analogue) in *methanol*. Further dilute suitably with mobile phase to produce a solution containing 0.00015 per cent w/v of nitrosophenylpyridine analogue.

Reference solution (d). Mix equal volume of *reference solution (b),(c) and mobile phase.*

Reference solution (e). Mix equal volume of *reference solution (a), (b) and (c).*

Chromatographic system.

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A mixture of 50 volumes of *water*, 25 volumes of *methanol* and 25 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 25 µl.

Inject reference solution (e). The peaks appear in the order of nitrophenylpyridine analogue, nitrosophenylpyridine analogue and nifedipine. The test is not valid unless, in the chromatogram obtained with reference solution (e), the resolution factor between the peaks due to nitrophenylpyridine analogue and nitrosophenylpyridine analogue is greater than 1.5, and the resolution between the peaks due to nitrosophenylpyridine analogue and nifedipine is greater than 1.0. The test is not valid unless the relative standard deviation for replicate injections is not more than 10.0 per cent.

Inject the reference solution (d) and the test solution. In the chromatogram obtained with the test solution, area of any peak corresponding to nitrophenylpyridine analogue is not more than the corresponding peak obtained with reference solution (d) (2.0 per cent) and area of any peak corresponding to nitrosophenylpyridine analogue is not more than the corresponding peak obtained with reference solution (d) (0.5 per cent).

Uniformity of content. Complies with the test stated under Tablets

Shake one tablet with *methanol* in a 200-ml volumetric flask, dilute to volume with *methanol*, mix and filter. Complete the Assay beginning at the words "Measure the absorbance...." and calculate the content of $C_{17}H_{18}N_2O_6$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of Nifedipine into a 200-ml volumetric flask. Dissolve with the aid of 50 ml of *methanol*. Dilute to volume with *methanol*, mix and filter. Dilute 20.0 ml of

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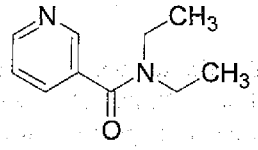
the solution to 100.0 ml with *methanol* and mix. Measure the absorbance of the resulting solution at the maximum at about 350 nm (2.4.7). Calculate the content of $C_{17}H_{18}N_2O_6$ from the absorbance obtained by repeating the operation with a 0.005 per cent w/v solution of *nifedipine IPRS* in *methanol*.

Storage. Store protected from light and moisture.

Nikethamide



$C_{10}H_{14}N_2O$ Mol. Wt. 178.2

Nikethamide is *N,N*-diethylpyridine-3-carboxamide.

Nikethamide contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{10}H_{14}N_2O$, calculated on the anhydrous basis.

Category. CNS and Respiratory stimulant.

Description. A colourless or slightly yellowish, oily liquid or crystalline mass.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests C and D may be omitted if tests A and B are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nikethamide IPRS* or with the reference spectrum of nikethamide.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution shows an absorption maximum at about 263 nm is about 0.57.

C. Heat 0.1 g with 1 ml of *2 M sodium hydroxide*; *diethylamine*, recognisable by its odour, is evolved progressively; the fumes turn red litmus paper blue.

D. To 2 ml of a 0.1 per cent w/v solution add 2 ml of *cyanogen bromide solution* and 3 ml of a 2.5 per cent w/v solution of *aniline* and mix; a yellow colour is produced.

Tests

Appearance of solution. The substance, in liquid form or liquefied by gentle heating, is clear (2.4.1), and not more intensely coloured than reference solution YS5 (2.4.1).

pH (2.4.24). 6.0 to 7.8, determined in 25.0 per cent w/v solution.

Congealing temperature (2.4.10). 23° to 24.5°.

Refractive index (2.4.27). 1.522 to1.526.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 75 volumes of *chloroform* and 25 volumes of *1-propanol*.

Test solution. Dissolve 0.4 g of the substance under examination in 10 ml of *methanol*.

Reference solution (a). A 0.04 per cent w/v solution of *ethylnicotinamide IPRS* in *methanol*.

Reference solution (b). A 0.004 per cent w/v solution of *ethylnicotinamide IPRS* in *methanol*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any spot corresponding to ethylnicotinamide in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (b).

Heavy metals (2.3.13). 2.0 g complies with limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.3 per cent, determined on 2.0 g.

Assay. Weigh 0.15 g, dissolve in 20 ml of *anhydrous glacial acetic acid* and 5 ml of *acetic anhydride*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01782 g of $C_{10}H_{14}N_2O$.

Storge. Store protected from light.

Nikethamide Injection

Nikethamide Injection is a sterile solution containing 25 per cent w/v solution of Nikethamide in Water for Injections.

Nikethamide Injection contains not less than 24.0 per cent and not more than 26.0 per cent w/v solution of nikethamide, $C_{10}H_{14}N_2O$.

Identification

A. Make 1 ml alkaline with *5 M sodium hydroxide*, extract with 5 ml of *dichloromethane* and evaporate the solvent.

On the residue determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nikethamide IPRS* or with the reference spectrum of nikethamide.

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B. Gives a voluminous precipitate with *alkaline potassium mercuri-iodide solution* and a greyish-brown flocculent precipitate with *tannic acid solution*. Gives no precipitate with *iodine solution* or with *potassium mercuri-iodide solution*.

C. Heat 1 ml with 0.2 g of *sodium hydroxide*; diethylamine, recognisable by its odour, is evolved.

Tests

pH (2.4.24). 6.0 to 8.0.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 75 volumes of *chloroform* and 25 volumes of *1-propanol*.

Test solution. Dilute 1 ml of the injection to 5 ml with *methanol*.

Reference solution (a). A 0.05 per cent w/v solution of *ethylnicotinamide IPRS* in *methanol*.

Reference solution (b). A 0.005 per cent w/v solution of *ethylnicotinamide IPRS* in *methanol*.

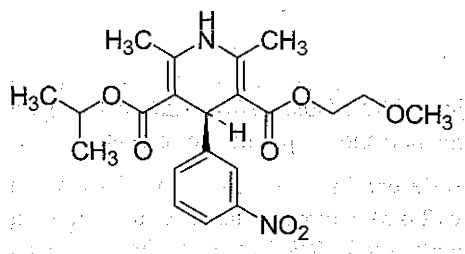
Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any spot corresponding to ethylnicotinamide in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (b).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Dilute 5.0 ml to 500.0 ml with *water*. To 5.0 ml of the solution add 5 ml of *1 M hydrochloric acid* and sufficient *water* to produce 500.0 ml. Measure the absorbance of the resulting solution at the maximum at about 263 nm (2.4.7). Calculate the content of $C_{10}H_{14}N_2O$ taking 282 as the specific absorbance at 263 nm.

Storage. Store protected from light, in single dose containers.

Nimodipine



and enantiomer

$C_{21}H_{26}N_2O_7$ Mol.Wt.418.4

Nimodipine is 2-methoxyethyl 1-methylethyl (4*RS*)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

Nimodipine contains not less than 98.5 per cent and not more than 101.5 per cent of $C_{21}H_{26}N_2O_7$, calculated on the dried basis.

Category. Calcium channel blocker.

Description. A light yellow or yellow, crystalline powder. It shows polymorphism (2.5.11). Exposure to ultraviolet light leads to the formation of a nitrophenylpyridne derivative.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nimodipine IPRS* or with the reference spectrum of nimodipine. If the spectra obtained in the solid state show differences, dissolve the substance under examination and the reference substance separately in the 2.0 per cent w/v solution of *methylene chloride* and using 0.2 mm cell for recording new spectra.

B. Specific optical rotation. See test.

Tes.ts

NOTE — Prepare solutions immediately before use either protected from light or under long-wavelength light (> 420 nm).

Appearance of solution. A 5.0 per cent w/v solution in *acetone* (Solution A) is clear (2.4.1).

Optical rotation (2.4.22). – 0.10° to + 0.10°, determined in solution A.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 40 mg of the substance under examination in 2.5 ml of *tetrahydrofuran* and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 8 mg of *nimodipine for peak identification IPRS* (containing impurity C) [*bis(2-methoxyethyl) 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate*] in 0.5 ml of *tetrahydrofuran* and dilute to 5.0 ml with the mobile phase.

Reference solution (c). Dilute 0.5 ml of the test solution to 25.0 ml with the mobile phase. Mix 0.5 ml of the solution with 0.5 ml of *nimodipine impurity A IPRS [2-methoxyethyl 1-methylethyl 2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate]* and dilute to 10.0 ml with the mobile phase.

Chromatographic system

– a stainless steel column 12.5 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),

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- column temperature: 40°,
- mobile phase: a mixture of 20 volumes of *methanol*, 20 volumes of *tetrahydrofuran* and 60 volumes of *water*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 235 nm,
- injection volume: 20 µl.

The relative retention time with reference to nimodipine (retention time about 7 minutes) for nimodipine impurity C is about 0.5 and for nimodipine impurity A is about 0.9.

Inject reference solution (c). The test is not valid unless the peak to valley ratio is not less than 4.0, where Hp is the height above the baseline of the peak due to nimodipine impurity A and Hv is the height above the baseline of the lowest point of the curve separating this peak from the peak due to nimodipine.

Inject reference solution (a), (b) and the test solution. Run the chromatogram 4.5 times the retention time of the principal peak for the test solution. In the chromatogram obtained with the test solution, the area of peak corresponding to nimodipine impurity C is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Weigh accurately about 0.18 g and dissolve with gentle heating in a mixture of 25 ml of *2-methyl-2-propanol* and 25 ml of *perchloric acid solution*. Add 0.1 ml of *ferroin solution* and titrate with *0.1 M ceric ammonium sulphate*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration. *[NOTE — Prepare solutions immediately before use either protected from light or under long-wavelength light (> 420 nm)].*

1 ml of *0.1 M ceric ammonium sulphate sulphate* is equivalent to 0.02092 g of $C_{21}H_{26}N_2O_7$.

Storage. Store protected from light.

Nimodipine Tablets

Nimodipine Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of nimodipine, $C_{21}H_{26}N_2O_7$. The tablets are coated.

Usual strength. 30 mg.

NOTE —Carry out the following procedures protected from light or under long-wavelength light (greater than 420 nm). Prepare the solutions immediately before use and protect them from light.

Identification

A. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 40 volumes of *ethyl acetate* and 60 volumes of *cyclohexane*.

Test solution. Disperse a quantity of the powdered tablets containing 60 mg of nimodipine with 10 ml of *ethyl acetate* for 15 minutes, centrifuge and use the supernatant liquid.

Reference solution (a). A 0.6 per cent w/v solution of *nimodipine IPRS* in *ethyl acetate*.

Reference solution (b). A mixture of equal volumes of test solution and reference solution (a).

Apply to the plate 2 µl of each solution. Allow the mobile phase to raise 15 cm. Dry the plate in air and examine under the ultraviolet light at 254 nm. Spray the plate with a freshly prepared 0.1 per cent w/v solution of *2,6-dichloroquinone-chlorimide* in *ethanol* and heat at 110° for about 3 minutes. The principal spot in the chromatogram obtained with the test solution correspond in position and colour to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *acetate buffer pH 4.5* prepared by dissolving 3.0 g of *sodium acetate* in 50 ml of *water*, add 1.75 g of *glacial acetic acid* and dilute to 1000.0 ml with *water*, containing 0.3 per cent w/v solution of *sodium dodecyl sulphate*,

Speed and time. 75 rpm for 30 minutes.

Withdraw a suitable volume of the medium and filter. Reject the first few ml of the filtrate and dilute a suitable volume of the filtrate with dissolution medium. Measure the absorbance of the resulting solution at the maximum at about 340 nm (2.4.7). Calculate the content of nimodipine, $C_{21}H_{26}N_2O_7$ in the medium from the absorbance obtained from a solution of known concentration of *nimodipine IPRS* in the dissolution medium.

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Q. Not less than 80 per cent of the stated amount of $C_{21}H_{26}N_2O_7$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 60 mg of nimodipine with 50 of *methanol* with the aid of ultrasound for 5 minutes and dilute to 100.0 ml with *methanol*. Centrifuge and use the supernatant liquid.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase and further dilute 2.0 ml of this resulting solution to 10.0 ml with the mobile phase.

Reference solution (b). A 0.0003 per cent w/v solution of *nimodipine impurity A IPRS* [(2-methoxyethyl 1-methylethyl 2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate] in the mobile phase.

Reference solution (c). A 0.0002 per cent w/v solution of *nimodipine IPRS* and *nimodipine impurity A IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 13 volumes of *acetonitrile*, 26 volumes of *tetrahydrofuran* and 60 volumes of *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 235 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to nimodipine and nimodipine impurity A is not less than 1.5 and the tailing factor for nimodipine peak is not more than 2.0.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to nimodipine impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of areas of all secondary peaks other than nimodipine impurity A is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances using following modifications.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 60 mg of Nimodipine with 50 ml of *methanol*, mix with the aid of ultrasound for 5 minutes and dilute to 100.0 ml with *methanol*. Centrifuge and use the supernatant liquid.

Reference solution (a). A 0.06 per cent w/v solution of *nimodipine IPRS* in *methanol*.

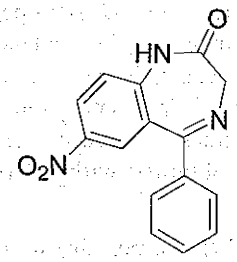
Reference solution (b). A solution containing 0.002 per cent w/v each of *nimodipine IPRS and nimodipine impurity A IPRS* in *methanol*.

Inject reference solution (b). The test is not valid unless the resolution between nimodipine and nimodipine impurity A is not less than 1.5 and the tailing factor for nimodipine peak is not more than 2.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{21}H_{26}N_2O_7$ in the Tablets.

Nitrazepam



$C_{15}H_{11}N_3O_3$ Mol. Wt. 281.3

Nitrazepam is 1,3-dihydro-7-nitro-5-phenyl-2*H*-1,4-benzodiazepin-2-one.

Nitrazepam contains not less than 99.0 per cent and not more than 101.0 per cent $C_{15}H_{11}N_3O_3$, calculated on the dried basis.

Category. Hypnotic; sedative.

Description. A yellow, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nitrazepam IPRS* or with the reference spectrum of nitrazepam.

B. *Carry out the following procedure in subdued light.*

When examined in the range 230 nm to 360 nm (2.4.7), a freshly prepared 0.0005 per cent w/v solution in a 0.5 per cent w/v

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solution of *sulphuric acid* in *methanol* shows an absorption maximum only at about 280 nm; absorbance at about 280 nm, about 0.45.

C. Dissolve 10 mg in 1 ml of *methanol*, warming if necessary, and add 0.05 ml of *2 M sodium hydroxide*; an intense yellow colour is produced.

D. Dissolve 20 mg in a mixture of 5 ml of *hydrochloric acid* and 10 ml of *water*, boil for 5 minutes, cool and add 2 ml of a 0.1 per cent w/v solution of *sodium nitrite*. Allow to stand for 1 minute, add 1 ml of a 0.5 per cent w/v solution of *sulphamic acid*, mix, allow to stand for 1 minute, add 1 ml of a 0.1 per cent w/v solution of *N-(1-naphthyl)ethylenediamine dihydrochloride*; a red colour is produced.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Carry out the test protected from light.

Test solution. Dissolve 50 mg of the substance under examination in *acetonitrile* and dilute to 20.0 ml with *acetonitrile*.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile*. Dilute 1.0 ml of the solution to 10.0 ml with *acetonitrile*.

Reference solution (b). A 0.002 per cent w/v solution of *clonazepam IPRS* in *acetonitrile*. Dilute 1.0 ml of the solution to 10.0 ml with the test solution.

Chromatographic system

- a stainless steel column 25 cm x 4 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. a 0.78 per cent w/v solution of *sodium dihydrogen phosphate*, adjusted to pH 3.0 with *orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	65	35
3	65	35
10	50	50
20	50	50
22	65	35

The relative retention time with reference to nitrazepam (retention time is about 9 minutes) for clonazepam is about 1.1.

Inject reference solution (b). The test is not valid unless the peak-to-valley ratio is not less than 4.0 where H_p is the height above the baseline of the peak due to clonazepam and H_v is the height above the baseline of the lowest point of the curve separating this peak from the peak due to nitrazepam.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 4 hours.

Assay. Weigh 0.25 g, dissolve in 50 ml of *acetic anhydride*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02813 g of $C_{15}H_{11}N_3O_3$.

Storage. Store protected from light and moisture.

Nitrazepam Tablets

Nitrazepam Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nitrazepam, $C_{15}H_{11}N_3O_3$.

Usual strengths. 5 mg; 10 mg.

Identification

Carry out the following procedure in subdued light.

A. When examined in the range 230 nm to 360 nm (2.4.7), the final solution obtained in the Assay shows an absorption maximum at about 280 nm.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 100 volumes of *chloroform* and 10 volumes of *methanol*.

Test solution. Shake a quantity of the powdered tablets with sufficient *methanol* to produce a solution containing 5 mg of Nitrazepam per ml, allow to settle and decant the supernatant liquid.

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Reference solution. A 0.5 per cent w/v solution of *nitrazepam IPRS* in *methanol*.

Apply to the plate 2 µl of each solution. After development, dry the plate in air, spray it with *ethanolic sulphuric acid (10 per cent v/v)*, heat at 105° for 10 minutes and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

C. To a quantity of the powdered tablets containing 5 mg of Nitrazepam add 5 ml of *hydrochloric acid* and 10 ml of *water*, heat on a water-bath for 15 minutes, filter and cool. To the clear filtrate add 1 ml of a 0.1 per cent w/v solution of *sodium nitrite*, allow to stand for 3 minutes and add 1 ml of a 0.5 per cent w/v solution of *sulphamic acid*. Allow to stand for 3 minutes and add 1 ml of a 0.1 per cent w/v solution of *N-(1-naphthyl) ethylenediamine dihydrochloride*; a red colour is produced.

Tests

Dissolution (2.5.2).

NOTE — Carry out the following procedure in subdued light.

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted if necessary, at the maximum at about 280 nm (2.4.7). Calculate the content of $C_{15}H_{11}N_3O_3$, in the medium from the absorbance obtained from a solution of known concentration of *nitrazapam IPRS* in the same medium.

Q. Not less than 70 per cent of the stated amount of $C_{15}H_{11}N_3O_3$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 40 volumes of *nitromethane*, 40 volumes of *toluene* and 20 volumes of *chloroform*.

Test solution. Shake a quantity of the powdered tablets containing 40 mg of Nitrazepam with 25 ml of *chloroform*, filter, carefully evaporate the filtrate to dryness and dissolve the residue in 2 ml of *chloroform*.

Reference solution. Dilute 1 volume of the test solution to 200 volumes with *chloroform*.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Uniformity of content. Complies with the test stated under Tablets.

NOTE — Carry out the following procedure in subdued light.

Powder 1 tablet, add 5 ml of *water*, mix and allow to stand for 15 minutes protected from light. Add 70 ml of a 0.5 per cent v/v solution of *hydrochloric acid* in *methanol*, shake for 15 minutes protected from light, add sufficient of the *hydrochloric acid solution* to produce 100.0 ml and filter. Dilute 10.0 ml of the filtrate with sufficient of the *hydrochloric acid solution* to produce a solution containing 0.0005 per cent w/v solution of Nitrazepam. Measure the absorbance of the resulting solution immediately at the maximum at about 280 nm (2.4.7). Calculate the content of $C_{15}H_{11}N_3O_3$ in the tablet taking 910 as the specific absorbance at 280 nm.

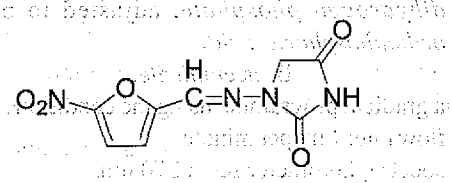
Other tests. Comply with the tests stated under Tablets.

Assay. *Carry out the following procedure in subdued light.*

Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 5 mg of Nitrazepam, add 5 ml of *water*, mix and allow to stand for 15 minutes protected from light. Add 70 ml of a 0.5 per cent v/v solution of *hydrochloric acid* in *methanol*, shake for 15 minutes protected from light, add sufficient of the *hydrochloric acid solution* to produce 100.0 ml and filter. Dilute 10.0 ml of the filtrate to 100.0 ml with the same solvent and measure the absorbance of the resulting solution immediately at the maximum at about 280 nm (2.4.7). Calculate the content of $C_{15}H_{11}N_3O_3$ taking 910 as the specific absorbance at 280 nm.

Storage. Store protected from light and moisture.

Nitrofurantoin



$C_8H_6N_4O_5$ Mol. Wt. 238.2 (anhydrous)
$C_8H_6N_4O_5,H_2O$ Mol. Wt. 256.2 (hydrous)

Nitrofurantoin is 1-(5-nitrofurfurylideneamino)hydantoin. It is anhydrous or contains one molecule of water of hydration.

Nitrofurantoin contains not less than 98.0 per cent and not more than 102.0 per cent of $C_8H_6N_4O_5$, calculated on the anhydrous basis.

NOTE —Nitrofurantoin and solutions of it are discolored by alkali and by exposure to light and are decomposed upon contact with metals other than stainless steel and aluminium.

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Category. Antibacterial.

Description. Lemon yellow crystals or a crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nitrofurantoin IPRS* or with the reference spectrum of nitrofurantoin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Limit of Nitrofurfural Diacetate. Determine by thin-layer chromatography (2.4.17), coating the plate with silica gel G.

Spray reagent. Dissolve 0.75 g of *phenylhydrazine hydrochloride* in 50 ml of *water*, and decolorize with activated charcoal. Add 25 ml of *hydrochloric acid*, and mix with water to produce 200 ml.

Mobile phase. A mixture of 90 volumes of *chloroform* and 10 volumes of *methanol*.

Test solution. Dissolve 100 mg of *nitrofurantoin* in 1 ml of *dimethylformamide*, and dilute to 10.0 ml with *acetone*.

Reference solution. Dissolve 10 mg of *nitrofurfurral diacetate IPRS* in 10 ml of *dimethylformamide* and dilute to 100.0 ml with *acetone*.

Apply to the plate 10 µl of each solution. After development, dry the plate at 105° for 5 minutes, while it is still warm, spray with spray reagent and examine in day light. Any secondary spot in the chromatogram obtained with the test solution, at R_f value of 0.7, is not more intense than the spot in the chromatogram obtained with the reference solution at the same Rf value (1.0 per cent).

Limit of Nitrofurazone. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in 2.0 ml of *dimethylformamide*, and add 20.0 ml *water*. Mix, and allow to stand for 15 minutes to allow a precipitate to form. Filter the solution through 0.45 µm nylon filter paper.

Reference solution (a). A 0.0005 per cent w/v solution of *nitrofurazone IPRS* in *dimethylformamide*. To 2.0 ml of the solution in 25-ml volumetric flask and add 20.0 ml of *water*.

Reference solution (b). A solution containing 0.0005 per cent w/v, each of, *nitrofurantoin IPRS* and *nitrofurazone IPRS* in *dimethylformamide*. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (3 to 10 µm),
- mobile phase: a mixture of 90 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 900 ml of *water*, add 30 ml of *1M sodium hydroxide*, adjusted to pH 7.0 with *1M sodium hydroxide* and dilute to 1000 ml with *water* and 10 volumes of *tetrahydrofuran*,
- flow rate: 1.6 ml per minute,
- spectrophotometer set at 375 nm,
- injection volume: 60-100 µl.

NOTE — Adjust the operating parameters so that the nitrofurazone peak has a retention time of about 10.5 minutes and its height is about 0.1 full-scale.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to nitrofurantoin and nitrofurazone is not less than 4.0 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 2.0 per cent, determined from the peak height, in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the height of any peak corresponding to nitrofurazone is not more than the height of the peak in the chromatogram obtained with the reference solution (a) (0.01 per cent).

Water (2.3.43). Not more than1.0 per cent (anhydrous form) and 6.5 to 7.5 per cent (hydrous form), using method 3, determined on sample previously dried at 140° for 30 minutes.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Determine by liquid chromatography (2.4.14).

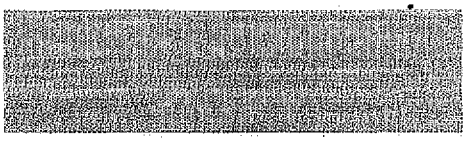
Internal standard solution. A 0.1 per cent w/v solution of *acetanilide* in *water*.

Test solution. Dissolve 50 mg of the substance under examination in 40.0 ml of *dimethylformamide*, and add 50.0 ml of internal standard solution.

Reference solution. Dissolve 50 mg of *nitrofurantoin IPRS* in 40.0 ml of *dimethylformamide*, and add 50.0 ml of internal standard solution.

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (3 to 10 µm),
- mobile phase: a mixture of 88 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 900 ml of *water*, add 30 ml of *1M sodium hydroxide*, adjusted to pH 7.0 with *1M sodium hydroxide* and dilute to 1000 ml with *water* and 12 volumes of *acetonitrile*,



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– spectrophotometer set at 254 nm,
– injection volume: 5-10 µl.

NOTE —Adjust the operating parameters so that the retention time of the nitrofurantoin peak is about 8 minutes and the peak heights is about half full-scale.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to acetanilide and nitrofurantoin is not less than 3.0, and the relative standard deviation from the ratio of the peak responses for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_6N_4O_5$.

Storage. Store protected from light and moisture.

Labelling. The label states whether the material is anhydrous or hydrous.

Nitrofurantoin Tablets

Nitrofurantoin Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nitrofurantoin, $C_8H_6N_4O_5$.

Usual strengths. 50 mg;100 mg.

Identification

A. Disperse a quantity of the powdered tablets containing 100 mg of nitrofurantoin in 10 ml of *6 M glacial acetic acid*. Boil for few minutes and filter while hot, cool to room temperature and dry the nitrofurantoin precipitate for 1 hour at 105°. The precipitate complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6) in mineral oil. Compare the spectrum with that obtained with *nitrofurantoin IPRS*, treated in the same manner or with the spectrum of nitrofurantoin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of *phosphate buffer pH 7.2*,
Speed and time. 100 rpm, 60 minutes and 120 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the medium if necessary, at the maximum at about 375 nm (2.4.7). Calculate the content of $C_8H_6N_4O_5$ in the medium from the absorbance obtained from a solution of known concentration of *nitrofurantoin IPRS* prepared by dissolving in minimum amount of *dimethylformamide* and diluted with the dissolution medium.

Q. Not less than 25 per cent of the stated amount of $C_8H_6N_4O_5$ in 60 minutes, and Not less than 85 per cent of the stated amount of $C_8H_6N_4O_5$ in 120 minutes.

Limit of Nitrofurazone. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 100 mg of Nitrofurantoin in 2.0 ml of *dimethylformamide*, and add 20.0 ml *water*. Mix, and allow to stand for 15 minutes to allow a precipitate to form. Filter the solution through 0.45 µm nylon filter paper.

Reference solution (a). A 0.0005 per cent w/v solution of *nitrofurazone IPRS* in *dimethylformamide*. To 2.0 ml of the solution in 25-ml volumetric flask and add 20.0 ml of *water*.

Reference solution (b). A solution containing 0.0005 per cent w/v, each of, *nitrofurantoin IPRS* and *nitrofurazone IPRS* in *dimethylformamide*. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system
– a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (3 to 10 µm),
– mobile phase: a mixture of 90 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 900 ml of *water*, add 30 ml of *1M sodium hydroxide*, adjusted to pH 7.0 with *1M sodium hydroxide* and dilute to 1000 ml with *water* and 10 volumes of *tetrahydrofuran*,
– flow rate: 1.6 ml per minute,
– spectrophotometer set at 375 nm,
– injection volume: 60-100 µl.

NOTE — Adjust the operating parameters so that the nitrofurazone peak has a retention time of about 10.5 minutes and its height is about 0.1 full-scale.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to nitrofurantoin and nitrofurazone is not less than 4.0 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 2.0 per cent, determined from the peak height, in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the height of any peak corresponding to nitrofurazone is not more than the height of the peak in the chromatogram obtained with the reference solution (a) (0.01 per cent).

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Assay. Determine by liquid chromatography (2.4.14).

Internal standard solution. A 0.1 per cent w/v solution of *acetanilide* in *water*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of Nitrofurantoin in 40.0 ml of *dimethylformamide,* and add 50.0 ml of internal standard solution.

Reference solution. Dissolve 50 mg of *nitrofurantoin IPRS* in 40.0 ml of *dimethylformamide,* and add 50.0 ml of internal standard solution.

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (3 to 10 µm),
- mobile phase: a mixture of 88 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 900 ml of *water*, add 30 ml of *1M sodium hydroxide*, adjusted to pH 7.0 with *1M sodium hydroxide* and dilute to 1000 ml with *water* and 12 volumes of *acetonitrile*,
- spectrophotometer set at 254 nm,
- injection volume: 5-10 µl.

NOTE — Adjust the operating parameters so that the retention time of the nitrofurantoin peak is about 8 minutes and the peak heights is about half full-scale.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to acetanilide and nitrofurantoin is not less than 3.0, and the relative standard deviation from the ratio of the peak responses for replicate injections is not more than 2.0 per cent.

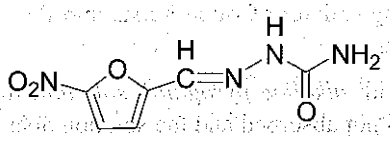
Inject the reference solution and the test solution.

Calculate the content of $C_8H_6N_4O_5$ in the tablets.

Storage. Store protected from light and moisture.

Nitrofurazone

Nitofural



$C_6H_6N_4O_4$ Mol. Wt. 198.1

Nitrofurazone is 5-nitro-2-furaldehyde semicarbazone.

Nitrofurazone contains not less than 97.0 per cent and not more than 103.0 per cent of $C_6H_6N_4O_4$, calculated on the dried basis.

Category. Antibacterial.

Description. A yellow to brownish-yellow, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nitrofurazone IPRS* or with the reference spectrum of nitrofurazone.

B. Dissolve 1 mg in 1 ml of *dimethylformamide* and add 0.05 ml of *1 M ethanolic potassium hydroxide*; a ruby red colour is produced.

Tests

pH (2.4.24). 5.0 to 7.0, determined in the filtrate obtained by shaking 1.0 g with 100 ml of *carbon dioxide-free water* and filtering.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in 100.0 ml of the mobile phase.

Reference solution (a). A 0.0005 per cent w/v solution of *(5-nitro-2-furyl)methylene diacetate* (Nitrofurazone impurity B) in the mobile phase.

Reference solution (b). Dissolve 10 mg each of *nitrofural IPRS* and *nitrofurantoin* in 100.0 ml of the mobile phase. Dilute 5 ml of the solution to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 40 volumes of *acetonitrile* and 60 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 310 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to nitrofural and nitrofurantoin is not less than 2.0.

Inject reference solution (a) and the test solution. Run the chromatogram 10 times the retention time of nitrofural. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peaks with the area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.025 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

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Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. *Carry out the following procedure in subdued light.*

Weigh 60 mg, add 20.0 ml of *dimethylformamide*, swirl to dissolve and add sufficient *water* to produce 500.0 ml. Dilute 5.0 ml of the solution to 100.0 ml with *water* and mix. Measure the absorbance of the resulting solution at the maximum at about 375 nm (2.4.7). Calculate the content of $C_6H_6N_4O_4$ taking 822 as the specific absorbance at 375 nm.

Storage. Store protected from light and moisture.

Nitroglycerin Injection

Diluted Glyceryl Trinitrate Injection

Nitroglycerin Injection is a sterile solution of Diluted Nitroglycerin, which may contains alcohol and propylene glycol in Water for Injections.

Nitroglycerin Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nitroglycerin, $C_3H_5N_3O_9$.

Description. A clear colourless solution.

Usual strengths. 5 mg per 5 ml; 5 mg per 10 ml.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 3.0 to 6.5, the solution prepared by diluting 5 ml of the injection with 5 ml of *water* and 1 drop of *saturated potassium chloride solution*.

Ethanol (2.3.45). Not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ethanol, determined by method I.

Bacterial endotoxins (2.2.3). Not more than 100 Endotoxin units per mg of nitroglycerin.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of the injection containing 5.0 mg of Nitroglycerin in 75 ml of mobile phase, shake well and dilute to 100.0 ml with the mobile phase.

Reference solution. Dissolve a quantity of *diluted nitroglycerin IPRS* to obtain 0.005 per cent w/v of nitroglycerin in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of equal volumes of *methanol* and *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.5 and the relative standard deviation is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_3H_5N_3O_9$.

Storage. Store protected from light at a temperature between 15° and 30°.

Labelling. It is to be diluted before use where necessary.

Nitrous Oxide

N_2O Mol. Wt. 44.0

Nitrous Oxide contains not less than 98.0 per cent v/v of N_2O.

NOTE — Carry out the following tests on a full cylinder from which no gas has been withdrawn. The cylinder from which the gas is taken should be kept at room temperature for not less than 6 hours before carrying out the tests. Keep the cylinder in the vertical position with the outlet valve uppermost and deliver the gas at a rate of 4 litres per hour, unless otherwise directed. The test for Carbon monoxide should be carried out on the first portion of gas drawn from the cylinder and that for Nitric oxide and nitrogen dioxide immediately thereafter.

Category. General anaesthetic.

Description. A colourless gas.

Identification

A. A glowing splinter of wood bursts into flame on contact with the gas.

B. Shake with *alkaline pyrogallol solution*; the gas being examined is not absorbed and the solution does not become brown.

Tests

Acidity or alkalinity. Use hermetically-closed, flat-bottomed, glass cylinders with dimensions such that 50 ml of liquid reaches a height of 12 to 14 cm, fitted with an outlet tube and

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with an inlet tube with an orifice of 1 mm in internal diameter reaching to within 2 mm of the bottom of the cylinder. For solution (1) pass 2.0 litres of the gas under examination through a mixture of 0.1 ml of *0.01 M hydrochloric acid* and 50 ml of *carbon dioxide-free water*. For solution (2) use 50 ml of *carbon dioxide-free water*. For solution (3) add 0.2 ml of *0.01 M hydrochloric acid* to 50 ml of *carbon dioxide-free water*. To each solution add 0.1 ml of 0.02 per cent w/v solution of *methyl red* in *ethanol (70 per cent)*. The intensity of the colour of solution (1) is between those of solution (2) and (3).

Arsine and phosphine. Through a *mercuric chloride paper* attached to a glass tube as in the limit test for arsenic (2.3.10), pass 2.0 litres of the gas; no visible stain is produced.

Carbon dioxide. Not more than 300 ppm v/v determined by the following method. Use the apparatus describedunder test for Acidity or alkalinity. Pass 1.0 litre through 50 ml of clear *barium hydroxide solution*. Any turbidity produced in the resulting solution is not more than that obtained in a reference solution prepared at the same time by adding 1 ml of 0.11 per cent w/v solution of *sodium bicarbonate* in *carbon dioxide-free water* to 50 ml of *barium hydroxide solution*.

Carbon monoxide. Not more than 10 ppm v/v, determined by the following method. Connect in series a U-tube containing *silica gel* impregnated with *chromium trioxide*, a drechsel bottle containing 100 ml of a 40 per cent w/v solution of *potassium hydroxide*, a U-tube containing pellets of *potassium hydroxide*, a U-tube containing *phosphorus pentoxide* dispersed on previously granulated, fused pumice, a tube containing *iodine pentoxide* in granules, previously dried at 200° and kept at a temperature of 120°, packed in 1-cm columns separated by 1-cm columns of glass wool giving an effective length of 5 cm, and a flask containing 2.0 ml of *1 M potassium iodide* and 0.15 ml of *starch solution*.

Flush the apparatus with 5.0 litres of carbon dioxide-free air and, if necessary, discharge the blue colour in the iodide solution by adding a small quantity of freshly prepared *0.002 M sodium thiosulphate*. Continue flushing until not more than 0.045 ml of *0.002 M sodium thiosulphate* is required after passing 5.0 litres of carbon dioxide-free air. Pass 5.0 litres of the gas under examination through the apparatus and flush the last traces of liberated iodine into the reaction flask by passing through the apparatus 1.0 litre of carbon monoxide-free air. Titrate the liberated iodine with *0.002 M sodium thiosulphate*. Carry out a blank titration under the same conditions, using 5.0 litres of carbon dioxide-free air. The difference between the volumes of *0.002 M sodium thiosulphate* used in the two titrations is not greater than 1.0 ml.

Halogens and hydrogen sulphide. Pass a volume containing 1.0 litre measured at 25° and at 101.3 kPa through a mixture of 100 ml of *water* and 1 ml of *silver nitrate solution*; neither opalescence nor darkening is produced.

Nitric oxide and nitrogen dioxide. Not more than 2 ppm v/v in both the liquid and gaseous phases, determined by the following method. Use two of the cylinders described under test for Acidity or alkalinity connected in series. Examine separately both the liquid and gaseous phases of the gas under examination. To obtain the liquid phase invert the cylinder. The liquid vaporises on leaving the valve.

For solution A dissolve 1 g of *sulphanilic acid* in a mixture of 10 ml of *glacial acetic acid* and 180 ml of *water*. For solution B dissolve 0.2 g of *N-(1-naphthyl)ethylenediamine dihydrochloride* in 10 ml of a 50 per cent v/v solution of *glacial acetic acid*, heating gently, and dilute to 200 ml with *water*. Mix 9 volumes of solution A with 1 volume of solution B (reagent A).

In the first cylinder place 15 ml of a solution containing 2.5 per cent w/v solution of *potassium permanganate* and 1.2 per cent v/v of *sulphuric acid (96 per cent)*. Place 20 ml of reagent A in the second cylinder and connect the outlet tube of the first cylinder to the inlet tube of the second cylinder. Pass 2.5 litres of the gas under examination through the reagents at a rate of 15 litres per hour. Prepare a reference solution by adding 0.25 ml of a 0.00616 per cent w/v solution of *sodium nitrite* to 20 ml of reagent A. Allow both the sample and reference solutions to stand for 10 minutes. For both liquid and gaseous phases, any red colour in the sample solution is not more intense than that in the reference solution.

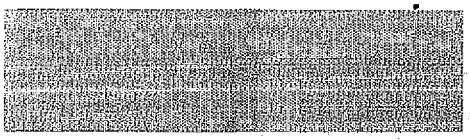
Oxidising substances. Pass a volume containing 2.0 litres measured at 25° and at 101.3 kPa through a freshly prepared solution of 0.5 g of *soluble starch* and 0.5 g of *potassium iodide* in 100 ml of *water* containing 0.05 ml of *glacial acetic acid*; the colour of the liquid is not changed.

Water. Pass a measured quantity at a rate of 6 litres per hour through an absorption tube containing *magnesium perchlorate*; the increase in weight of the tube does not exceed 2 mg per litre of gas, the initial and final weighings of the tube being made when the air in it has been displaced by the nitrous oxide.

Assay. Carry out the assay of nitrous oxide (2.3.32), using 100 ml of the gas under examination. Use a cylinder of the gas under examination from which at least 1 per cent w/w of the contents have been removed.

Storage. Store under pressure in metal cylinders of the type conforming to the appropriate safety regulations and at a temperature not exceeding 37°.

Labelling. The cylinder is painted blue and carries a label stating "Nitrous Oxide". In addition, "Nitrous Oxide" or the symbol "N_2O" should be stencilled in paint on the shoulder of the cylinder.



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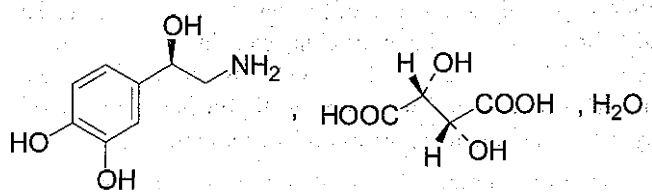
Noradrenaline Bitartrate

Noradrenaline Acid Tartrate; Levarterenol Bitartrate; Norepinephrine Bitartrate



$C_8H_{11}NO_3,C_4H_6O_6,H_2O$ Mol. Wt. 337.3

Noradrenaline Bitartrate is (*R*)-2-amino-1-(3,4-dihydroxyphenyl) ethanol tartrate monohydrate.

Noradrenaline Bitartrate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_8H_{11}NO_3,C_4H_6O_6$, calculated on the anhydrous basis.

Category. Sympathomimetic.

Description. A white or almost white, crystalline powder. It gradually darkens on exposure to air and light.

Identification

Test A may be omitted if tests B, C, D, E and F are carried out. Tests C, D, E may be omitted if tests A, B and F are carried out.

A. Dissolve 0.2 g in 2 ml of *water* containing about 10 mg of *sodium sulphite* and add sufficient *dilute ammonia solution* to give an alkaline reaction. Keep the mixture at about 4° for 1 hour and filter.

On the residue (residue R) determine by infrared absorption spectrophotometry (2,4,6). Compare the spectrum with that obtained with *noradrenaline acid tartrate IPRS* treated in the same manner.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.005 per cent w/v solution in *0.01 M hydrochloric acid* shows an absorption maximum at about 279 nm; absorbance at about 279 nm, about 0.40.

C. Wash residue R obtained in test A with three quantities, each of 2 ml, of *water*, followed by 5 ml of *ethanol (95 per cent)* and 5 ml of *ether* and dry the precipitate under pressure of 1.5 to 2.5 kPa for 3 hours. The specific optical rotation (2.4.22), determined in a 2.0 per cent w/v solution of the dried precipitate in *0.5 M hydrochloric acid* is – 48° to – 44°.

D. To 1 ml of a 1 per cent w/v solution, add 0.05 ml of *ferric chloride solution*; an intense green colour is produced. Add, drop by drop, *sodium bicarbonate solution*; the colour changes to blue and then red.

E. To 1 ml of a 0.1 per cent w/v solution add 10 ml of *phthalate buffer pH 3.6*, add 1 ml of *0.05 M iodine*, set aside for 5 minutes and add 2 ml of *0.1 M sodium thiosulphate*; not more than a faint red colour is produced. Repeat the test using *buffer solution pH 6.6* instead of *phthalate buffer pH 3.6*; a strong reddish violet colour is produced (distinction from adrenaline and isoprenaline).

F. The filtrate obtained in test A gives the reactions of tartrates (2.3.1).

Tests

Appearance of solution. A freshly prepared 2.0 per cent w/v solution is clear (2.4.1), and not more intensely coloured than reference solution BY*S5* (2.4.1).

Melting range (2.4.21). 100° to 106°, with decomposition.

Adrenaline. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 50 volumes of *acetone*, 50 volumes of *dichloromethane* and 0.5 volume of *anhydrous formic acid*.

NOTE—Prepare the following solutions immediately before use.

Test solution. Dissolve 0.25g of the substance under examination in 10 ml of *water*.

Reference solution (a). A 0.125 per cent w/v solution of *adrenaline tartrate IPRS* in *water*.

Reference solution (b). A 0.025 per cent w/v solution of *adrenaline tartrate IPRS* in *water*.

Reference solution (c). A mixture of equal volumes of the test solution and reference solution (b).

Apply to the plate 6 µl of each of test solution, reference solution (a) and (b) and 12 µl of reference solution (c) as bands 20 mm by 2 mm. Allow the applied bands to dry in air, spray them with a saturated solution of *sodium bicarbonate*, allow to dry in air and spray the bands twice with *acetic anhydride*, drying between the two sprayings. Heat the plate at 50° for 90 minutes and develop the chromatograms. After removal of the plate, allow it to dry in air and spray with a freshly prepared mixture of 8 volumes of *methanol*, 2 volumes of *ethylenediamine* and 2 volumes of a 0.5 per cent w/v solution of *potassium ferricyanide*. Dry the plate at 60° for 10 minutes and examine under ultraviolet light at 254 and 365 nm. In the chromatogram obtained with the test solution any band with a slightly higher R_f value than the principal band is not more intense than the corresponding band in the chromatogram obtained with reference solution (b). The chromatogram obtained with reference solution (c) shows a clearly separated band corresponding to the most intense band in the chromatogram obtained with reference solution (a) at a higher R_f value than the most intense band.

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Noradrenalone. Absorbance of a 0.2 per cent w/v solution in *0.01 M hydrochloric acid* at 310 nm, not more than 0.40 (2.4.7).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). 4.5 to 5.8 per cent, determined on 0.5 g.

Assay. Weigh 0.6 g, dissolve in 50 ml of *anhydrous glacial acetic acid*, warming if necessary. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator, until a bluish green colour is obtained. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03193 g of $C_8H_{11}NO_3,C_4H_6O_6$.

Storage. Store protected from moisture.

Noradrenaline Bitartrate Injection

Noradrenaline Acid Tartrate Injection; Noradrenaline Injection; Levarterenol Bitartrate Injection; Norepinephrine Bitartrate Injection

Noradrenaline Bitartrate Injection is a sterile solution of Noradrenaline Bitartrate. It is prepared by diluting Sterile Noradrenaline Concentrate to 250 times its volume with Sodium Chloride and Dextrose Injection or with Dextrose Injection (5 per cent w/v) immediately before use.

Noradrenaline Bitartrate Injection contains in 1 ml 8 µg of Noradrenaline Bitartrate equivalent to approximately 4 µg of noradrenaline.

Tests

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Sterile Noradrenaline Concentrate

Sterile Noradrenaline Concentrate is a sterile, isotonic solution containing 0.2 per cent w/v of Noradrenaline Bitartrate in Water for Injections.

Sterile Noradrenaline Concentrate contains not less than 0.18 per cent and not more than 0.23 per cent w/v of noradrenaline bitartrate, $C_8H_{11}NO_3,C_4H_6O_6,H_2O$.

Identification

Mix 0.5 ml with 10 ml of *phthalate buffer pH 3.6*, add 1 ml of *0.05 M iodine*, allow to stand for 5 minutes and add 2 ml of *0.1 M sodium thiosulphate*; not more than a very faint red colour is produced. Repeat the test using *phosphate buffer pH 6.6* instead of *phthalate buffer pH 3.6*; a strong reddish violet colour is produced.

Tests

pH (2.4.24). 3.0 to 4.6.

Bacterial endotoxins (2.2.3). Not more than 83.4 Endotoxin Units per mg of noradrenaline.

Other tests. Comply with the tests stated under Parenteral Preparations (Concentrated Solutions for Injection).

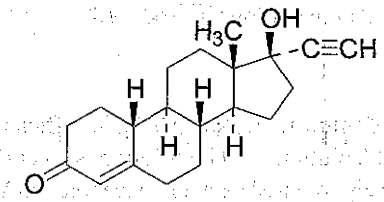
Assay. Dilute 5.0 ml to 200.0 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 279 nm (2.4.7). Calculate the content of $C_8H_{11}NO_3,C_4H_6O_6,H_2O$ taking 80 as the specific absorbance at 279 nm.

Storage. Store protected from light, in single dose containers.

Labelling. The label states (1) "Sterile Noradrenaline Concentrate"; (2) that 1 volume of the solution diluted to 250 volumes with Sodium Chloride and Dextrose Injection or with Dextrose Injection (5 per cent w/v) produces Noradrenaline Bitartrate Injection, which must be used immediately after preparation; (3) that if the solution is brown it should not be used.

Norethisterone

Norethindrone



$C_{20}H_{26}O_2$ Mol. Wt. 298.4

Norethisterone is 17β-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one.

Norethisterone contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{20}H_{26}O_2$, calculated on the dried basis.

Category. Progestogen.

Description. A white or yellowish-white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *norethisterone IPRS* or with the reference spectrum of norethisterone.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. A mixture of 90 volumes of *acetone* and 10 volumes of *formamide*.

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Mobile phase. A mixture of 40 volumes of *hexane* and 10 volumes of *dioxan*.

Test solution. Dissolve 10 mg of the substance under examination in 10 ml of *chloroform*.

Reference solution. Dissolve 10 mg of *norethisterone IPRS* in 10 ml of *chloroform*.

Place the dry plate in a tank containing a shallow layer of the solvent mixture, allow the solvent mixture to ascend to the top, remove the plate from the tank and allow the solvent to evaporate. Use within 2 hours, with the flow of the mobile phase in the direction in which the aforementioned treatment was done.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of warm air, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulphuric acid (20 per cent v/v)*. Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to and exhibits fluorescence similar to that in the chromatogram obtained with the reference solution.

C. Dissolve about 2 mg in 2 ml of *ethanol (95 per cent)* and add 1 ml of a 1 per cent w/v solution of *butylated hydroxytoluene* in *ethanol (95 per cent)* and 2 ml of *1 M sodium hydroxide*. Heat in a water-bath for 30 minutes and cool; a yellowish pink colour is produced.

Tests

Appearance of solution. Dissolve 0.2 g in sufficient *dioxan* to produce 10 ml (solution A). The solution is clear (2.4.1), and not more intensely coloured than reference solution YS6 (2.4.1).

Specific optical rotation (2.4.22). – 37.0° to – 33.0°, determined in a solution prepared by diluting 5.0 ml of solution A to 10.0 ml with *dioxan*.

Light absorption. Dissolve 10 mg in sufficient *ethanol (95 per cent)* to produce 100.0 ml, dilute 10.0 ml of the solution to 100.0 ml with *methanol (98 per cent)*. Absorbance of the resulting solution at the maximum at about 240 nm, 0.55 to 0.59 (2.4.7).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. A mixture of 40 volumes of *water* and 60 volumes of *acetonitrile*.

Test solution. Dissolve 25 mg of the substance under examination in 10 ml of the solvent mixture.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with end capped octylsilane bonded to porous silica (5 µm),
- mobile phase: A. *water*,
  B. *acetonitrile*,
- a gradient programme using the condition given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent w/v)	Mobile phase B (per cent w/v)
0	63	37
20	63	37
25	20	80
35	20	80
36	63	37
50	63	37

Inject the reference solution the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (0.3 per cent). Ignore any peaks with the area less than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Weigh 0.4 g, dissolve in 40 ml of *tetrahydrofuran*, add 10 ml of a 10 per cent w/v solution of *silver nitrate* and titrate with *0.1 M sodium hydroxide* using 2 ml of *bromocresol green solution* as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of *sodium hydroxide* required.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.02984 g of $C_{20}H_{26}O_2$.

Storage. Store protected from light and moisture.

Norethisterone Tablets

Norethindrone Tablets

Norethisterone Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of norethisterone, $C_{20}H_{26}O_2$.

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Usual strength. 5 mg.

Identification

Place a quantity of the powdered tablets containing 25 mg of Norethisterone on a small filter, wash with three quantities, each of 5 ml, of *light petroleum (60° to 80°)* and discard the washings. Extract the residue with 15 ml of *chloroform*, evaporate the extract to dryness and recrystallise from aqueous *methanol*. The residue complies with the following test.

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. A mixture of 90 volumes of *acetone* and 10 volumes of *1,2-propanediol*.

Mobile phase. A mixture of 40 volumes of *cyclohexane* and 10 volumes of *toluene*.

Test solution. Dissolve 25 mg of the substance under examination in 10 ml of the solvent mixture.

Reference solution (a). Dissolve 25 mg of *norethisterone IPRS* in 10 ml of the solvent mixture.

Reference solution (b). Mix equal volumes of the test solution and reference solution (a).

Place the dry plate in a tank containing a shallow layer of the solvent mixture, allow the solvent mixture to ascend to the top, remove the plate from the tank and allow the solvent to evaporate. Use within 2 hours, with the flow of the mobile phase in the direction in which the aforementioned treatment was done.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of warm air, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulphuric acid (20 per cent v/v)*. Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

Tests

Uniformity of content. Complies with the test stated under Tablets.

Carry out the procedure described under Assay but using the following test solution.

Test solution. Powder one tablet and dissolve as completely as possible in 2 ml of *water* with the aid of ultrasound for 15 minutes and dilute to 5.0 ml with *methanol*. Centrifuge for 15 minutes and use the clear supernatant liquid.

Calculate the content of $C_{20}H_{26}O_2$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 5 mg of Norethisterone in 10 ml of *water*; sonicate for 15 minutes and dilute to 25.0 ml with *methanol*, filter.

Reference solution. A 0.02 per cent w/v solution of *norethisterone IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (10 µm) (Such as Spherisorb ODS 1),
- mobile phase: a mixture of 28 volumes of *water* and 72 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{26}O_2$ in the tablets.

Storage. Store protected from light and moisture.

Norethisterone and Ethinyl Estradiol Tablets

Northindrone and Ethinyl Estradiol Tablets

Norethisterone and Ethinyl Estradiol Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of norethisterone $C_{20}H_{26}O_2$ and ethinyl estradiol $C_{20}H_{24}O_2$.

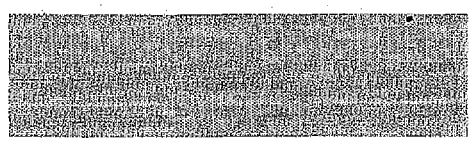
Usual strengths. Ethinyl Estradiol, 35 mcg and Norethisterone, 0.5 mg; Ethinyl Estradiol, 35 mcg and Norethisterone, 0.75 mg; Ethinyl Estradiol, 35 mcg and Norethisterone, 1.0 mg.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 40 volumes of *toluene* and 10 volumes of *ethyl acetate*.

Test solution. Powder one tablet, mix with 2 ml of *ethanol* in a centrifuge tube, and warm to 50° for 10 minutes with gentle swirling. Cool and centrifuge to obtain a clear solution.



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Reference solution. A solution containing 0.05 per cent w/v of *norethisterone IPRS* and 0.005 per cent w/v of *ethinyl estradiol IPRS* in *ethanol.*

Apply to the plate 40 µl of each solution. Allow the mobile phase to rise 10 cm, dry the plate in air and spray the plate with *sulphuric acid-methanol*, prepared by cautiously adding 70 ml of *sulphuric acid* in small increments to 30 ml of *methanol* chilled in an ice-bath, and mixing. The two principal spots in the chromatogram obtained with the test solution correspond to those in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

NOTE — Exercise care in filtering and handling solutions containing ethinyl estradiol to prevent adsorptive loss of the drug. Centrifugation may be used instead of filtration with non adsorptive membrane filters. Withdraw dissolution aliquots with glass or polytef pipets or syringes that have been checked for adsorptive loss. Use glass dissolution vessels and polytef-coated or solid polytef paddles.

Apparatus No. 2 (Paddle),
Medium. 500 ml of a buffer solution prepared by dissolving 0.45 g of *sodium lauryl sulphate* in 500 ml of *0.1 M hydrochloric acid*,
Speed and time. 75 rpm, 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *norethisterone IPRS* and *ethinyl estradiol IPRS* in the medium to obtain a solution having a known concentration similar to the test solution.

NOTE — A volume of methanol not exceeding 4.0 per cent of the total final volume of the reference solution may be used in preparing the reference solution.

Chromatographic system
- a stainless steel column 8.3 cm x 5 mm, packed with octadecyl silane bonded to porous silica (3µm),
- mobile phase: a mixture of 65 volumes of *0.02 M phosphate buffer* pH 6.0, and 35 volumes of *acetonitrile.*
- flow rate: 1 ml per minute,
- spectrophotometer set at 200 nm,
- injection volume: 100 µl.

The relative retention time with respect to ethinylestradiol for norethisterone is 0.9.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to norethisterone and ethinyl estradiol is not less than 1.5, the theoretical plates for the ethinyl estradiol peaks is not less than 7000, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 3.0 per cent for both peaks.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{26}O_2$, and $C_{20}H_{24}O_2$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{20}H_{26}O_2$, and $C_{20}H_{24}O_2$.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), using the chromatographic conditions as described under Assay.

Test solution. Transfer one tablet to a 10.0 ml volumetric flash, add 0.4 ml *water*, allow the tablet to stand for 2 minutes and swirl to disperse the tablet. Add 8 ml of *acetonitrile* and mix with the aid of ultrasound for 10 minutes. Cool and dilute to volume with *acetonitrile*. Shake to mix and centrifuge. Dilute 5.0 ml of the solution to 10.0 ml with solvent mixture and mix.

Reference solution (a). A 0.0035 per cent w/v solution of *ethinyl estradiol IPRS* in the *acetonitrile.*

Reference solution (b). A 0.05 per cent w/v solution of *norethisterone IPRS* in the *acetonitrile.*

Reference solution (c). Dilute reference solution (a) and reference solution (b) with the solvent mixture to obtain a solution having a known concentration similar to the test solution.

Inject reference solution (c) and the test solution.

Calculate the content of $C_{20}H_{26}O_2$, and $C_{20}H_{24}O_2$ in the tablets.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. A mixture of 45 volumes of *acetonitrile* and 55 volumes of *water.*

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.35 mg of ethinyl estradiol in *acetonitrile*, mix with the aid of ultrasound for 3 minutes. Cool and add 10.0 ml reference solution (a) and dilute to 100.0 ml with the same solvent. Centrifuge and dilute 5.0 ml with the solvent mixture to 10.0 ml.

Reference solution (a). Dissolve 15 mg of valerophenone in 125 ml of *acetonitrile,* and dilute to 250.0 ml with *water* and mix.

Reference solution (b). A 0.035 per cent w/v solution of *ethinyl estradiol IPRS in acetonitrile.*

Reference solution (c). A 0.05 per cent w/v solution of *norethisterone IPRS in acetonitrile.*

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Reference solution (d). Transfer 5.0 ml of each reference solution (a), reference solution (b) and reference solution (c) into a 100-ml volumetric flask to make a concentration similar to the test solution. Add (26 - X) ml of *acetonitrile*, X being the total volume of the, reference solution (b) and reference solution (c) taken. Dilute with the solvent mixture to volume, and mix.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecyl silane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of *acetonitrile* and 40 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 200 nm,
- injection volume: 25 µl.

The relative retention time with respect to ethinylestrodiol for norethisterone is 0.9.

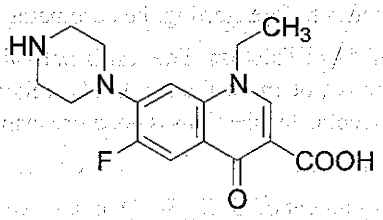
Inject reference solution (a) and (d). The test is not valid unless the resolution between the peaks due to norethisterone and ethinylestradiol is not less than 2.0 in the chromatogram obtained with reference solution (d), the theoretical plates are not less than 8000 in the chromatogram obtained with reference solution (a) and the relative standard deviation for replicate peaks is not more than 2.0 per cent for both the peaks in the chromatogram obtained with reference solution (d).

Inject reference solution (d) and the test solution.

Calculate the content of $C_{20}H_{26}O_2$, and $C_{20}H_{24}O_2$ in the tablets.

Storage. Store protected from light and moisture.

Norfloxacin



$C_{16}H_{18}FN_3O_3$ Mol. Wt. 319.3

Norfloxacin is 1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid.

Norfloxacin contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{16}H_{18}FN_3O_3$, calculated on the dried basis.

Category. Antibacterial.

Description. A white to pale yellow, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *norfloxacin IPRS* or with the reference spectrum of norfloxacin.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.0005 per cent w/v solution in *0.1 M sodium hydroxide* shows an absorption maximum at about 273 nm.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solution A. A mixture of 95 volumes of *water*, adjusted to pH 2.0 with *orthophosphoric acid* and 5 volumes of *acetonitrile*.

Test solution. Dissolve 20 mg of the substance under examination in 50.0 ml of solution A.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with solution A. Dilute 1.0 ml of the solution to 10.0 ml with solution A.

Reference solution (b). Dissolve 4 mg of *norfloxacin for system suitability IPRS* (containing norfloxacin impurities A, E and H) in 10.0 ml of solution A.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with end capped hexadecylamidylsilane bonded to porous silica (5 µm),
- column temperature: 60°,
- mobile phase: A. *water*, adjusted to pH 2.0 with *orthophosphoric acid*,
  B. *acetonitrile*,
- flow rate: 1.4 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 265 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
5	95	5
7	93	7
10	87	13
15	47	53
20	10	90
22	95	5

The relative retention time with reference to norfloxacin for norfloxacin impurity E (7-chloro-1-ethyl-4-oxo-6-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid)is about 0.97, for norfloxacin impurity A (7-chloro-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid) is about 1.5 and for

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norfloxacin impurity H (7-[4-(ethoxycarbonyl)piperazin-1-yl]-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid) is about 1.6.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to norfloxacin impurity A and norfloxacin impurity H is not less than 3.0 and peak-to-valley ratio is not less than 5.0, where H_p is the height above the baseline of the peak due to norfloxacin impurity E and H_v is the height above the baseline of the lowest point of the curve separating this peak from the peak due to norfloxacin.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of the peak due to norfloxacin impurity E is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 1.33 g complies with the limit test for heavy metals, Method B (15 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 0.5 g by drying in an oven at 100° at a pressure not exceeding 0.7 kPa.

Assay. Weigh 0.3 g and dissolve in 100 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25) and using a suitable anhydrous electrode system. (The electrode system may be rendered anhydrous by filling the electrode with *0.1 M lithium perchlorate* in *acetic anhydride* after removing any aqueous solution contained in it). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03193 g of $C_{16}H_{18}FN_3O_3$.

Storage. Store protected from light and moisture.

Norfloxacin Eye Drops

Norfloxacin Eye Drops are a sterile solution of Norfloxacin in Purified water.

Norfloxacin Eye Drops contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of norfloxacin, $C_{16}H_{18}FN_3O_3$.

Usual strength. 0.3 per cent w/v.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.6 to 5.5.

Other tests. Comply with the tests stated under Eye Drops.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a suitable volume of the eye drops with a 0.1 per cent v/v solution of *orthophosphoric acid* to produce a solution containing 0.005 per cent w/v of Norfloxacin.

Reference solution. A 0.005 per cent w/v solution of *norfloxacin IPRS* in 0.1 per cent v/v solution of *orthophosphoric acid*.

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (10 µm) (Such as Bondapack C18),
- column temerature. 50°,
- mobile phase: a mixture of 300 volumes of *methanol* and 700 volumes of 0.1 per cent v/v *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Precondition the column using *0.01 M anhydrous sodium dihydrogen orthophosphate*, adjusted to pH 4.0 with *orthophosphoric acid*, at a flow rate of 0.5 ml per minute for 8 hours. Equilibrate the column with the mobile phase for about 30 minutes before starting the chromatography.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0. The relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject alternately the test solution and the reference solution.

Calculate the content of $C_{16}H_{18}FN_3O_3$ in the eye drops.

Storage. Store protected from light.

Norfloxacin Tablets

Norfloxacin Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of norfloxacin, $C_{16}H_{18}FN_3O_3$.

Usual strengths. 200 mg; 400 mg; 800 mg.

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Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 40 volumes of *chloroform*, 40 volumes of *methanol*, 20 volumes of *toluene*, 14 volumes of *diethylamine* and 8 volumes of *water*.

Test solution. Shake a quantity of the finely powdered tablets containing 75 mg of Norfloxacin with 50 ml of a mixture of equal volumes of acidified *methanol* (containing 0.9 per cent v/v of *hydrochloric acid*) and *dichloromethane*, centrifuge and use the clear supernatant solution.

Reference solution. A 0.15 per cent w/v solution of *norfloxacin IPRS* in the same solvent mixture.

Apply to the plate 50 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to norfloxacin in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 750 ml of *acetate buffer pH 4.0*,

Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with *acetate buffer pH 4.0*, if necessary, at the maximum at about 278 nm (2.4.7). Concomitantly measure the absorbance of a solution of known concentration of *norfloxacin IPRS* in the same medium. Calculate the total content of $C_{16}H_{18}FN_3O_3$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{16}H_{18}FN_3O_3$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Add 80 ml of the mobile phase to a weighed quantity of the powdered tablets containing about 100 mg of Norfloxacin, mix with the aid of ultrasound for 10 minutes, dilute with a 0.1 per cent v/v solution of *orthophosphoric acid* to 200.0 ml and mix. Dilute 10.0 ml of the solution to 25.0 ml with the mobile phase, mix and filter.

Reference solution. A 0.02 per cent w/v solution of *norfloxacin IPRS* in the mobile phase.

Chromatographic system

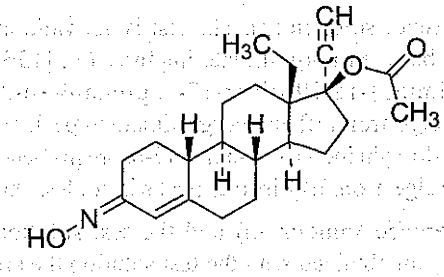
- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: 85 volumes of a 0.1 per cent v/v solution of *orthophosphoric acid* and 15 volumes of *acetonitrile*,
- temperature. column 40° ± 1°, after preconditioning with degassed *0.01 M sodium dihydrogen phosphate* adjusted to pH 4.0 with *phosphoric acid* flowing at a rate of 0.5 ml per minute for 8 hours,
- flow rate: 2 ml per minute,
- spectrophotometer set at 275 nm,
- injection volume: 20 µl.

Inject the reference solution the test solution. The assay is not valid unless the capacity factor is not less than 2, the column efficiency is not less than 1500 theoretical plates, the tailing factor for the norfloxacin peak is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Calculate the content of $C_{16}H_{18}FN_3O_3$ in the tablets.

Storage. Store protected from light and moisture.

Norgestimate



$C_{23}H_{31}NO_3$ Mol. Wt. 369.5

Norgestimate is (3EZ)-13α-Ethyl-3-(hydroxyimino)-18, 19-dinor-17α-pregn-4-en-20-yn-17-yl acetate.

Norgestimate contains not less than 98.5 per cent and not more than 101.5 per cent of $C_{23}H_{31}NO_3$, calculated on the dried basis.

Category. Progestogen.

Description. A white or almost white powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *norgestimate IPRS* or with the reference spectrum of norgestimate.

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Tests

Specific optical rotation (2.4.22). +42° to +50°, determined in a 1.0 per cent w/v solution in *methylene chloride*.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 1 volume of *water* and 4 volumes of *methanol*.

Test solution. Dissolve 25 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). Dilute 2.0 ml of the test solution to 100.0 ml with the solvent mixture. Further dilute 1.0 ml of the solution to 20.0 ml with the same solvent.

Reference solution (b). Dissolve 2 mg of *norgestimate impurity A IPRS* [13β-ethyl-3-oxo-18,19-dinor-17-α-pregn-4-en-20yn-17-yl acetate (levonorgestrel acetate)] in 4 ml of solvent mixture.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with end capped octadecylsilane silica gel (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 18 volumes of *acetonitrile*, 25 volumes of *tetrahydrofuran* and 60 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 244 nm,
- injection volume: 25 µl.

Inject reference solution (b). The test is not valid unless the resolution between norgestimate impurity (E) [13β-ethyl-3-(hydroxyimino)-18,19-dinor-17-α-pregn-4-en-20yn-17-ol((E))norelgestromin)] and norgestimate impurity (Z) [13β-ethyl-3-(hydroxyimino)-18,19-dinor-17-α-pregn-4-en-20yn-17-ol((Z)) norelgestromin)]- isomer peaks is not less than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of the peak corresponding to norgestimate impurity A is not more than twice the area of the corresponding peak norgestimate (E) and (Z) isomer multiplied with correction factor 1.33 in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any other secondary peak is not more than the area of the corresponding peak norgestimate (E) and (Z) isomer multiplied with correction factor 1.33 in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 3 times the area of the corresponding peak norgestimate (E) and (Z) isomer multiplied with correction factor 1.33 in the chromatogram obtained with reference solution (a) (0.3 per cent). Ignore any peak with an area less than 0.5 times the area of the corresponding peak norgestimate (E) and (Z) isomer multiplied with correction factor 1.33 in the chromatogram obtained with reference solution (a) (0.05 per cent).

Ratio of (E) to (Z) isomer. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modification.

Inject the test solution . Calculate the (E) to (Z) isomer ratio by dividing the area of the peak corresponding to the (E) isomer by 1.33 times the area of the peak due to the (Z) isomer. The ratio is 1.27 to 1.78.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 0.5 g by drying in an oven at 105° for 3 hours.

Assay. Weigh accurately about 0.3 g and dissolve in a mixture of 40.0 ml of *tetrahydrofuran* and 10 ml of *silver nitrate (10 per cent w/v)* and titrate with *0.1 M sodium hydroxide* determining the end-point potentiometrically (2.4.25). Record the volume used between the two inflections.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.03695 g of $C_{23}H_{31}NO_3$.

Norgestimate and Ethinyl Oestradiol Tablets

Norgestimate and Ethinyl Estradiol Tablets

Norgestimate and Ethinyl Oestradiol Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of norgestimate, $C_{23}H_{31}NO_3$, and ethinyl estradiol, $C_{20}H_{24}O_2$.

Category. Oral contraceptive.

Usual strengths. Norgestimate, 180 µg and Ethinyl Oestradiol, 35 µg; Norgestimate, 215 µg and Ethinyl Oestradiol, 35 µg; Norgestimate, 250 µg and Ethinyl Oestradiol, 35 µg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with reference solution (b).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Use mobile phase, test solution and reference solution (b) as described under Assay.

Chromatographic system

- a stainless steel column 5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 50 µl.

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Name	Relative retention time
Ethinyl Estradiol	0.5
(Z)- norgestimate	1.0
(E)- norgestimate	1.2

Inject reference solution (b). The test is not valid unless the resolution between the (z)- norgestimate and (e)- norgestimate peaks is not less than 1.5, and the relative standard deviation for replicate injections is not more than 2.0.

Inject the test solution. In the chromatogram obtained with the test solution the sum of the areas of impurity having relative retention of about 0.2 and 0.4 peaks is not more than 4.0 per cent multiplying 1.54 as correction factor calculated by area normalization.

Uniformity of content. Complies with the test stated under tablets.

Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Disperse one tablet in 20.0 ml of the *water*, with the aid of ultrasound for 5 minutes and dilute to 100.0 ml with *methanol*.

Reference solution (a). A 0.00018 per cent w/v solution of *norgestimate IPRS* in a 100-ml volumetric flask in a mixture of 20 ml of *water* and 80 ml of *methanol*.

Reference solution (b). A 0.000035 per cent w/v solution of *ethinyl oestradiol IPRS* in a 100-ml volumetric flask in a mixture of 20 ml of *water* and 80 ml of *methanol*.

Reference solution (c). Mix reference solution (a) and reference solution (b) to produce a solution having similar concentration to that of test solution in a mixture of 20 ml of *water* and 80 ml of *methanol*.

Chromatographic system as described under Assay.

Inject reference solution (c) and the test solution.

Calculate the contents of $C_{23}H_{31}NO_3$ and $C_{20}H_{24}O_2$ in the tablets.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 0.35 mg of Ethinyl Estradiol in a 50-ml volumetric flask, add 10 ml of *water* and 40 ml of reference solution (a), mix. Sonicate for 5 minutes and filter.

Reference solution (a). A 0.005 per cent w/v solution of *dibutyl phthalate IPRS* in *methanol*.

Reference solution (b). Weigh a quantity of *ethinyl estradiol IPRS* and *norgestimate IPRS* in reference solution (a) equivalent to 80 per cent of final volume and 20 per cent of *water*. Mix to obtain a known concentration of about 0.0007 per cent w/v solution of ethinyl estradiol and a known concentration of norgestimate similar to the expected concentration of test solution.

Chromatographic system

- a stainless steel column 5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 5 volumes of *tetrahydrofuran*, 2 volumes of *methanol* and 13 volumes of *water*,
- flow rate: 2.1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 25 µl.

Name	Relative retention time
Ethinyl Estradiol	0.5
(Z)- norgestimate	1.0
(E)- norgestimate	1.2
Dibutyl phthalate	1.5

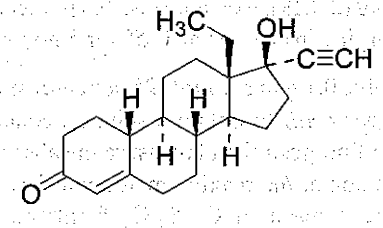
Inject reference solution (b). The test is not valid unless the resolution between the (z)- norgestimate and (e)- norgestimate peaks is not less than 1.5, and the relative standard deviation for replicate injections is not more than 2.0.

Inject reference solution (b) and the test solution.

Calculate the contents of $C_{23}H_{31}NO_3$ and $C_{20}H_{24}O_2$ in the tablets.

Storage. Store protected from moisture.

Norgestrel



$C_{21}H_{28}O_2$ Mol. Wt. 312.5

Norgestrel is *rac*-13-ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one.

Norgestrel contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{21}H_{28}O_2$, calculated on the dried basis.

Category. Progestogen.

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *norgestrel IPRS* or with the reference spectrum of norgestrel.

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B. When examined in the range 220 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *methanol* shows an absorption maximum only at about 240 nm.

C. Melting range (2.4.21). 205° to 212°, but the range between beginning and end of melting does not exceed 4°.

Tests

Optical rotation (2.4.22). −0.1° to + 0.1°, determined in a 5.0 per cent w/v solution in *chloroform*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 80 volumes of *dichloromethane* and 20 volumes of *ethyl acetate*.

Test solution. Dissolve 0.2 g of the substance under examination in 10 ml of *chloroform*.

Reference solution (a). A 0.01 per cent w/v solution of *norgestrel IPRS* in *chloroform*.

Reference solution (b). A 0.004 per cent w/v solution of *norgestrel IPRS* in *chloroform*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and spray with *phosphomolybdic acid solution*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than two such spots are more intense than the spot in the chromatogram obtained with reference solution (b).

Sulphated ash. (2.3.18) Not more than 0.3 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 5 hours.

Assay. Dissolve 0.1 g in *ethanol (95 per cent)* and dilute with *ethanol (95 per cent)* to obtain a solution containing 0.001 per cent w/v of norgestrel and measure the absorbance of the resulting solution at the maximum at about 241 nm, (2.4.7). Calculate the content of $C_{21}H_{28}O_2$ from the absorbance obtained with a 0.001 per cent w/v solution of *norgestrel IPRS* in *ethanol (95 per cent)*.

Storage. Store protected from moisture.

Norgestrel and Ethinyloestradiol Tablets

Norgestrel and Ethinyloestradiol Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of norgestrel, $C_{21}H_{28}O_2$ and ethinyloestradiol, $C_{20}H_{24}O_2$.

Category. Oral contraceptive.

Usual strength. Norgestrel, 300 µg and Ethinyloestradiol, 30 µg.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 96 volumes of *dichloromethane* and 4 volumes of *ethanol (95 per cent)*.

Test solution. Powder 20 tablets finely, triturate with 20 ml of *dichloromethane*, allow the solids to sediment and use the clear supernatant liquid.

Reference solution. A solution containing 0.06 per cent w/v solution of *norgestrel IPRS* and 0.006 per cent w/v solution of *ethinyloestradiol IPRS*.

Apply to the plate 40 µl of each solution. After development, dry the plate in air, spray with *ethanolic sulphuric acid (80 per cent v/v)*, heat at 110° for 10 minutes and examine under ultraviolet light at 365 nm. The principal spots in the chromatogram obtained with the test solution correspond to the spots for norgestrel (red fluorescence) and ethinylo-estradiol (orange-yellow fluorescence) in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 500 ml of 0.0005 per cent w/v of polysorbate 80,
Speed and time. 75 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter through 0.7-µm borosilicate microfiber filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *norgestrel IPRS* and *ethinyloestradiol IPRS* in *ethanol* (not more than 2 per cent of total volume) and dilute with the dissolution medium to obtain a solution of known concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 40 volumes of *acetonitrile* and 60 volumes *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 247 nm for norgestrel,

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– spectrofluorometer detector with an excitation wavelength of 285 nm and emission wavelength of 310 nm for ethinyloestradiol,
– injection volume: 100 µl.

The relative retention time with reference to norgestrel for ethinyloestradiol is about 0.7.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent for both the peaks.

Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{28}O_2$ and $C_{20}H_{24}O_2$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{21}H_{28}O_2$ and $C_{20}H_{24}O_2$.

Uniformity of content. Complies with the test stated under Tablets.

Carry out the procedure described under Assay but using the following test solution.

Test solution. Add 2.0 ml of *methanol (70 per cent)* and 2.0 ml of a 0.00002 per cent w/v solution of *diphenyl* in *methanol (70 per cent)* (internal standard solution) to one tablet, shake for 20 minutes, centrifuge, filter the supernatant liquid through a membrane filter with a pore size of not more than 0.2 mm and use the filtrate.

Calculate the contents of norgestrel $C_{21}H_{28}O_2$, and ethinyloestradiol, $C_{20}H_{24}O_2$, in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14) using the chromatographic system described under Uniformity of content.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder equivalent to one tablet add 2.0 ml of *methanol (70 per cent)* and 2.0 ml of a 0.00002 per cent w/v solution of *diphenyl* in *methanol (70 per cent)* (internal standard solution), shake for 20 minutes, centrifuge, filter the supernatant liquid through a membrane filter with a pore size of not more than 0.2 mm and use the filtrate.

Reference solution. A solution in *methanol (70 per cent)* containing 0.15 mg per ml of *norgestrel IPRS* and 0.015 mg per ml of *ethinyloestradiol IPRS*. Take 2.0 ml of the solution and add 2.0 ml of a 0.00002 per cent w/v solution of *diphenyl* in *methanol (70 per cent)* and use the resulting solution.

Chromatographic system
– a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 to 7 µm),
– mobile phase: a mixture of 35 volumes of *acetonitrile*, 15 volumes of *methanol* and 45 volumes of *water*,
– flow rate: 1 to1.5 ml per minute,
– spectrophotometer set at 215 nm,
– injection volume: 20 µl.

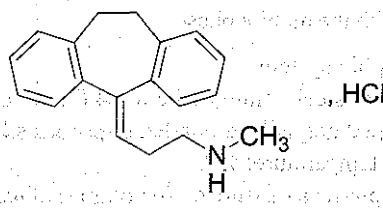
Inject the reference solution. The resolution between the two major peaks is not less 2.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution the test solution. The relative retention times are about 0.7 for ethinyloestradiol and about 1.0 for norgestrel.

Calculate the contents of norgestrel, $C_{21}H_{28}O_2$, and ethinyloestradiol, $C_{20}H_{24}O_2$ in the tablets.

Storage. Store protected from light.

Nortriptyline Hydrochloride



$C_{19}H_{21}N,HCl$ Mol. Wt. 299.8

Nortriptyline Hydrochloride is 3-(10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohept-5-ylidene)propyl(methyl)amine hydrochloride.

Nortriptyline Hydrochloride contains not less than 98.0 per cent and not more than 101.5 per cent of $C_{19}H_{21}N,HCl$, calculated on the dried basis.

Category. Antidepressant.

Description. A white to off-white powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and, D are carried out.

A. Dissolve 0.1 g in 10 ml of *water*, make alkaline with *1 M sodium hydroxide*, extract with 5 ml of *chloroform* and evaporate to dryness using a current of nitrogen.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nortriptyline hyrdochloride IPRS* treated in the same manner or with the reference spectrum of nortriptyline.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *methanol* shows an absorption maximum only at about 239 nm; absorbance at about 239 nm, about 0.48.

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C. To about 50 mg dissolved in 3 ml of warm *water*, add 1 drop of a 2.5 per cent w/v solution of *quinhydrone* in *methanol*; a red colour is produced after a few minutes (distinction from amitriptyline).

D. It gives the reactions of chlorides (2.3.1).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Protect the solutions from light.

Test solution. Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with endcapped octylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: a mixture of 70 volumes of *methanol* and 30 volumes of a solution prepared by dissolving 3.25 ml of 40 per cent w/v solution of *tetrabutylammonium hydroxide* and 0.68 g of *potassium dihydrogen phosphate* in 900 ml of *water*, adjusted to pH 7.5 with *orthophosphoric acid* and dilute to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

Name	Relative retention time	Correction factor
Nortriptyline impurity A[1]	0.5	—
Nortriptyline impurity D[2]	0.8	1.7
Nortriptyline impurity B[3]	0.9	-
Nortriptyline (Retention time: about 13 minutes)	1.0	---

[1]dibenzosuberone,

[2]5-[3-(dimethylamino)propyl]-10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-ol,

[3]norcyclobenzaprine.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to nortriptyline impurity D is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent), the area of any peak corresponding to nortriptyline impurity A is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Weigh 0.25 g and dissolve in 25 ml of *anhydrous glacial acetic acid*, warm slightly, if necessary, to effect solution. Cool, add 5 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02998 g of $C_{19}H_{21}N,HCl$.

Storage. Store protected from light and moisture.

Nortriptyline Tablets

Nortriptyline Hydrochloride Tablets

Nortriptyline Tablets contain Nortriptyline Hydrochloride equivalent to less than 90.0 per cent and not more than 110.0 per cent of the stated amount of nortriptyline, $C_{19}H_{21}N$. The tablets are coated.

Usual strengths. 10 mg; 25 mg.

Identification

A. Shake a quantity of the powdered tablets containing about 5 mg of nortriptyline with 20 ml of *methanol* and filter. To 1 ml of the filtrate add 1 ml of a 2.5 per cent w/v solution of *sodium bicarbonate*, 1 ml of a 2 per cent w/v solution of *sodium periodate* and 1 ml of a 0.3 per cent w/v solution of *potassium permanganate*. Allow to stand for 15 minutes, acidify with *1 M sulphuric acid* and extract with 10 ml of *2,2,4-trimethylpentane*.

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When examined in the range 230 nm to 360 nm (2.4.7), the resulting trimethylpentane solution shows an absorption maximum only at about 265 nm.

B. Triturate a quantity of the powdered tablets containing 0.1 g of nortriptyline with 10 ml of *chloroform*, filter and evaporate the filtrate to a low volume. Add *ether* until a turbidity is produced and allow to stand. Dissolve 50 mg of the precipitate in 3 ml of warm *water*, cool and add 1 drop of a 2.5 per cent w/v solution of *quinhydrone* in *methanol*; a red colour is produced after a few minutes (distinction from amitriptyline).

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 85 volumes of *cyclohexane*, 15 volumes of *ethyl acetate* and 3 volumes of *diethylamine*.

Test solution. Extract a quantity of the powdered tablets containing 20 mg of nortriptyline with 5 ml of a mixture of 9 volumes of *ethanol (95 per cent)* and 1 volume of *2 M hydrochloric acid*, centrifuge and use the supernatant liquid.

Reference solution. A 0.001 per cent w/v solution of *dibenzosuberone IPRS* in *ethanol (95 per cent)* prepared in subdued light.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 14 cm in an unsaturated tank protected from light. Dry the plate in air, spray with a freshly prepared solution of *sulphuric acid* containing 4 per cent v/v of *formaldehyde solution* and examine immediately under ultraviolet light at 365 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14).

Test solution. Transfer one tablet to 100.0 ml volumetric flask, add about 5 ml of *water* and disperse with the aid of ultrasound. Add about 50.0 ml of *methanol*, mix with the aid of ultrasound for 30 minutes and dilute to 100.0 ml with *water*. Centrifuge and use the supernatant liquid, dilute if necessary.

Reference solution. A 0.01 per cent w/v solution of *nortriptyline hydrochloride IPRS* in *methanol (50 per cent)*.

Follow the procedure given in the Assay. Calculate the content of $C_{19}H_{21}N$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Shake vigorously 20 tablets with 50 ml of *water* until the tablets disintegrate completely, add 100.0 ml of *methanol* and shake for 30 minutes. Add sufficient *water* to produce 200.0 ml, filter and dilute a volume of the filtrate containing about 25 mg of nortriptyline to 100.0 ml with *methanol (50 per cent)*.

Reference solution. A 0.025 per cent w/v solution of *nortriptyline hydrochloride IPRS* in *methanol (50 per cent)*.

Chromatographic system
- a stainless steel column 20 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a 0.56 per cent w/v solution of *sodium hexanesulphonate* in a mixture of equal volumes of *water* and *acetonitrile* adjusted to pH 4.5 with *glacial acetic acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 239 nm,
- injection volume: 20 µl.

Inject the reference solution the test solution.

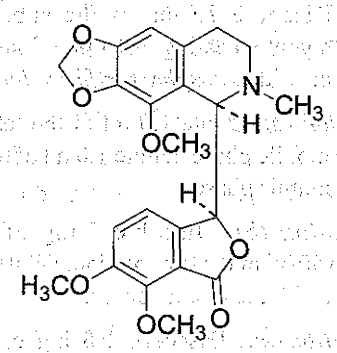
Calculate the content of $C_{19}H_{21}N$ in the tablets.

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of equivalent amount of nortriptyline.

Noscapine

Narcotine



$C_{22}H_{23}NO_7$ Mol. Wt. 413.4

Noscapine is (3*S*)-6,7-dimethoxy-3-[(5*R*)-5,6,7,8-tetrahydro-4-methoxy-6-methyl-1,3-dioxolo[4,5-*g*]isoquinolin-5-yl]phthalide, an alkaloid obtained from opium.

Noscapine contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{22}H_{23}NO_7$, calculated on the dried basis.

Category. Cough suppressant.

Description. Colourless crystals or a white crystalline powder.

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Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *noscapine IPRS* or with the reference spectrum of noscapine.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.005 per cent w/v solution in *methanol* shows absorption maxima at about 291 nm and 310 nm; ratio of absorbance at the maximum at about 310 nm to that at the maximum at about 291 nm, 1.2 to 1.3.

C. To 0.1 g in a porcelain dish add a few drops of *sulphuric acid* and stir; a greenish-yellow solution is formed which on warming becomes red and finally violet.

D. Dissolve 50 mg in 5 ml of *5 M hydrochloric acid*, add 10 ml of a mixture of equal volumes of *ethanol (95 per cent)* and a saturated solution of *sodium acetate*, mix and allow to stand for about 3 minutes; shining crystals separate.

Tests

Appearance of solution. A 2.0 per cent w/v solution in *acetone* examined immediately after preparation is clear (2.4.1), and not more intensely coloured than reference solution YS6 (2.4.1).

Specific optical rotation (2.4.22). + 42.0° to + 48.0°, determined at 20° in a solution prepared by dissolving 0.5 g in sufficient *0.1 M hydrochloric acid* to produce 25.0 ml.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination with gentle heating in 14 ml of *methanol*, cool, and dilute to 20 ml with *phosphate buffer solution pH 6.0*.

Reference solution (a). Dilute 1.0 ml of the test solution to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *papaverine hydrochloride* in 50 ml of the test solution. Dilute 1.0 ml of the solution to 20 ml with the mobile phase.

Reference solution (c). Dissolve 1.5 mg of *papaverine hydrochloride* in 10 ml of the test solution and dilute to 50 ml with the mobile phase.

Chromatographic system
- a stainless steel column 12.5 cm x 4.6 mm, packed with nitrile groups chemically bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of *methanol* and 65 volumes of *phosphate buffer pH 6.0*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to noscapine and papaverine (noscapine impurity A) is not less than 2.0. The relative retention time with reference to noscapine for noscapine impurity A is about 1.3.

Inject reference solution (a), (b) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of the peak corresponding to noscapine impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of the any other secondary peak is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), and the sum of the areas of secondary peaks other than noscapine impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Morphine. Dissolve 0.1 g in 10 ml of *0.1 M hydrochloric acid*. To 1 ml of the resulting solution add a mixture of 1 ml of *potassium ferricyanide solution*, 0.05 ml of *ferric chloride test solution* and 4 ml of *water*; no blue or dark green colour develops within 1 minute.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh 0.35 g, dissolve in 40 ml of *anhydrous glacial acetic acid*, warming gently. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.04134 g of $C_{22}H_{23}NO_7$.

Storage. Store protected from light and moisture.

Noscapine Linctus

Narcotine Linctus

Noscapine Linctus is a solution of Noscapine in a suitable flavoured vehicle. It may contain up to 1 per cent w/v solution of Citric Acid.

Noscapine Linctus contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of noscapine, $C_{22}H_{23}NO_7$.

Usual strength. 7 mg per 5 ml.

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Identification

To a quantity containing 60 mg of Noscapine add 20 ml of *water*, 2 g of *sodium chloride* and 2 ml of *5 M sodium hydroxide*. Extract with successive quantities of 50, 50, 25 and 25 ml of *ether*. Combine the extracts, wash with three quantities, each of 5 ml, of *water* and evaporate to dryness. Dissolve the residue in 20 ml of *chloroform*. Wash with three quantities, each of 20 ml, of *water*, dry the chloroform layer with *anhydrous sodium sulphate*, filter and evaporate the solvent. If necessary, induce crystallisation by scratching with a glass rod. The crystals comply with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *noscapine IPRS* or with the reference spectrum of noscapine.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.005 per cent w/v solution in *methanol* shows absorption maxima at about 291 nm and 310 nm; ratio of absorbance at the maximum at about 310 nm to that at the maximum at about 291 nm, 1.2 to 1.3.

Tests

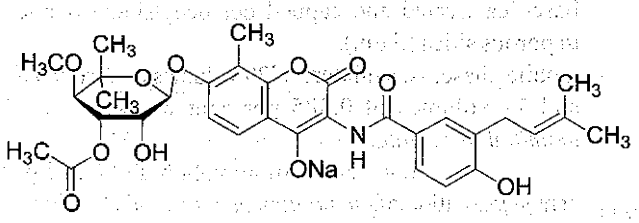
Other tests. Comply with the tests stated under Oral Liquids.

Assay. Weigh a quantity containing 60 mg of Noscapine, add 20 ml of *water*, 2 g of *sodium chloride* and 2 ml of *5 M sodium hydroxide* and extract with successive quantities of 50, 50, 25 and 25 ml of *ether*. Combine the extracts, wash with three quantities, each of 5 ml, of *water* and evaporate to dryness. To the residue add 50.0 ml of *0.1 M hydrochloric acid*, warm on a water-bath to dissolve and to remove any traces of ether and dilute to 100.0 ml with *water*. Dilute 3.0 ml to 50.0 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 310 nm (2.4.7). Calculate the content of $C_{22}H_{23}NO_7$ taking 90.7 as the specific absorbance at 310 nm.

Determine the weight per ml of the linctus (2.4.29), and calculate the content of $C_{22}H_{23}NO_7$, weight in volume.

Storage. Store protected from light and moisture.

Novobiocin Sodium



$C_{31}H_{35}N_2NaO_{11}$ Mol. Wt. 634.6

Novobiocin Sodium is the monosodium salt of novobiocin, *N*-[7-{3-*O*-(aminocarbonyl)-6-deoxy-5-*C*-methyl-4-*O*-methyl-β-L-*lyxo*-hexopyranosyl}-oxy-4-hydroxy-8-methyl-2-oxo-2*H*-1-benzopyran-3-yl]-4-hydroxy-3-(3-methyl-2- butenyl)-benzamide, an antimicrobial substance produced by the growth of certain strains of *Streptomyces niveus* or related organisms or by other means.

Novobiocin Sodium contains the equivalent of not less than 850 µg of novobiocin per mg, calculated on the dried basis.

Category. Antibacterial.

Description. A white or yellowish white, crystalline powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G F254*.

Mobile phase. A mixture of 75 volumes of *chloroform*, 25 volumes of *methanol* and 1 volume of *strong ammonia solution*.

Test solution. Dissolve a quantity of the substance under examination in *methanol* so as to obtain a solution containing 0.1 per cent w/v solution of novobiocin.

Reference solution. A 0.1 per cent w/v solution of *novobiocin IPRS* in *methanol*.

Apply to the plate 1 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in a 0.4 per cent w/v solution of *potassium hydroxide* shows an absorption maximum only at about 307 nm.

C. The residue obtained by igniting it gives the tests for sodium salts (2.3.1).

Tests

pH (2.4.24). 6.6 to 8.5, determined in a 2.5 per cent w/v solution.

Specific optical rotation (2.4.22). −58.0° to −50.0°, determined in a 5.0 per cent w/v solution in *methanol* containing 1 per cent v/v of *hydrochloric acid*.

Loss on drying (2.4.19). Not more than 6 per cent, determined on 0.2 g by drying in an oven at 60° over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa for 3 hours.

Assay. Determine by the microbiological assay of antibiotics, Method A (2.2.10), and express the results in µg of novobiocin per mg.

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Novobiocin Sodium intended for use in the manufacture of Parenteral Preparations complies with the following additional requirements.

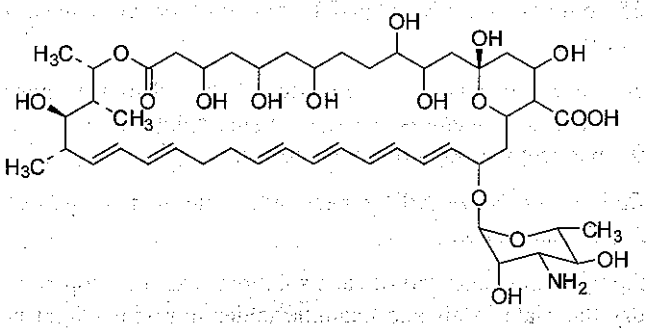
Bacterial endotoxins (2.2.3). Not more than 0.7 Endotoxin units per mg.

Sterility. Complies with the test for sterility (2.2.11).

Storage. Store protected from light and moisture at a temperature not exceeding 30°. If it is intended for use in the manufacture of parenteral preparations, the container should be sterile and sealed so as to exclude micro-organisms.

Labelling. The label states whether or not the contents are intended for use in the manufacture of parenteral preparations.

Nystatin



$C_{47}H_{75}NO_{17}$ Mol. Wt. 926.1

Nystatin is an antifungal substance produced by the growth of certain strains of *Streptomyces noursei* or by any other means. It consists mainly of polyenes, the principal component being nystatin A_1.

Nystatin has a potency of not less than 4400 Units per mg, calculated on the dried basis.

Category. Antifungal.

Description. A yellow to slightly brown powder; hygroscopic.

Identification

A. When examined in the range 220 nm to 360 nm (2.4.7), the final solution obtained in the test for Light absorption shows absorption maxima at about 230 nm, 291 nm, 305 nm and 319 nm. The ratios of the absorbances at the maxima at about 291 nm and about 319 nm to the absorbance at the maximum at about 305 nm are 0.61 to 0.73 and 0.83 to 0.96, respectively. Use as the blank a solution prepared in the same manner without the substance under examination.

B. Shake 30 mg with 5 ml of *water* for 2 minutes, add 2 ml of *sodium molybdotungstophosphate solution*, and allow to stand for 1 hour; the green colour produced is darker than that produced by repeating the test without the substance under examination.

C. Shake 30 mg with 5 ml of *water* for 2 minutes, add 2 ml of a solution prepared by dissolving 0.1 g of *pyrogallol* in 100 ml of *decolorised magenta solution*, heat on a water-bath until a dark pink colour is produced, cool and allow to stand for 1 hour; the pink colour is retained.

D. To 2 mg add 0.1 ml of *hydrochloric acid*; a brown colour is produced.

E. To 2 mg add 0.1 ml of *sulphuric acid*; a brown colour is produced which becomes violet on standing.

Tests

pH (2.4.24). 6.5 to 8.0, determined in a 3.0 per cent w/v suspension in *water*.

Light absorption (2.4.7). Dissolve 0.1 g in a mixture of 5.0 ml of *glacial acetic acid* and 50 ml of *methanol*, add sufficient *methanol* to produce 100.0 ml and dilute 1.0 ml of the resulting solution to 100.0 ml with *methanol*. Absorbance of the resulting solution, measured within 30 minutes of preparation, at the maximum at about 305 nm, not less than 0.60. Use as the blank a solution prepared in the same manner without the substance under examination.

Composition. Determine by liquid chromatography (2.4.14).

NOTE—Carry out the test protected from light.

Test solution. Dissolve 20 mg of the substance under examination in 50 ml of *dimethylsulphoxide*.

Reference solution (a). A 0.04 per cent w/v solution of *nystatin IPRS* in *dimethylsulphoxide*.

Reference solution (b). Dissolve 20 mg of the substance under examination in 25 ml of the *methanol* and dilute to 50 ml with *water*. To 10.0 ml of the solution add 2.0 ml of *dilute hydrochloric acid*. Allow to stand at room temperature for 1 hour.

Reference solution (c). Dilute 1.0 ml of reference solution (a) in 100.0 ml with *dimethylsulphoxide*. Dilute 1.0 ml of the solution to 10.0 ml with *dimethylsulphoxide*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with base-deactivated end capped octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 29 volumes of *acetonitrile* and 71 volumes of 0.385 per cent w/v solution of *ammonium acetate*,

  B. a mixture of 40 volumes of 0.385 per cent w/v solution of *ammonium acetate* and 60 volumes of *acetonitrile*.
- a gradient programme using the conditions given below,

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- flow rate: 1 ml per minute,
- spectrophotometer set at 305 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent w/v)	Mobile phase B (per cent w/v)
0	100	0
25	100	0
35	0	100
45	0	100
50	100	0
55	100	0

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 3.5.

Inject reference solution (a), (c) and the test solution. In the chromatogram obtained with the test solution the area of the peak due to nystatin A1 is not less than 85 per cent the area of the principal peak in the chromatogram obtained with reference solution (a), the area of the any other compound is not more than 4.0 per cent the area of the principal peak in the chromatogram obtained with reference solution (a). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 3.5 per cent.

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g by drying in an oven at 60° at a pressure not exceeding 0.1 kPa for 3 hours.

Assay *NOTE— Protect the solution from light throughout the assay.*

Weigh 75 mg and dissolve in sufficient *dimethylformamide* to produce 50.0 ml; dilute 10.0 ml of the resulting solution to 200.0 ml with *buffer solution No 4* (2.2.10).

Determine by the microbiological assay of antibiotics (2.2.10).

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of the number of Units of Nystatin per mg.

Nystatin Ointment

Nystatin Ointment is a dispersion of Nystatin in microfine powder in a suitable ointment base.

Nystatin Ointment contains not less than 90.0 per cent and not more than 130.0 per cent of the stated number of Units of nystatin.

Usual strength. 100,000 Units per g.

Identification

Disperse a quantity containing 25,000 Units in 10 ml of *chloroform*, add 40 ml of *methanol* and shake. Filter and dilute 1 ml of the filtrate to 25 ml with *methanol*.

When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows absorption maxima at about 291 nm, 305 nm and 319 nm. The ratios of the absorbances at the maxima at about 291 nm and about 319 nm to the absorbance at the maximum at about 305 nm are 0.61 to 0.73 and 0.83 to 0.96, respectively. Use as the blank a solution prepared exactly in the same manner without the substance under examination.

Tests

Other tests. Comply with the tests stated under Ointments.

Assay. *Protect the solution from light throughout the assay.*

Weigh a quantity containing 400,000 Units, disperse in 20 ml of *ether* in a stoppered flask, add 70 ml of *dimethylformamide*, shake for a few minutes, add 10 ml of *water*, shake vigorously for a few minutes and add sufficient *dimethylformamide* to produce 100.0 ml. Mix well, filter and dilute 10.0 ml of the filtrate to 100.0 ml with *buffer solution No 4* (2.2.10).

Determine by the microbiological assay of antibiotics (2.2.10).

Storage. Store protected from moisture.

Labelling. The label states the strength in terms of the number of Units of Nystatin per g.

Nystatin Pessaries

Nystatin Vaginal Tablets

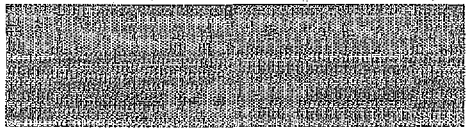
Nystatin Pessaries contain not less than 90.0 per cent and not more than 130.0 per cent of the stated number of Units of nystatin.

Usual strength. 100,000 Units.

Identification

Extract a quantity of the powdered pessaries containing 300,000 Units with a mixture of 50 ml of *methanol* and 5 ml of *glacial acetic acid*, add sufficient *methanol* to produce 100 ml and filter. Dilute 1 ml of the filtrate to 100 ml with *methanol*. The resulting solution complies with the follwing test.

Disperse a quantity containing 25,000 Units in 10 ml of *chloroform*, add 40 ml of *methanol* and shake. Filter and dilute 1 ml of the filtrate to 25 ml with *methanol*.



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When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows absorption maxima at about 291 nm, 305 nm and 319 nm. The ratios of the absorbances at the maxima at about 291 nm and about 319 nm to the absorbance at the maximum at about 305 nm are 0.61 to 0.73 and 0.83 to 0.96, respectively. Use as the blank a solution prepared exactly in the same manner without the substance under examination.

Tests

Other tests. Comply with the tests stated under Pessaries.

Loss on drying (2.4.19). Not more than 5 per cent, determined on 1.0 g of the powdered pessaries by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 3 hours.

Assay. *NOTE— Protect the solution from light throughout the assay.*

Weigh and powder 20 pessaries. Weigh a quantity of the powder containing 200,000 Units and shake with 50.0 ml of *dimethylformamide* for 1 hour. Centrifuge, dilute 10.0 ml of the clear, supernatant liquid to 200.0 ml with *buffer solution No 4* (2.2.10).

Determine by the microbiological assay of antibiotics (2.2.10).

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of the number of Units of Nystatin.

Nystatin Tablets

Nystatin Tablets contain not less than 90.0 per cent and not more than 130.0 per cent of the stated number of Units of nystatin. The tablets are coated.

Usual strength. 500,000 Units.

Identification

Extract a quantity of the powdered tablets containing 300,000 Units with a mixture of 50 ml of *methanol* and 5 ml of *glacial acetic acid*, add sufficient *methanol* to produce 100 ml and filter. Dilute 1 ml of the filtrate to 100 ml with *methanol*. The resulting solution complies with the follwing test.

When examined in the range 230 nm to 360 nm (2.4.7), the solution shows absorption maxima at about 291 nm, 305 nm and 319 nm. The ratios of the absorbances at the maxima at about 291 nm and about 319 nm to the absorbance at the maximum at about 305 nm are 0.61 to 0.73 and 0.83 to 0.96, respectively. Use as the blank a solution prepared exactly in the same manner without the substance under examination.

Tests

Disintegration (2.5.1). 30 minutes, but using a 0.6 per cent v/v solution of *hydrochloric acid* in place of *water*. If the tablets fail to disintegrate, wash them rapidly by immersion in *water* and continue the test using *phosphate buffer pH 6.8*; the tablets then disintegrate within a further 30 minutes.

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g of the powdered tablets by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 3 hours.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 200,000 Units and shake with 50.0 ml of *dimethylformamide* for 1 hour. Centrifuge, dilute 10.0 ml of the clear, supernatant liquid to 200.0 ml with *buffer solution No 4* (2.2.10).

Determine by the microbiological assay of antibiotics (2.2.10).

Storage. Store protected from moisture.

Labelling. The label states the strength in terms of the number of Units of Nystatin.

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O

Octyldodecanol 3099
Oestradiol Benzoate 3099
Oestradiol Injection 3100
Ofloxacin 3101
Ofloxacin Infusion 3102
Ofloxacin Opthalmic Solution 3102
Ofloxacin Oral Suspension 3103
Ofloxacin Tablets 3103
Ofloxacin and Ornidazole Tablets 3104
Olanzapine 3105
Olanzapine Tablets 3106
Olanzapine and Fluoxetine Tablets 3107
Oleic Acid 3108
Olmesartan Medoxomil 3109
Olmesartan Medoxomil Tablets 3110
Olmesartan Medoxomil and Hydrochlorothiazide Tablets 3112
Olopatadine Hydrochloride 3113
Olopatadine Ophthalmic Solution 3114
Olopatadine Tablets 3116
Omeprazole 3116
Omeprazole Gastro-resistant Capsules 3118
Omeprazole and Domperidone Capsules 3119
Ondansetron 3120
Ondansetron Hydrochloride 3121
Ondansetron Injection 3122
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Ormeloxifene Hydrochloride 3129
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Ornidazole Injection 3131
Ornidazole Tablets 3132
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Oxcarbazepine 3149
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Oxybutynin Prolonged-release Tablets 3154
Oxybutynin Tablets 3155
Oxygen 3156
Oxygen 93 Per cent 3157
Oxymetazoline Hydrochloride 3157
Oxymetazoline Hydrochloride Nasal Solution 3158
Oxytetracycline 3159
Oxytetracycline Injection 3161
Oxytetracycline Hydrochloride 3162
Oxytetracycline Capsules 3164
Oxytetracycline Eye Ointment 3165
Oxytetracycline Hydrochloride Injection 3166
Oxytocin 3167
Oxytocin Injection 3169
Oxytocin Nasal Solution 3170
Ozagrel Hydrochloride 3171

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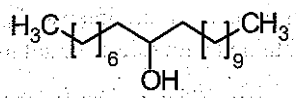
Octyldodecanol



$C_{20}H_{42}O$ Mol. Wt. 298.6

Octyldodecanol is 2-octyl-1-dodecanol.

Octyldodecanol contains not less than 90 per cent of $C_{20}H_{42}O$, the remainder consisting mainly of related alcohols.

Category. Pharmaceutical aid.

Description. A clear, colourless or yellowish, oily liquid.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 5 volumes of *ethyl acetate* and 95 volumes of *toluene*.

Test solution. Dissolve 0.2 g of the substance under examination in *toluene* and dilute to 20.0 ml with *toluene*.

Reference solution. A 1.0 per cent w/v solution of *octyldodecanol IPRS* in *toluene*.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in air and spray with about 7 ml of a mixture of 1 volume of a 2.5 per cent w/v solution of *vanillin* in *ethanol (95 per cent)* and 4 volumes of *sulphuric acid* and heat at 130° for 10 minutes. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Acidity or alkalinity. Mix 5.0 g thoroughly for 1 minute with a mixture of 0.1 ml of *bromothymol blue solution*, 2 ml of *heptane* and 10 ml of *water*. If the aqueous layer is blue, not more than 0.15 ml of *0.01 M hydrochloric acid* is required to change the colour of the indicator to yellow. If the aqueous layer is yellow, add 0.45 ml of *0.01 M sodium hydroxide* and shake vigorously. After standing to ensure complete separation, the aqueous layer is blue.

Relative density (2.4.29). About 0.84.

Refractive index (2.4.27). About 1.455.

Optical rotation (2.4.22). – 0.10° to + 0.10°, determined in a 10.0 per cent w/v solution in *ethanol (95 per cent)*.

Hydroxyl value (2.3.27). 175 to 190, using Method A.

Iodine value (2.3.28). Not more than 8.0, using Method A.

Peroxide value (2.3.35). Not more than 5.0.

Saponification value (2.3.37). Not more than 5.0.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.5 per cent, determined on 2.0 g.

Assay. Determine by gas chromatography (2.4.13).

Internal standard solution. Dissolve 0.4 g of *tetradecane* in *hexane* and dilute to 100.0 ml with *hexane*.

Test solution. Dissolve 0.1 g of the substance under examination in the internal standard solution and dilute to 10.0 ml with the internal standard solution.

Reference solution. A 1.0 per cent w/v solution of *octyldodecanol IPRS* with internal standard solution.

Chromatographic system

- a stainless steel column 60 m x 0.25 mm, packed with poly(dimethyl)(diphenyl)(divinyl)siloxane (film thickness 0.25 µm),
- temperature:
- temperature:

column	Time (in min.)	Temperature (°)
	0 - 2	180
	2 - 22	180→280
	22 - 52	280

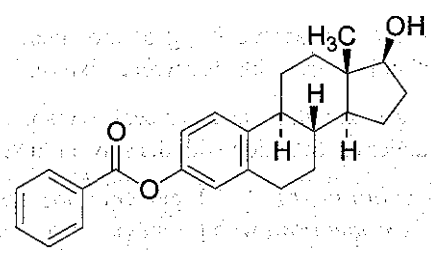
- inlet port at 290° and detector at 300°,
- flame ionization detector,
- flow rate: 0.68 ml per minute, using helium or nitrogen as the carrier gas.
- split ratio: 1:50.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{42}O$.

Storage. Store protected from light.

Oestradiol Benzoate



$C_{25}H_{28}O_3$ Mol. Wt. 376.5

Oestradiol Benzoate is 17β-hydroxyestra-1,3,5(10)-trien-3-yl benzoate.

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Oestradiol Benzoate contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{25}H_{28}O_3$, calculated on the dried basis.

Category. Oestrogen.

Description. Colourless crystals or a white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Test C may be omitted if tests A and B are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oestradiol benzoate IPRS* or with the reference spectrum of oestradiol benzoate.

B. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (b) when examined in daylight and under ultraviolet light at 365 nm.

C. To about 1 mg add 0.5 ml of a 5 per cent w/v solution of *ammonium molybdate* in *sulphuric acid*; a yellowish green colour develops which exhibits an intense green fluorescence when examined under ultraviolet light at 365 nm. Add 1 ml of *sulphuric acid* and 9 ml of *water*; the solution becomes pink with a yellowish fluorescence.

Tests

Specific optical rotation (2.4.22). + 57.0° to + 63.0°, determined in a 1.0 per cent w/v solution in *dioxan*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. 90 volumes of *chloroform* and 10 volumes of *methanol*.

Mobile phase. A mixture of 90 volumes of *toluene* and 10 volumes of *ethanol (95 per cent)*.

Test solution (a). Dissolve 0.2 g of the substance under examination in 10.0 ml with the solvent mixture.

Test solution (b). Dissolve 0.1 g of the substance under examination in 100.0 ml with the solvent mixture.

Reference solution (a). A 0.02 per cent w/v solution of the substance under examination with the solvent mixture.

Reference solution (b). A 0.1 per cent w/v solution of *oestradiol benzoate IPRS* with the solvent mixture.

Apply to the plate 5 µl of each solution. After development, dry the plate in air until odour of the solvent is no longer detectable, heat at 110° for 10 minutes, spray the plate while hot with *ethanolic sulphuric acid (20 per cent)*, heat again at 110° for 10 minutes and examine under ultraviolet light at 365 nm. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a).

Sulphated ash (2.3.18). Not more than 0.2 per cent, determined on 0.5 g.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 0.5 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 25 mg in sufficient *ethanol (95 per cent)* to produce 250.0 ml. Dilute 10.0 ml to 100.0 ml with *ethanol (95 per cent)* and measure the absorbance of the resulting solution at the maximum at about 231 nm (2.4.7). Calculate the content of $C_{25}H_{28}O_3$ taking 500 as the specific absorbance at 231 nm.

Storage. Store protected from light and moisture.

Oestradiol Injection

Oestradiol Benzoate Injection

Oestradiol Injection is a sterile solution of Oestradiol Benzoate in Ethyl Oleate or other suitable ester, in a suitable fixed oil or in any mixture of these. It may contain suitable alcohols.

Oestradiol Benzoate Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of oestradiol benzoate, $C_{25}H_{28}O_3$.

Usual strength. 1 mg per ml.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 80 volumes of *toluene* and 20 volumes of *ethyl acetate*.

Test solution. Add 10 ml of *2,2,4-trimethylpentane* to a volume of the injection containing 2 mg of Oestradiol Benzoate and extract with three quantities, each of 10 ml, of *ethanol (70 per cent)*. Wash the combined extracts with 15 ml of *2,2,4-trimethylpentane*, evaporate the ethanolic extract to dryness using a rotary evaporator and dissolve the residue in 2 ml of *chloroform*.

Reference solution. A 0.1 per cent w/v solution of *oestradiol benzoate IPRS* in *chloroform*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air, spray with *ethanolic sulphuric acid (20 per cent)*, heat at 105° for 10 minutes and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

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Tests

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution (a). Dilute a measured quantity of the injection containing about 1 mg of Oestradiol Benzoate to 10.0 ml with a mixture of 90 volumes of *cyclohexane* and 10 volumes of *dioxan*.

Test solution (b). Add 1 ml of a solution prepared by dissolving 15 mg of *4-hydroxybenzaldehyde* (internal standard) in 10.0 ml of *dioxan*, adding sufficient *cyclohexane* to produce 100.0 ml (solution A), to a measured quantity of the injection containing about 1 mg of Oestradiol Benzoate and dilute to 10.0 ml with sufficient of a mixture of 90 volumes of *cyclohexane* and 10 volumes of *dioxan*.

Reference solution. Add 10 ml of solution A to 10 mg of *oestradiol benzoate IPRS*, weighed, and dilute to 100.0 ml with the same solvent mixture.

Chromatographic system
- a stainless steel column 30 cm x 4 mm, packed with porous silica particles (10 µm),
- mobile phase: a mixture of 90 volumes of *cyclohexane* and 10 volumes of *dioxan*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject test solution (a) for identification of oestradiol benzoate peak.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to oestradiol benzoate and the internal standard is not less than 1.5.

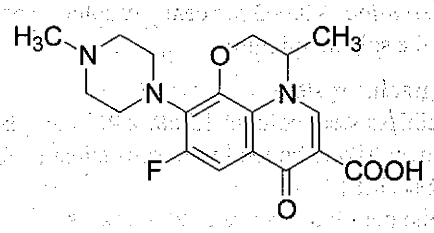
Inject the reference solution and test solution (b).

Calculate the content of $C_{25}H_{28}O_3$ in the injection.

Storage. Store protected from light.

Labelling. The label states (1) the nature and composition of the solvent; (2) that it is meant for intramuscular injection only; (3) that any solid matter that may have separated on standing should be redissolved by warming before use.

Ofloxacin



$C_{18}H_{20}FN_3O_4$ Mol. Wt. 361.4

Ofloxacin is (*RS*)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3,-*de*]-1,4-benzoxazine-6-carboxylic acid.

Ofloxacin contains not less than 98.5 per cent and not more than 101.5 per cent of $C_{18}H_{20}FN_3O_4$, calculated on the dried basis.

Category. Antibacterial.

Description. A pale yellow or bright yellow, crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ofloxacin IPRS* or with the reference spectrum of ofloxacin.

Tests

Light absorption. Absorbance at 440 nm (2.4.7), of 0.5 per cent w/v solution in *0.1 M hydrochloric acid* is not more than 0.25.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 10 mg of the substance under examination in 10.0 ml of *methanol*.

Reference solution (a). A 0.1 per cent w/v solution of *ofloxacin IPRS* in *methanol*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *methanol*.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 10 volumes of *acetonitrile* and 90 volumes of *phosphate buffer pH 2.4* prepared by dissolving 2.72 g of *monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 2.4 with *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 294 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the column efficiency in not less than 1400 theoretical plates.

Inject reference solution (b) and the test solution. Run the chromatogram three times of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent).

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Heavy Metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.2 per cent, determined on 1.0 g by drying in an oven at 105° for 4 hours.

Assay. Dissolve 0.3 g in 100 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid,* determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03614 g of $C_{18}H_{20}FN_3O_4$.

Storage. Store protected from light and moisture.

Ofloxacin Infusion

Ofloxacin Infusion is a sterile solution of Ofloxacin in 5 per cent Dextrose Injection or in Sodium Chloride Injection.

Ofloxacin Infusion contains not less than 90.0 per cent and not more than 120.0 per cent of the stated amount of ofloxacin, $C_{18}H_{20}FN_3O_4$.

Usual strengths. 25 mg; 50 mg; 100 mg; 200 mg; 400 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 3.8 to 7.5.

Bacterial endotoxins (2.2.3). Not more than 0.88 Endotoxin Units per mg of Ofloxacin.

Other tests. Comply with the tests stated under Parenteral Preparation (Infusions).

Assay. Determine by liquid chromatography (2.4.14).

NOTE —Protect the solutions from the light.

Test solution. Measure a volume containing 50 mg of Ofloxacin in 100.0 ml with mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with mobile phase.

Reference solution. A 0.005 per cent w/v solution of *ofloxacin IPRS* in mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 80 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* and 0.47 g *sodium 1-hexane sulphonate* in 1000 ml of *water*, add 1 ml of *triethylamine* and adjusted to pH 3.0 with *orthophosphoric acid* and 20 volumes of *acetonitrile,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 294 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{20}FN_3O_4$ in infusion.

Ofloxacin Ophthalmic Solution

Ofloxacin Ophthalmic Solution is a sterile aqueous solution of Ofloxacin.

Ofloxacin Ophthalmic Solution contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ofloxacin, $C_{18}H_{20}FN_3O_4$.

Usual strength. 0.3 per cent w/v.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 6.0 to 7.2.

Sterility (2.2.11). Comply with the test for sterility.

Other tests. Comply with the tests stated under Eye Drops.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Phosphate buffer pH 7.3 prepared by dissolving 3.56 g of *disodium hydrogen phosphate* in 1000 ml *water,* adjusted to pH 7.3 using *orthophosphoric acid* or *sodium hydroxide solution.*

Test solution. Dilute a volume of Opthalmic Solution containing 30 mg of Ofloxacin in 100.0 ml of solvent mixture. Dilute 1.0 ml of the solution to 10.0 with the solvent mixture.

Reference solution. A 0.003 per cent w/v solution of *ofloxacin IPRS* with the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as TSK GEL),
- mobile phase: a mixture of 20 volumes of *acetonitrile,* 80 volumes of phosphate buffer pH 7.25 prepared by

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dissolving 2.54 g of *tetrabutyl ammonium hydrogen sulphate* and 3.56 g of *disodium hydrogen phosphate* in 1000 ml *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 294 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{20}FN_3O_4$.

Storage. Store protected from light.

Ofloxacin Oral Suspension

Ofloxacin Oral Suspension is a suspension of Ofloxacin in a suitable aqueous vehicle.

Ofloxacin Oral Suspension contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ofloxacin, $C_{18}H_{20}FN_3O_4$.

Usual strengths. 50 mg per 5 ml; 100 mg per 5 ml.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the oral suspension containing 50 mg of Ofloxacin in 25 ml of *glacial acetic acid* with the aid of ultrasound and dilute to 50.0 ml with *water*. Dilute 5.0 ml of the solution to 50.0 ml with *water*.

Reference solution. Disperse 50 mg of *ofloxacin IPRS* in 25 ml of *glacial acetic acid* with the aid of ultrasound and dilute to 50.0 ml with *water*. Dilute 5.0 ml of the solution to 50.0 ml with *water*.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 90 volumes of buffer solution prepared by dissolving 2.72 g of *potassium dihydrogen orthophosphate* anhydrous in 1000 ml of *water*, adjusted to pH 2.4 with *orthophosphoric acid* and 10 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 294 nm,
- Injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Determine the weight per ml (2.4.29) of the suspension and calculate the content of $C_{18}H_{20}FN_3O_4$, in the oral suspension.

Storage. Store protected from light and moisture.

Ofloxacin Tablets

Ofloxacin Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ofloxacin, $C_{18}H_{20}FN_3O_4$.

Usual strengths. 200 mg; 400 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloride acid*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the medium if necessary, at the maximum at about 294 nm (2.4.7). Calculate the content of $C_{18}H_{20}FN_3O_4$ in the medium from the absorbance obtained from a solution of known concentration of *ofloxacin IPRS* in the same medium.

Q. Not less than 75 per cent of the stated amount of $C_{18}H_{20}FN_3O_4$ in the medium.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 100 mg of Ofloxacin, disperse in 60 ml of *methanol* and dilute to 100.0 ml with *methanol* and filter.

Reference solution (a). A 0.1 per cent w/v solution of *ofloxacin IPRS* in *methanol*.

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Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *methanol*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 8 volumes of *acetonitrile* and 92 volumes of *phosphate buffer* prepared by dissolving 2.72 g of *monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 2.4 with *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 294 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the column efficiency in not less than 1400 theoretical plates.

Inject reference solution (b) and the test solution. Run the chromatogram three times of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

Other tests. Comply with the tests stated under the Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 25 mg of Ofloxacin in 60 ml of *methanol* and dilute to 100 ml with *methanol* and filter.

Reference solution. A 0.025 per cent w/v solution of *ofloxacin IPRS* in *methanol*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 92 volumes of buffer solution prepared by dissolving 2.72 g of *potassium dihydrogen phosphate* in 1000 ml of *water* and adjusted to pH 2.4 with *orthophosphoric acid* and 8 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 294 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{20}FN_3O_4$.

Storage. Store protected from light and moisture.

Ofloxacin and Ornidazole Tablets

Ofloxacin and Ornidazole Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of Ofloxacin, $C_{18}H_{20}FN_3O_4$ and Ornidazole $C_7H_{10}ClN_3O_3$.

Usual strength. Ofloxacin 200 mg and Ornidazole 500 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1M hydrochloric acid*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter, discarding the first few ml of filtrate.

Determine by liquid chromatography (2.4.14)

Solvent mixture. Equal volumes of *acetonitrile* and *water*.

Test solution. Use the filtrate, dilute if necessary, with dissolution medium.

Reference solution. Weigh about 55 mg of *ornidazole IPRS* and 22 mg of *ofloxacin IPRS* into a 100-ml volumetric flask, add about 60.0 ml of solvent mixture and sonicate to dissolve, make the volume up to the mark with solvent mixture and mix. Dilute 5.0 ml of the solution to 50.0 ml with the dissolution medium.

Use the chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{20}FN_3O_4$ and $C_7H_{10}ClN_3O_3$.

Q. Not less than 75 per cent of the stated amount of $C_{18}H_{20}FN_3O_4$ and $C_7H_{10}ClN_3O_3$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. A mixture of equal volumes of *acetonitrile* and *water*.

Test solution. Disperse a quantity of the powdered tablets containing 200 mg of Ofloxacin into a 100-ml volumetric flask add about 10.0 ml *0.1M hydrochloric acid* and sonicate for 15 minutes with shaking and add 30.0 ml of solvent mixture and sonicate for 60 minutes, after sonication cool to room temperature and make the volume up to the mark with solvent mixture and mix. Filter through 0.45 mm filter. Dilute 5.0 ml of the solution to 25.0 ml with solvent mixture.

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Reference solution. Weigh and transfer about 50 mg of *ornidazole IPRS* and 20 mg of *ofloxacin IPRS* into a 100-ml volumetric flask. Add about 5.0 ml *0.1M hydrochloric acid* and 50.0 ml of solvent mixture and sonicate to dissolve, make the volume up to the mark with solvent mixture and mix. Dilute 5.0 ml of the solution to 50.0 ml with the *solvent mixture* and mix. Dilute 5.0 ml of the solution to 25.0 ml with solvent mixture.

Chromatographic system

- a stainless steel column 15cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 87 volumes of a buffer solution prepared by dissolving 3.07 g *ammonium acetate* and 5.38 g *sodium perchlorate* in 1000 ml of *water*, adjusted to pH 4.0 with *orthophosphoric acid*, and 13 volumes of *acetonitrile*.
- flow rate: 1 ml per minute,
- spectrophotometer set at 300 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency for both the analytes is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 for ofloxacin peak and is not more than 2.5 for ornidazole peak and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. Run the chromatogram three times the retention time of ofloxacin peak. In the chromatogram obtained with test solution the area of any secondary peak due to ornidazole at Relative retention time about 0.23 and 0.75 is not more than 0.5 times the area of the ornidazole peak in the chromatogram obtained with reference solution (0.5 per cent) and sum of the areas of all the secondary peaks due to ornidazole is not more than the area of the ornidazole peak in the chromatogram obtained with reference solution (1.0 per cent). In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the ofloxacin peak in the chromatogram obtained with reference solution (1.0 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area for the ofloxacin peak in the chromatogram obtained with reference solution (2.0 per cent). Ignore any peak with an area 0.05 times the area of the ofloxacin peak in the chromatogram obtained with reference solution (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. A mixture of equal volumes of *acetonitrile* and *water*.

Test solution. Weight and powder 20 tablets. Transfer powder containing 200 mg of Ofloxacin into 100-ml volumetric flask add about 10 ml *0.1M hydrochloric acid* and sonicate for 15 minutes with occasional shaking and add 30 ml of solvent mixture and sonicate for 60 minutes, after sonication cool to room temperature and make the volume up to the mark with solvent mixture and mix. Filter through 0.45 µm filter. Dilute 2.0 ml of the solution to 200.0 ml with solvent mixture.

Reference solution. Weigh and transfer accurately about 25 mg of *ofloxacin IPRS* and 62.5 mg of *ornidazole IPRS* into a 250-ml volumetric flask. Add about 5.0 ml *0.1M hydrochloric acid* and 50.0 ml of solvent mixture, sonicate to dissolve. Make the volume up to the mark with solvent mixture and mix. Dilute 5.0 ml of the solution to 25.0 ml with solvent mixture.

Chromatographic system

- a stainless steel column, 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5µm), (Such as Inertsil ODS-2)
- column temperature: 40°.
- mobile phase: a mixture of 87 volumes of a buffer solution prepared by dissolving 3.07 g *ammonium acetate* and 5.38 g *sodium perchlorate* in 1000 ml of *water*, adjusted to pH 2.2 with *orthophosphoric acid* and 13 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 300 nm,
- injection volume: 10 µl.

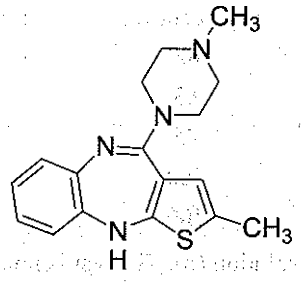
Inject the reference solution. The test is not valid unless the column efficiency for both the principal peaks is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{20}FN_3O_4$ and $C_7H_{10}ClN_3O_3$ in the tablets.

Storage. Store protected from light and moisture.

Olanzapine



$C_{17}H_{20}N_4S$ Mol. Wt. 312.4

Olanzapine is 2-methyl-4-(4-methyl-1-piperazinyl)-10*H*-thieno[2,3-*b*][1,5]benzodiazepine.

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Olanzapine contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{17}H_{20}N_4S$, calculated on the anhydrous basis.

Category. Antipsychotic.

Description. A yellow crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *olanzapine IPRS* or with the reference spectrum of olanzapine.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 40 volumes of *water* and 60 volumes of *acetonitrile*.

Test solution. Dissolve 50 mg of the substance under examination in 25 ml of solvent mixture.

Reference solution (a). A 0.2 per cent w/v solution of *olanzapine IPRS* with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 80 volumes of *buffer solution pH 6.8* prepared by dissolving 4.83 g of *sodium dihydrogen orthophosphate monohydrate* in 1000 ml of *water*, adjusted to pH 6.8 with 10 per cent w/v of *sodium hydroxide* and 20 volumes of *acetonitrile*,
  B. *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
20	63	37
30	45	55
32	100	0
38	100	0

Inject reference solution (b). The test is not valid unless the tailing factor is not more than 2.0 and the column efficiency is not less than 2000 theoretical plates.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b)(0.5 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 1.0 g.

Assay. Dissolve 0.2 g in 40 ml of *glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01562 g of $C_{17}H_{20}N_4S$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Olanzapine Tablets

Olanzapine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of olanzapine, $C_{17}H_{20}N_4S$.

Usual strengths. 2.5 mg; 5 mg; 7.5 mg; 10 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.01 M hydrochloric acid*,
Speed and time. 50 rpm for 45 minutes.

Withdraw a suitable volume of the medium and filter. Determine by liquid chromatography (2.4.14).

NOTE — Protect all the solutions from light.

Test solution. Use the filtrate, dilute if necessary, with dissolution medium.

Reference solution. Weigh 16 mg of *olanzapine IPRS*, dissolve in about 2.5 ml of *acetonitrile* and dilute to 25 ml with *0.01 M hydrochloric acid*. Dilute suitably to get 0.00016 per cent w/v in dissolution medium.

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Chromatographic system as described under Assay, using Injection volume 50 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the column efficiency is not less than 2500 theoretical plates.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{20}N_4S$.

Q. Not less than 70 per cent of the stated amount of $C_{17}H_{20}N_4S$ in the medium.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay with the following modification.

Test solution. Disperse one tablet in suitable volume of *acetonitrile* (25 per cent of the final volume) and dilute with *0.01 M hydrochloric acid* to obtain a 0.01 per cent w/v solution of Olanzapine.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Transfer 10 intact tablets to a suitable volumetric flask, disperse in a mixture of 25 volumes of *acetonitrile* and 75 volumes of *0.01M hydrochloric acid* and dilute with the same mixture to obtained a solution containing 0.01 per cent w/v of Olanzapine.

Reference solution. Dissolve 10 mg of *olanzapine IPRS* in 25 ml of *acetonitrile* and dilute to 100.0 ml with *0.01 M hydrochloric acid*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 70 volumes of a buffer solution prepared by dissolving 3 g of *ammonium dihydrogen orthophosphate* in 900 ml *water*, add 2 ml of *triethylamine* and dilute to 1000 ml with *water*, adjusted to pH 2.5 with *orthophosphoric acid* and 30 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency is not less than 2500 theoretical plates. The relative standard deviation for replicate injections is not more than 2 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{20}N_4S$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Olanzapine and Fluoxetine Tablets

Olanzapine and Fluoxetine Hydrochloride Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of olanzapine, $C_{17}H_{20}N_4S$ and fluoxetine, $C_{17}H_{18}F_3NO$.

Usual strengths. 5 mg olanzapine and 20 mg fluoxetine;10 mg olanzapine and 20 mg fluoxetine,

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with reference solution (c).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 500 ml of a *sodium acetate* buffer prepared by dissolving 4.1g of *sodium acetate* in 1000 ml of *water*, adjusted to pH 4.5 with *orthophosphoric acid*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with dissolution medium.

Reference solution. Dissolve an accurately weighed amount of *olanzapine IPRS* and *fluoxetine hydrochloride IPRS* with the dissolution medium having similar concentration of the test solution.

Use the chromatographic system as described under Assay except injection volume 50 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

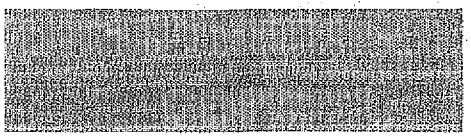
Calculate the content of $C_{17}H_{20}N_4S$ and $C_{17}H_{18}F_3NO$ in the medium.

Q. Not less than 70 per cent of the stated amounts of $C_{17}H_{20}N_4S$ and $C_{17}H_{18}F_3NO$.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), using the chromatographic system as described under Assay.

Test solution. Disperse one tablet in 60 ml of mobile phase, sonicate for 25 minutes, centrifuge at 2500 rpm for 10 minutes and dilute to 100.0 ml with the mobile phase. Dilute a volume of the solution with the mobile phase to obtain a solution containing 0.002 per cent w/v of olanzapine.



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Reference solution (a) A 0.002 per cent w/v solution of *olanzapine IPRS* in the mobile phase.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{20}N_4S$ in the tablet.

Related substances. Determine by liquid chromatography (2.4.14).

Use the chromatographic system, mobile phase, and test solution as described under Assay.

Inject the test solution. The area of any secondary peak is not more than 1.0 per cent and the sum of the areas of all the secondary peaks is not more than 2.0 per cent, calculated by area normalization.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14)

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 10 mg of Olanzapine add 60 ml of mobile phase, sonicate for 25 minutes, centrifuge at 2500 rpm for 10 minutes and dilute to 100.0 ml in the mobile phase. Dilute a volume of the solution with the mobile phase to obtain a solution containing 0.002 per cent w/v of olanzapine.

Reference solution (a). A 0.1 per cent w/v solution of *olanzapine IPRS* in the mobile phase.

Reference solution (b). A 0.45 per cent w/v solution of *fluoxetine hydrochloride IPRS* in the mobile phase.

Reference solution (c). Dilute reference solution (a) and (b) with the mobile phase to obtain a solution having a known concentration similar to the test solution.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of a buffer solution prepared by dissolving 7.09 g of *disodium hydrogen phosphate* in 1000 ml of *water*, 30 volumes of *acetonitrile*, 20 volumes of *methanol* and 0.1 volume of *triethylamine* and adjusted to pH 7.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 227 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

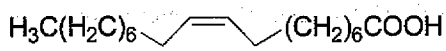
Inject reference solution (c) and the test solution.

Calculate the contents of $C_{17}H_{20}N_4S$ and $C_{17}H_{18}F_3NO$ in the tablets.

Storage. Store protected from moisture.

Labelling. The label states the strength in terms of the equivalent amount of fluoxetine.

Oleic Acid



$C_{18}H_{34}O_2$ Mol. Wt. 282.5

Oleic Acid consists mainly of (*Z*)-octadec-9-enoic acid, $C_{18}H_{34}O_2$, together with varying amounts of saturated and other unsaturated fatty acids and is obtained by the hydrolysis of fats or fixed oils and separation of the liquid acids by expression or other suitable means. It may contain a suitable antioxidant.

Category. Pharmaceutical aid (emulsion adjuvant).

Description. A clear, yellowish to pale brown, oily liquid. On exposure to air it darkens in colour.

Identification

A. To 1 ml add 1 ml of *ethanol (95 per cent);* the solution is clear. It turns orange or red on addition of 0.1 ml of *methyl orange solution.*

B. Take a mixture of 1 ml of *nitric acid* and 1 ml of *water* in a test-tube with an internal diameter of about 12.5 mm and add 1 ml of the substance under examination on the surface of the mixture. Introduce 0.5 g of *copper* turnings into the lower layer and allow to stand under a hood for 4 hours; the upper layer solidifies.

C. Complies with the test for Iodine value (2.3.28).

Tests

Weight per ml (2.4.29). 0.889 g to 0.895 g.

Peroxide value (2.3.35). Not more than 10.0.

Acid value (2.3.23). 195 to 202, determined on 0.5 g.

Iodine value (2.3.28). 85 to 95, determined by Method A.

Water-soluble acids. Shake 5 ml with 5 ml of *water* for 2 minutes, allow the liquids to separate and filter the aqueous layer through paper moistened with *water*. To the filtrate add 0.05 ml of *methyl orange solution*; the liquid does not become red.

Neutral fats and mineral oils. Boil 1 ml with 5 ml of *0.5 M sodium carbonate* and 25 ml of *water* in a large flask. The solution, while still hot, is not more opalescent than opalescence standard OS2 (2.4.1).

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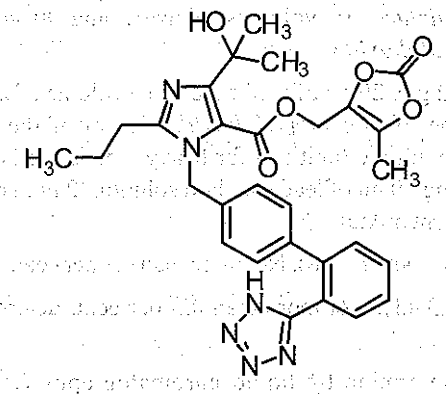
Congealing point. Dry about 10 ml by heating to 110° with frequent stirring, transfer to a test-tube about 20 mm in diameter, cool and when at 15° immerse the tube in a suitable water-bath so that the cooling takes place at the rate of 2° per minute. Stir the sample with a thermometer; it does not become cloudy until the temperature has fallen to 10°. On further cooling it congeals to a white solid or semi-solid mass at about 4°.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Storage. Store protected from light and moisture in a refrigerator (8° to 15°).

Labelling. The label states the name and concentration of any added antioxidant.

Olmesartan Medoxomil



$C_{29}H_{30}N_6O_6$ Mol.Wt. 558.6

Olmesartan Medoxomil is (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl 4-(1-hydroxy-1-methylethyl)-2-propyl-1-[[2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-imidazole-5-carboxylate.

Olmesartan Medoxomil contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{29}H_{30}N_6O_6$, calculated on the anhydrous basis.

Category. Antihypertensive

Production

As Nitrosamines are classified as probable human carcinogens, their presence in olmesartan medoxomil should be avoided or limited as much as possible. For this reason, manufacturers of olmesartan medoxomil for human use are expected to perform an assessment of the risk of *N*-nitrosamine formation and contamination during their manufacturing process; if this assessment identifies a potential risk, the manufacturing process should be modified to minimise contamination and a control strategy implemented to detect and control *N*-nitrosamine impurities in olmesartan medoxomil. The general chapter 5.11. Nitrosamine Impurities is available to assist manufacturers.

Description. A white or almost white, crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *olmesartan medoxomil IPRS* or with the reference spectrum of olmesartan medoxomil.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution (a). Dissolve about 25 mg of the substance under examination in *acetonitrile* and dilute to 25.0 ml with *acetonitrile*.

Test solution (b). Dissolve 25 mg of the substance under examination in *acetonitrile* and dilute to 50.0 ml with *acetonitrile*.

Reference solution (a). Dilute 1.0 ml of test solution (a) to 50.0 ml with *acetonitrile*. Dilute 1.0 ml of the solution to 10.0 ml with *acetonitrile*.

Reference solution (b). A 0.05 per cent w/v solution of *olmesartan medoxomil IPRS* in *acetonitrile*.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm packed with octylsilane bonded to porous silica (3.5 µm),
- column temperature: 40°,
- mobile phase: A. a mixture 80 volumes of buffer solution prepared by dissolving 2.04 g of *potassium dihydrogen orthophosphate* in 1000 ml *water*, adjusted to pH 3.4 with *orthophosphoric acid* and 20 volumes of *acetonitrile*,

  B. a mixture of 20 volumes of buffer solution and 80 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 250 nm,
- injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	75	25
10	75	25
35	0	100
45	0	100
48	75	25

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Name	Relative retention time
Olmesartan medoxomil impurity A[1]	0.2
Olmesartan medoxomil impurity B[2]	0.7
Olmesartan medoxomil (Retention time about 10 minutes)	1.0
Olmesartan medoxomil impurity C[3]	1.5

[1]4-(1-hydroxy-1-methylethyl)-2-propyl-1-[[2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-imidazole-5-carboxylic acid (olmesartan),

[2]6,6-dimethyl-2-propyl-3-[[2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-3,6-dihydro-4*H*-furo[3,4-*d*]imidazol-4-one,

[3](5-methyl-2-oxo-1,3-dioxol-4-yl)methyl 4-(1-methylethenyl)-2-propyl-1-[[2'-(1*H*- tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-imidazole-5-carboxylate.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (a) and test solution (a). In the chromatogram obtained with test solution (a), the area of any peak due to olmesartan medoxomil impurity A is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent), the area of any peak corresponding to olmesartan medoxomil impurity C is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a). (0.3 per cent), the area of any other secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent). Ignore any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Acetone. Not more than 0.6 per cent.

Determine by gas chromatography (2.4.13).

Internal standard solution. Dilute 1.0 ml of *butanol* to 100.0 ml with *dimethylsulphoxide*.

Test solution. Dissolve 0.25 g the substance under examination in *dimethylsulphoxide*, add 2.0 ml of the internal standard solution and dilute to 10.0 ml with *dimethylsulphoxide*.

Reference solution. Dilute 0.5 ml of *acetone* to 200.0 ml with *dimethylsulphoxide*. Dilute 15.0 ml of the solution to 100.0 ml with *dimethylsulphoxide*. To 25.0 ml of the solution add 10.0 ml of the internal standard solution and dilute to 50.0 ml with *dimethylsulphoxide*.

Chromatographic system

- a fused silica column 30 m x 0.53 mm, coated with macrogol 20 000 (1 µm),
- temperature:

column	time (in min.)	temperature (°)
	5	50
	5 – 18	50-180
	18 – 23	180

- inlet port at 200° and detector at 200°,
- split ratio: 1:5,
- flow rate: 4 ml per minute, using nitrogen or helium as the carrier gas.

Head-space injection conditions:

- equilibration time: 30 minutes,
- equilibration temperature: 80°,

Inject 1 ml of each solution.

Calculate the content of acetone, taking its relative density to be 0.79 at 20°.

Heavy metals (2.3.13).

Solvent mixture. 10 volumes of *water* and 90 volume of *dimethylsulphoxide*

Dissolve 2 g in 20 ml of a mixture of 10 volume of *water* and 90 volume of *dimethylsulphoxide*. 12 ml of the solution complies with the limit test for heavy metals Method D (20 ppm), using 10 ml of lead standard solution (2 ppm pb) in the same solvent mixture.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.5 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14) as described under Related substances with the following modifications.

Chromatographic system

- mobile phase. a mixture of 75 volumes of mobile phase A and 25 volume of mobile phase B.

Inject reference solution (b) and test solution (b).

Calculate the content of $C_{29}H_{30}N_6O_6$.

Olmesartan Medoxomil Tablets

Olmesartan Medoxomil Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of olmesartan medoxomil, $C_{29}H_{30}N_6O_6$.

Usual strengths. 20 mg; 40 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution correspond to the peak in the chromatogram obtained with the reference solution.

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Tests

Dissolution (2.5.2)

NOTE — Prepare the solution immediately before use.

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1M hydrochloric acid,*
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with dissolution medium.

Reference solution. Dissolve a quantity of *olmesartan medoxomil IPRS* in *acetonitrile* and dilute with the dissolution medium to obtain a solution of known concentration similar to the expected concentration of the test solution.

Use the chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{29}H_{30}N_6O_6$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{29}H_{30}N_6O_6$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Use freshly prepared test solution.

Solvent mixture. Equal volumes of *acetonitrile* and buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water,* add 1 ml of *triethylamine,* adjusted to pH 2.5 with *orthophosphoric acid.*

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 100 mg of Olmesartan Medoxomil in 100 ml of the solvent mixture, with the aid of ultrasound for 20 minutes, and dilute to 200.0 ml with the solvent mixture and filter.

Reference solution. A 0.00025 per cent w/v solution of *olmesartan medoxomil IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Intersil ODS 3V),
- mobile phase: a mixture of 65 volumes of buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water,* add 1 ml of *triethylamine,* adjusted to pH 2.5 with *orthophosphoric acid* and 35 volumes of *acetonitrile.*
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

The relative retention time of olmesartan with respect to olmesartan medoxomil is 0.26 and the correction factor is 0.83.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 1500 theoretical plates, the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of peak corresponding to olmesartan impurity A is not more than 6 times the area of the principal peak in the chromatogram obtained with the reference solution (3.0 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks other than olmesartan impurity A peak is not more than four times the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *acetonitrile* and buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water,* add 1 ml of *triethylamine.* adjusted to pH 2.5 with *orthophosphoric acid* .

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 200 mg of Olmesartan Medoxomil in 150 ml of the solvent mixture, with the aid of ultrasound for 20 minutes, and dilute to 250.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution. A 0.004 per cent w/v solution of *olmesartan medoxomil IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Intersil ODS 3V),
- mobile phase: a mixture of 55 volumes of buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water,* add 1 ml of *triethylamine.* adjusted to pH 2.5 with *orthophosphoric acid* and 45 volumes of *acetonitrile,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 1500 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

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Calculate the content of $C_{29}H_{30}N_6O_6$ in the tablets.

Storage. Store protected from moisture at a temperature not exceeding 30°.

Olmesartan Medoxomil and Hydrochlorothiazide Tablets

Olmesartan Medoxomil and Hydrochlorothiazide Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of olmesartan medoxomil, $C_{29}H_{30}N_6O_6$ and hydrochlorothiazide $C_7H_8ClN_3O_4S$.

Usual strengths. Olmesartan Medoxomil 20 mg and Hydrochlorothiazide 12.5 mg; Olmesartan Medoxomil 40 mg and Hydrochlorothiazide 12.5 mg; Olmesartan Medoxomil 40 mg and Hydrochlorothiazide 25 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

Tests

NOTE — The solutions should be used within 3 hours of preparation.

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid,*
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Solvent mixture. A mixture of 50 volumes of a buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water,* adjusted to pH 2.2 with *orthophosphoric acid* and 50 volumes of *acetonitrile.*

Test solution. Use the filtrate, dilute if necessary, with dissolution medium.

Reference solution (a). Dissolve a quantity of *olmesartan medoxomil IPRS* in the solvent mixture and dilute with dissolution medium to obtain a solution having a known concentration similar to the test solution.

Reference solution (b). Dissolve a quantity of *hydrochlorothiazide IPRS* in the solvent mixture and dilute with dissolution medium to obtain a solution having a known concentration similar to the test solution.

Reference solution (c). Dilute a volume of reference solution (a) and reference solution (b) with dissolution medium to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of a buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water,* adjusted to pH 2.2 with *orthophosphoric acid,* 30 volumes of *acetonitrile* and 5 volumes of *methanol,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the column efficiency is not less than 2500 theoretical plates, the tailing factor not more than 2.0, and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (c) and the test solution.

Q. Not less than 75 per cent of the stated amount of olmesartan medoxomil, $C_{29}H_{30}N_6O_6$ and hydrochlorothiazide, $C_7H_8ClN_3O_4S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of hydrochlorothiazide in 160 ml of the mobile phase, with the aid of ultrasound for 25 minutes and dilute to 200.0 ml with the mobile phase, centrifuge at 2500 rpm for 15 minutes. Dilute 5.0 ml of supernatant to 50.0 ml with the mobile phase.

Reference solution (a). A 0.05 per cent w/v solution of *olmesartan medoxomil IPRS* in the mobile phase.

Reference solution (b). A 0.07 per cent w/v solution of *hydrochlorothiazide IPRS* in the mobile phase.

Reference solution (c). Dilute 4.0 ml of reference solution (a) and 2.0 ml of reference solution (b) to 50.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS-3),
- mobile phase: a mixture of 60 volumes of buffer solution prepared by dissolving 1 ml of *triethylamine* in 1000 ml of *water,* adjusted to pH 3.0 with *orthophosphoric acid* and 40 volumes of *acetonitrile,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 226 nm,
- injection volume: 20 µl.

The retention time of hydrochlorothiazide peak is about 4.2 minutes and olmesartan medoxomil is about 15 minutes.

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Inject reference solution (c). The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and relative standard deviation for replicate injections is not more than 2.0 per cent for both the peaks.

Inject the test solution. The area of any secondary peak is not more than 1.0 per cent and the sum of areas of all secondary peaks is not more than 2.0 per cent, calculated by area normalization.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. A mixture of 50 volumes of a buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 2.2 with *orthophosphoric acid* and 50 volumes of *acetonitrile*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 125 mg of Hydrochlorothiazide in to 200-ml volumetric flask, add about 120 ml of the solvent mixture and sonicate for about 30 minutes with occasional shaking. Cool the solution to room temperature and make the volume up to the mark with solvent mixture and mix. Filter the test solution with 0.45 µ Nylon membrane filter. Dilute 2.0 ml of the solution to 50.0 ml with solvent mixture.

Reference solution (a). A 0.04 per cent w/v solution of *olmesartan medoxomil IPRS* in solvent mixture.

Reference solution (b). A 0.025 per cent w/v solution of *hydrochlorothiazide IPRS* in solvent mixture.

Reference solution (c). Dilute a volume of reference solution (a) and (b) with solvent mixture to obtain a solution having known concentration similar to the concentration of the test solution.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of a buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 2.2 with *orthophosphoric acid*, 30 volumes of *acetonitrile* and 5 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 20 µl

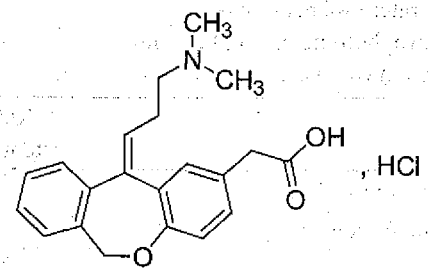
Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{29}H_{30}N_6O_6$ and $C_7H_8ClN_3O_4S$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Olopatadine Hydrochloride



$C_{21}H_{23}NO_3,HCl$ Mol. Wt. 373.9

Olopatadine Hydrochloride is 11-[(*Z*)-3-(Dimethylamino) propylidene]-6,11-dihydrodibenz[*b*,*e*]oxepin-2-acetic acid, hydrochloride.

Olopatadine Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{21}H_{23}NO_3,HCl$, calculated on dried basis.

Category. Antihistamine.

Description. A white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *olopatadine hydrochloride IPRS* or with the reference spectrum of olopatadine hydrochloride.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

C. It gives reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 2.0 to 4.0, determined in a 1.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution. A solution containing 0.02 per cent w/v of *olopatadine hydrochloride IPRS* and 0.002 per cent w/v of *olopatadine impurity B IPRS* in the mobile phase.

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Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 72 volumes of a buffer solution prepared by dissolving 13.6 g of *monobasic potassium phosphate* in *water* and dilute to 1000 ml with same solvent, add 1 ml of *triethylamine*, adjusted to pH 3.0 with *orthophosphoric acid* and 28 volumes of *acetonitrile,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 299 nm,
- injection volume: 30 µl.

Name	Relative retention time
α-Hydroxy olopatadine[1]	0.4
Olopatadine *E*-isomer[2]	0.7
Olopatadine	1.0
Olopatadine Impurity B[3]	1.2

[1](Z)-2-{11-[3-(Dimethylamino)propylidene]-6,11-dihydrodibenz[b,e]oxepin-2-yl}-2-hydroxyacetic acid,

[2][11-[(E)-3-(Dimethylamino)propylidene]-6,11-dihydrodibenz[b,e]oxepin-2-acetic acid,

[3](Z)-3-{2-(Carboxymethyl)dibenzo[b,e]oxepin-11(6H)-ylidene}-*N*,*N*-dimethylpropan-1-amine oxide.

Inject the reference solution. Run the chromatogram 2.5 times the retention time of principal peak. The test is not valid unless the resolution between the peaks due to olopatadine and olopatadine impurity B is not less than 2.0, the tailing factor is not more than 2.0, the column efficiency is not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent for the principal peak.

Inject the test solution. The area of peak corresponding to α-hydroxy olopatadine is not more than the 0.2 per cent, the area of peak corresponding to olopatadine *E*-isomer, multiplied with correction factor 0.77 is not more than 0.1 per cent. The area of any other secondary peak is not more than 0.1 per cent and the sum of areas of all the secondary peaks is not more than 0.25 per cent, calculated by area normalization.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.3 per cent, determined by drying in an oven at 105° for 1 hour.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances test with the following modifications.

Test solution. Dissolve about 10 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution. A 0.01 per cent w/v solution of *olopatadine hydrochloride IPRS* in the mobile phase.

Inject the reference solution.The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{23}NO_3$,HCl.

Storage. Store protected from light, at a temperature not exceeding 30°.

Olopatadine Ophthalmic Solution

Olopatadine Hydrochloride Ophthalmic Solution

Olopatadine Hydrochloride Ophthalmic Solution is a sterile aqueous solution of olopatadine hydrochloride. It may contain suitable antimicrobial agents.

Olopatadine Hydrochloride Ophthalmic Solution contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of olopatadine, $C_{21}H_{23}NO_3$.

Usual strengths. 0.1 per cent w/v; 0.2 per cent w/v; 0.5 per cent w/v; 1.0 per cent w/v.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 5.0 to 8.0.

Related substances. A. Determine by liquid chromatography (2.4.14).

NOTE — Protect the solutions from light.

Test solution. Dilute a volume of ophthalmic solution to obtain a solution containing 0.02 per cent w/v of olopatadine in the mobile phase.

Reference solution (a). A solution containing 0.022 per cent w/v of *olopatadine hydrochloride IPRS* and 0.002 per cent of *olopatadine impurity B IPRS* in the mobile phase.

Reference solution (b). A solution containing 0.022 per cent w/v of *olopatadine hydrochloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),

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- mobile phase: a mixture of 28 volumes of *acetonitrile*, 72 volumes of buffer prepared by dissolving 13.6 g of *monobasic potassium phosphate* in 1000 ml *water*, add 1 ml of *triethylamine* and mix, adjusted to pH 3.0 with *orthophosphoric acid*
- flow rate: 1 ml per minute,
- spectrophotometer set at 299 nm,
- injection volume: 30 µl.

Inject reference solution (a) and (b). Run the chromatogram 1.6 times the retention time of the principal peak. The test is not valid unless the resolution between the peaks due to olopatadine and olopatadine impurity B is not less than 2.0 obtained with reference solution (a), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent for principal peak obtained with reference solution (b).

Inject the test solution. In the chromatogram obtained with the test solution, the area of any peak due to olopatadine *E*-isomer is not more than 0.5 per cent. The area of any peak due to olopatadine impurity B is not more than 2.0 per cent. The area of any peak due to olopatadine carbaldehyde is not more than 0.5 per cent and area of any other secondary peak is not more than 0.5 per cent calculated by area normalisation. Ignore any peak at relative retention time more than 1.5.

Name	Relative retention time	Correction factor
Olopatadine *E*-isomer[1]	0.7	0.77
Olopatadine	1.0	---
Olopatadine Impurity B[2]	1.2	1.0
Olopatadine carbaldehyde[3]	1.3	0.22

[1]11-[(E)-3-(Dimethylamino)propylidene]-6,11-dihydrodibenz[b,e] oxepin-2-acetic acid,

[2](Z)-3-{2-(Carboxymethyl)dibenzo[b,e]oxepin-11(6H)-ylidene}-*N*,*N*-dimethylpropan-1-amine oxide,

[3](Z)-11-[3-(Dimethylamino)propylidene]-6,11-dihydrodibenzo[b,e] oxepine-2-carbaldehyde.

B. Determine by liquid chromatography (2.4.14), as described under Related substances test (A) with the following modifications.

NOTE — Protect the solutions from light.

Test solution. Dilute a volume of ophthalmic solution to obtain a solution containing 0.02 per cent w/v of olopatadine in the mobile phase.

Reference solution (a). A solution containing 0.0022 per cent w/v of *olopatadine hydrochloride IPRS* and 0.001 per cent *olopatadine impurity C IPRS* in the mobile phase.

Reference solution (b). A solution containing 0.001 per cent w/v of *olopatadine impurity C IPRS* (11-Oxo-6,11-dihydrodibenzo[b,e] oxepin-2-yl acetic acid) in the mobile phase.

- mobile phase: equal volumes of *acetonitrile* and buffer prepared by dissolving 13.6 g of *monobasic potassium phosphate* in 1000 ml *water*, add 1 ml of *triethylamine* and mix, adjusted to pH 3.0 with *orthophosphoric acid.*

Inject reference solution (a) and (b). Run the chromatogram 3 times the retention time of the olopatadine impurity C. The test is not valid unless the resolution between the peaks due to olopatadine and olopatadine impurity C is not less than 7.0 obtained with reference solution (a), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent for principal peak obtained with reference solution (b).

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to olopatadine impurity C is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), and area of any secondary peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). The sum of olopatadine impurity C, olopatadine *E*-isomer, olopatadine carbaldehyde and all other impurities found in the related substances test A and related substances test B is not more than 3.0 per cent. Ignore any peak at a relative retention time less than 0.7 with respect to the principal peak in the chromatogram obtained with reference solution (b).

Other tests. Comply with the tests stated under Eye Drops.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances test (A) with the following modifications.

NOTE — Protect the solutions from light.

Test solution. Dilute a volume of ophthalmic solution to obtain a solution containing 0.01 per cent w/v of Olopatadine in the mobile phase.

Reference solution. A solution containing 0.011 per cent w/v of *olopatadine hydrochloride IPRS* in the mobile phase.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{23}NO_3$.

Storage. Store protected from light and moisture, at a temperature between 4° to 25°.

Labelling. The label states the strength in terms of the equivalent amount of olopatadine.

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Olopatadine Tablets

Olopatadine Hydrochloride Tablets

Olopatadine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of olopatadine hydrochloride, $C_{21}H_{23}NO_3$,HCl.

Usual strengths. 5 mg; 10 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with dissolution medium.

Reference solution. Dissolve a quantity of *olopatadine hydrochloride IPRS* in the dissolution medium to obtain a solution of concentration similar to the expected concentration of the test solution.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 72 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 4.5 with *orthophosphoric acid* or *triethylamine* and 28 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution.

Q. Not less than 70 per cent of the stated amount of $C_{21}H_{23}NO_3$,HCl.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Disperse one tablet in 60 ml of the mobile phase, with the aid of ultrasound for 5 minutes and dilute to 100.0 ml with the mobile phase. Dilute further, if necessary, with the mobile phase, centrifuge at 2500 rpm for 10 minutes and use the supernatant liquid.

Calculate the content of $C_{21}H_{23}NO_3$,HCl in the tablet.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powder containing 25 mg of Olopatadine Hydrochloride with 60 ml of mobile phase, ultrasound for 10 minutes, and dilute to 100.0 ml with the mobile phase. Centrifuge at about 2500 rpm for 10 minutes, and dilute 5.0 ml of the solution to 25.0 ml with the mobile phase.

Use the chromatographic conditions as described under Dissolution.

Inject the test solution. The area of any secondary peak is not more than 1.0 per cent and the sum of area of all the secondary peaks is not more than 2.0 per cent, calculated by area normalization.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 25 mg of Olopatadine Hydrochloride with 60 ml of mobile phase, ultrasound for 10 minutes, and dilute to 100.0 ml with the mobile phase. Centrifuge at about 2500 rpm for 10 minutes, and dilute 5.0 ml of the solution to 25.0 ml with the mobile phase.

Reference solution. Dissolve 25 mg of *olopatadine hydrochloride IPRS* in 15 ml of mobile phase and dilute to 50.0 ml with mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Use the chromatographic condition as described under Dissolution.

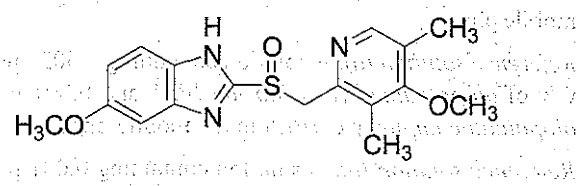
Inject the reference solution. The test is not valid unless the column efficiency in not less than 4000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{23}NO_3$,HCl in tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Omeprazole



$C_{17}H_{19}N_3O_3S$ Mol. Wt. 345.4

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Omeprazole is 5-methoxy-2-[[(4-methoxy-3,5-dimethyl-pyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazole.

Omeprazole contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{17}H_{19}N_3O_3S$, calculated on the dried basis.

NOTE — Perform the tests and assay in subdued light and use low-actinic glassware.

Category. Antiulcerative (proton pump inhibitor).

Description. A white or almost white powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *omeprazole IPRS* or with the reference spectrum of omeprazole.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution in *0.1 M sodium hydroxide* shows absorption maxima at about 276 nm and 305 nm; the ratio of the absorbance at about 305 nm to that at about 276 nm, 1.6 to 1.8.

C. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with reference solution (a).

Tests

Appearance of solution. A 2.0 per cent w/v solution in *dichloromethane* is clear (2.4.1).

Light absorption (2.4.7). Absorbances of a freshly prepared 2.0 per cent w/v solution in *dichloromethane* at 400 nm, 500 nm and 600 nm are not more than 0.25, 0.10 and 0.10 respectively.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Prepare the solutions immediately before use.

Test solution. Dissolve 3 mg of the substance under examination in 25.0 ml of the mobile phase.

Reference solution (a). A solution containing 0.01 per cent w/v each of *omeprazole IPRS* and *omeprazole sulphone IPRS (omeprazole impurity A IPRS)* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 12.5 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of and 73 volumes of a buffer solution prepared by dissolving 0.14 per cent w/v solution of *disodium hydrogen phosphate*, previously adjusted to pH 7.6 with *orthophosphoric acid* and 27 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 40 µl.

The relative retention time with reference to omeprazole for omeprazole impurity E (4-methoxy-2-[[(*RS*)-(5-methoxy-1*H*-benzimidazol-2-yl)sulphinyl]methyl]-3,5- dimethylpyridine 1-oxide) is about 0.6 and for omeprazole impurity D is about 0.8.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to omeprazole impurity D and the principal peak is not less than 3.0.

Inject reference solution (b) and the test solution. Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to omeprazole impurities D and E, each of, is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent) and the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent) and the sum of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). The residue obtained in the test for Sulphated ash, complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 0.2 per cent, determined on 1.0 g by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 4 hours.

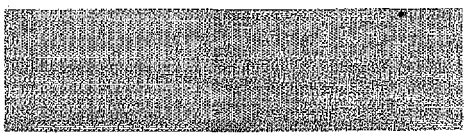
Assay. Determine by liquid chromatography (2.4.14).

Test solution. A 0.005 per cent w/v solution of the substance under examination in the mobile phase.

Reference solution. A 0.005 per cent w/v solution of *omeprazole IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of *phosphate buffer pH 7.4* and 35 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 302 nm,
- injection volume: 20 µl.



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Inject the reference solution. Repeat the procedure at least five times and measure the peak responses of the peak due to omeprazole. The relative standard deviation of the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{19}N_3O_3S$.

Storage. Store protected from light and moisture in a refrigerator (2° to 8°).

NOTE — A combination of elevated temperatures (37° to 50°) and high humidity degrades Omeprazole. It rapidly degrades under acidic conditions.

Omeprazole Gastro-resistant Capsules

Omeprazole Capsules

Omeprazole Gastro-resistant Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of omeprazole, $C_{17}H_{19}N_3O_3S$. They are made gastro-resistant by enteric-coating or by other means.

NOTE — Perform the tests and assay in subdued light and use low-actinic glassware.

Usual strength. 20 mg.

Identification

A. To a quantity of the contents of the capsules containing 50 mg of Omeprazole in a 100-ml volumetric flask add about 70 ml of *0.1 M sodium hydroxide*. Mix in an ultrasonic bath for about 5 minutes and heat on a water-bath for 10 minutes. Cool, make up to volume with *0.1 M sodium hydroxide* and filter. Dilute 2.0 ml of the filtrate to 100.0 ml with *0.1 M sodium hydroxide*.

When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows absorption maxima at about 276 nm and 305 nm.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to omeprazole in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

A. Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 100 rpm and 2 hours.

Determine by liquid chromatography (2.4.14).

Test solution. Withdraw the medium completely without any loss of residue. Transfer the residue into 100-ml volumetric flask, dissolve in 20 ml of *0.1M sodium hydroxide*, with the aid of ultrasound and make up the volume with *0.1M sodium hydroxide*. Centrifuge at about 3000 rpm for 10 minutes. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.02 per cent w/v solution of *omeprazole IPRS* in *0.1M sodium hydroxide*. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{19}N_3O_3S$ released in the acid medium by subtracting the content of $C_{17}H_{19}N_3O_3S$ in the test solution from the total content of Omeprazole $C_{17}H_{19}N_3O_3S$ determined in the Assay.

Complies with the acceptance criteria given under acid stage.

B. Apparatus No. 2 (Paddle),
Medium. 900 ml of *phosphate buffer pH 6.8*,
Speed and time. 100 rpm and 45 minutes.

Transfer another 6 capsules and run the apparatus for 2 hours in *0.1 M hydrochloric acid*. Decant the medium without losing the residue, add phosphate buffer pH 6.8 and run the apparatus for 45 minutes. Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. To 5.0 ml of the filtrate, add immediately 1.0 ml of *0.1M sodium hydroxide*.

Reference solution. A 0.055 per cent w/v solution of *omeprazole IPRS* in *0.1M sodium hydroxide*. Dilute 2.0 ml of the solution to 50.0 ml with the dissolution medium. To 5.0 ml of the solution, add immediately 1.0 ml of *0.1M sodium hydroxide*.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{19}N_3O_3S$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{17}H_{19}N_3O_3S$.

Other tests. Comply with the tests stated under Capsules.

Loss on drying (2.4.19). Not more than 3.0 per cent, determined on 0.5 g of the contents of the capsules by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 4 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Mix the contents of 20 capsules. Weigh and transfer the granules containing about 20 mg of Omeprazole to a 100-ml volumetric flask, add 20 ml of *0.1 M sodium*

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hydroxide, mix with the aid of ultrasound and dilute to volume with *0.1 M sodium hydroxide*. Centrifuge for 5 minutes and dilute 5.0 ml of the clear supernatant liquid to 50.0 ml with the mobile phase.

Reference solution. Take 20 mg of *omeprazole IPRS* in 100-ml volumetric flask, add 20.0 ml of *0.1 M sodium hydroxide*, shake vigorously for 5 minutes and dilute to volume with *0.1 M sodium hydroxide*. Dilute 5.0 ml of the solution with the mobile phase to produce 50.0 ml.

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of *phosphate buffer pH 7.4* and 35 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 302 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation of the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{19}N_3O_3S$ in the capsules.

Storage. Store protected from light and moisture.

Omeprazole and Domperidone Capsules

Omeprazole and Domperidone Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of omeprazole $C_{17}H_{19}N_3O_3S$ and domperidone $C_{22}H_{24}ClN_5O_2$.

Usual strength. Domperidone, 10 mg and Omeprazole, 20 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Domperidone —

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 100 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with dissolution medium.

Reference solution. Weigh 20 mg of *domperidone IPRS*, transfer to a 200-ml volumetric flask, dissolve and dilute to volume with *methanol*. Dilute 2.0 ml of the solution to 20.0 ml with the dissolution medium.

Use chromatographic system as described under Assay.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Q. Not less than 75 per cent of the stated amount of $C_{22}H_{24}ClN_5O_2$.

Omeprazole —

A. Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 100 rpm and 2 hours.

Determine by liquid chromatography (2.4.14).

Test solution. Withdraw the medium completely without any loss of residue. Transfer the residue into 100-ml volumetric flask, dissolve in 20 ml of *0.1 M sodium hydroxide*, with the aid of ultrasound and make up the volume with *0.1 M sodium hydroxide*. Centrifuge at about 3000 rpm for 10 minutes. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.02 per cent w/v solution of *omeprazole IPRS* in *0.1 M sodium hydroxide*. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{19}N_3O_3S$ released in the acid medium by subtracting the content of $C_{17}H_{19}N_3O_3S$ in the test solution from the total content of Omeprazole $C_{17}H_{19}N_3O_3S$ determined in the Assay.

Complies with the acceptance criteria given under acid stage.

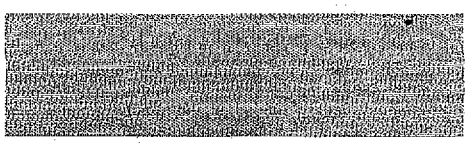
B. Apparatus No. 2 (Paddle),
Medium. 900 ml of *phosphate buffer pH 6.8*,
Speed and time. 100 rpm and 45 minutes.

Transfer another 6 capsules and run the apparatus for 2 hours in *0.1 M hydrochloric acid*. Decant the medium without losing the residue, add phosphate buffer pH 6.8 and run the apparatus for 45 minutes. Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. To 5.0 ml of the filtrate, add immediately 1.0 ml of *0.1 M sodium hydroxide*.

Reference solution. A 0.055 per cent w/v solution of *omeprazole IPRS* in *0.1 M sodium hydroxide*. Dilute 2.0 ml of



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the solution to 50.0 ml with the dissolution medium. To 5.0 ml of the solution, add immediately 1.0 ml of *0.1M sodium hydroxide*.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{19}N_3O_3S$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{17}H_{19}N_3O_3S$.

Uniformity of content. Complies with the test stated under capsules.

Determine by liquid chromatography (2.4.14), using the chromatographic system as described under Assay.

Test solution. Disperse one capsules in 60 ml of solvent mixture, sonicate for 45 minutes, and dilute to 100.0 ml with the mobile phase.

Reference solution (a). A 0.01 per cent w/v solution of *domperidone IPRS* in the mobile phase.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{24}ClN_5O_2$ in the capsule.

Other tests.Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14)

Solvent mixture.A mixture of 20 volumes of *water*, 40 volumes of *methanol* and 40 volumes of *acetonitrile*.

Test solution. Shake a quantity of the mixed contents of 20 capsules containing 40 mg of Omeprazole in 50 ml of the solvent mixture for 10 minutes and dilute to 100.0 ml with the solvent mixture and filter. Dilute 5.0 ml of the filtrate to 10.0 ml with the solvent mixture.

Reference solution. Weigh 40 mg of *omeprazole IPRS* and 20 mg of *domperidone IPRS*, transfer to a 200-ml volumetric flask, dissolve in the solvent mixture and dilute to volume with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of a buffer solution prepared by dissolving 1.4 g of *disodium hydrogen phosphate* in 1000 ml of *water*, adjusted to pH 7.5 with *orthophosphoric acid* and 35 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

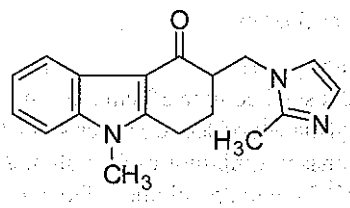
Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the contents of $C_{17}H_{19}N_3O_3S$ and $C_{22}H_{24}ClN_5O_2$ in the capsules.

Storage. Store protected from light and moisture.

Ondansetron



$C_{18}H_{19}N_3O$ Mol. Wt. 293.4

Ondensetron is 1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1*H*-imidazol-1-yl)methyl]-4*H*-carbazol-4-one.

Ondansetron contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{18}H_{19}N_3O$, calculated on the anhydrous basis.

Category. Antiemetic.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ondansetron IPRS* or with the reference spectrum of ondansetron.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Ondansetron impurity D. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 100.0 ml of the mobile phase.

Reference solution (a). A 0.00005 per cent w/v solution of *1, 2, 3, 9-tetrahydro-9- methyl-3-methylene-4H-carbazol-4-one IPRS (ondansetron impurity D IPRS)* in the mobile phase.

Reference solution (b). A solution containing 0.00005 per cent w/v of *ondansetron impurity D IPRS* and 0.0001 per cent w/v of *1,2,3,9-tetrahydro-9-methyl-4H –carbazol-4-one IPRS (ondansetron impurity C IPRS)* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with nitrile group bonded to porous silica (3 to 10 µm),

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- column temperature: 30°,
- mobile phase: a mixture of 80 volumes of phosphate buffer pH 5.4 prepared by dissolving 2.72 g of *monobasic potassium phosphate* in 900 ml of *water*, adjusted to pH 5.4 with *1 M sodium hydroxide* and dilute to 1000ml with *water* and 20 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 328 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between ondansetron impurity C and ondansetron impurity D is not less than 1.5. The relative retention time with reference to ondansetron impurity D for ondansetron impurity C is about 0.8.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any peak corresponding to ondansetron impurity D is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay.

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent), including ondansetron impurity D.

Chlorides (2.3.12). To 1 g of substance under examination, add 30 to 40 ml of *water* and warm gently, if necessary. Mix and pass through a filter paper that gives a negative test for chloride. Add 1 ml of *nitric acid* and 1 ml of *silver nitrate TS*. Dilute with *water* to 50 ml. Mix and allow to stand for 5 minutes protected from direct sunlight. Any turbidity formed is not more than that produced in a similarly treated control solution containing 0.3 ml of *0.02 M hydrochloric acid* (0.02 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 3.0 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 45 mg of the substance under examination in 50.0 ml of the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). A 0.009 per cent w/v solution of *ondansetron IPRS* in the mobile phase.

Reference solution (b). A solution containing 0.009 per cent w/v of *ondansetron IPRS* and 0.005 per cent w/v of *ondansetron impurity A IPRS* in the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with nitrile group bonded to porous silica (3 to 10 µm),
- column temperature: 30°,
- mobile phase: a mixture of 52 volumes of phosphate buffer pH 5.4 prepared by dissolving 2.72 g of *monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 5.4 with *1M sodium hydroxide* and 48 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 216 nm,
- injection volume: 10 µl.

The relative retention times with reference to ondansetron for ondansetron impurity A is about 1.1.

Inject reference solution (b). The test is not valid unless the resolution between ondansetron impurity A and ondansetron is not less than 1.5.

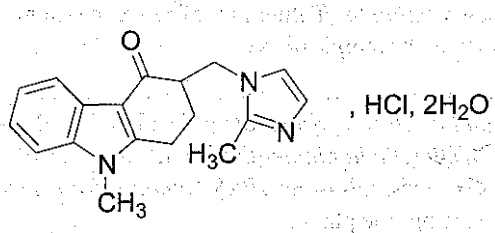
Inject reference solution (a). The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.5 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{18}H_{19}N_3O$.

Storage. Store protected from light, at a temperature not exceeding 30°.

Ondansetron Hydrochloride



$C_{18}H_{19}N_3O, HCl, 2H_2O$ Mol. Wt. 365.9

Ondansetron Hydrochloride is (*RS*)-9-methyl-3-[(2-methyl-1*H*-imidazol-1-yl)methyl]-1,2,3,9-tetrahydro-4*H*-carbazol-4-one hydrochloride dihydrate.

Ondansetron Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{18}H_{19}N_3O,HCl$, calculated on the anhydrous basis.

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Category. Antiemetic. (5-HT_3 receptor antagonist)

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6).Compare the spectrum with that obtained with *ondansetron hydrochloride IPRS* or with the reference spectrum of ondansetron hydrochloride.

B. It gives the reactions of chlorides (2.3.1).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Use the chromatographic system, as described under Assay using the test solution, reference solution (b), reference solution (c) and system suitability criteria for resolution.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.2 times the area of the peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 0.5 times the area of the peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). 9.0 to 10.5 per cent, determined on 0.2 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 50 ml of the mobile phase and filter. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). A 0.01 per cent w/v solution of *ondansetron hydrochloride IPRS* in the mobile phase.

Reference solution (b). Dilute 1 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). A solution containing 0.01 per cent w/v of *ondansetron hydrochloride IPRS* and 0.002 per cent w/v of *3[Dimethylaminomethyl]-1, 2, 3, 9-tetrahydro-9-methyl-4H-carbozol-4-one IPRS* (*ondansetron impurity A IPRS*) in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with porous silica (5 µm) with chemically bonded nitrile groups (Such as Supelco LC-CN),
- mobile phase: a mixture of 50 volumes of *0.02 M monobasic potassium phosphate* with the pH previously adjusted to pH 5.4 with *1 M sodium hydroxide* and 50 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 216 nm,
- injection volume: 10 µl.

Inject reference solution (c). The test is not valid unless the relative retention time and for ondansetron impurity A is about 1.1. The resolution between ondansetron and ondansetron impurity A is not less than 1.5.

Inject reference solution (a). The tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{18}H_{19}N_3O$,HCl.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Ondansetron Injection

Ondansetron Hydrochloride Injection

Ondansetron Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of ondansetron, $C_{18}H_{19}N_3O$.

Usual strength. 2 mg per ml.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

pH (2.4.24). 3.3 to 4.0.

Related substances. Determine by liquid chromatography (2.4.14).

Use the chromatographic system, test solution and reference solution (b) described under Assay.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.2 times the area of the peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 1.5 times the area of the peak in the chromatogram obtained with reference solution (b) (1.5 per cent).

Bacterial endotoxins (2.2.3). Not more than 9.9 Endotoxin Units per mg of ondansetron.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

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Assay. Determine by liquid chromatography (2.4.14).

Test solution. Transfer a measured volume of the injection containing about 2 mg of ondansetron to a 25-ml volumetric flask, dilute to volume with the mobile phase and mix.

Reference solution (a). A 0.01 per cent w/v solution of *ondansetron hydrochloride IPRS* in the mobile phase.

Reference solution (b). Dilute 1 ml of reference solution (a) to 100 ml with the mobile phase.

Reference solution (c). A solution containing 0.01 per cent w/v of *ondansetron hydrochloride IPRS* and 0.005 per cent w/v of *3[Dimethylaminomethyl]-1, 2, 3, 9-tetrahydro-9-methyl-4H-carbozol-4-one IPRS (ondansetron impurity A IPRS)* in the mobile phase.

Chromatographic system
- a stainless steel column 20 cm x 4.6 mm, packed with porous silica (5 µm) bonded to nitrile groups (Such as Supelco LC-CN),
- mobile phase: a mixture of 50 volumes of *0.02 M monobasic potassium phosphate* with the pH previously adjusted to pH 5.4 with *1 M sodium hydroxide* and 50 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 216 nm,
- injection volume: 10 µl.

Inject reference solution (c). The test is not valid unless the relative retention time and for ondansetron impurity A is about 1.1. The resolution between ondansetron and ondansetron impurity A is not less than 1.5.

Inject reference solution (a). The tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{18}H_{19}N_3O$ in the injection.

Storage. Store protected from light, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of ondansetron.

Ondansetron Orally Disintegrating Tablets

Ondansetron Orally Disintegrating Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ondansetron, $C_{18}H_{19}N_3O$.

Usual strengths. 4 mg; 8 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Disintegration (2.5.1). Not more than 30 seconds.

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 500 ml of *0.1 M hydrochloric acid.*
Speed and time. 50 rpm and 10 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the dissolution medium if necessary, at the maximum at about 310 nm (2.4.7). Calculate the content of $C_{18}H_{19}N_3O$ in the medium from the absorbance obtained by using a solution of known concentration of *ondansetron IPRS* in same medium.

Q. Not less than 80 per cent of the stated amount of $C_{18}H_{19}N_3O$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing about 40 mg of ondansetron with 100.0 ml of the mobile phase. Centrifuge a portion of the solution at 3000 rpm for 10 minutes. Use the supernatant.

Reference solution (a). A 0.004 per cent w/v solution of *1,2,3,9-tetrahydro-9-methyl-3-methylene-4H-carbazol-4-one IPRS (ondansetron impurity D IPRS)* in *acetonitrile.*

Reference solution (b). A 0.004 per cent w/v solution of *2-methylimidazole* in *acetonitrile.*

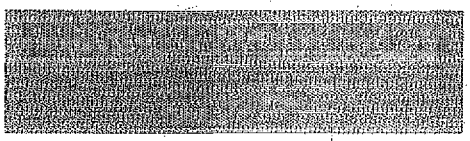
Reference solution (c). A 0.004 per cent w/v solution of *ondansetron IPRS* in *acetonitrile.*

Reference solution (d). Dilute 5.0 ml of reference solution (c) to 100.0 ml with the mobile phase.

Reference solution (e). Dilute 10.0 ml of reference solution (d) to 100.0 ml with the mobile phase.

Reference solution (f). Transfer 5.0 ml of each reference solution (a), (b) and (c) to a 100-ml volumetric flask, dilute to volume with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with porous silica chemically bonded to nitrile groups (5 µm),
- mobile phase: a mixture of 80 volumes of phosphate buffer prepared by dissolving 2.72 g of *monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 5.4 with *1 M sodium hydroxide* and 20 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,



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- spectrophotometer set at 216 nm,
- injection volume: 20 µl.

The relative retention time with reference to ondansetron for 2-methylimidazole is about 0.16 and for ondansetron impurity D is about 0.45.

Inject reference solution (e) and (f). The test is not valid unless the resolution between ondansetron and adjacent peak is not less than 1.5, the theoretical plates is not less than 8000 for ondansetron and tailing factor is not more than 2.0 in the chromatogram obtained with reference solution (f). In the chromatogram obtained with reference solution (e), the signal to noise ratio for the ondansetron peak is not less than 15.

Inject reference solution (d) and the test solution. In the chromatogram obtained with the test solution, the area of the peak corresponding to 2-methylimidazole, multiplied with correction factor 1.89, is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.15 per cent), the area of the peak corresponding to ondansetron impurity D, multiplied with correction factor 0.77, is not more than 0.24 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.12 per cent), the area of any other secondary peak is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent) and sum of areas of all the secondary peaks is not more than the area of principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Carry out the procedure described under Assay but using the following test solution.

Test solution. Disperse 1 tablet in *0.01 M hydrochloric acid* and filter, dilute if necessary, to 100.0 ml with *0.01 M hydrochloric acid* and filter.

Calculate the content of $C_{18}H_{19}N_3O$ in the tablet.

Other tests. Comply with the tests stated under tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 40 mg of ondansetron with about 60 ml of 0.01 *M hydrochloric acid* with the aid of ultrasound for about 10 minutes and dilute to 100.0 ml. Filter through polypropylene membrane of 0.45 µm pore size. Discard first few ml of the filtrate. Dilute 1.0 ml of the solution to 10.0 ml with *0.01 M hydrochloric acid*.

Reference solution (a). A 0.004 per cent w/v solution of *ondansetron IPRS* in *0.01 M hydrochloric acid*.

Reference solution (b). A 0.014 per cent w/v solution of *3[Dimethylamino methyl]-1,2,3,9-tetrahydro-9-methyl-H-carbozol-4-one-IPRS (ondansetron impurity A IPRS)* in *0.01 M hydrochloric acid*.

Reference solution (c). Transfer 8.0 ml each of reference solution (a) and (b) to 50-ml volumetric flask and dilute to volume with *0.01 M hydrochloric acid*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with porous silica bonded to nitrile groups (5 µm),
- mobile phase: a mixture of 52 volumes of 0.272 per cent w/v solution of *monobasic potassium phosphate*, adjusted to pH 5.4 with *1 M sodium hydroxide* and 48 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 216 nm,
- injection volume: 10 µl.

The relative retention time with reference to ondansetron for ondansetron impurity A is about 1.1.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to ondansetron and ondansetron impurity A is not less than 1.5 and the tailing factor is not more than 2.0 for ondansetron peak.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{18}H_{19}N_3O$ in the tablets.

Storage. Store protected form light and moisture, at a temperature not exceeding 30°.

Labelling. The label states the active ingredient in terms of equivalent amount of ondansetron.

Ondansetron Oral Solution

Ondansetron Hydrochloride Oral Solution

Ondansetron Oral Solution is a solution of Ondansetron Hydrochloride in a suitable vehicle.

Ondansetron Oral Solution contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of ondansetron, $C_{18}H_{19}N_3O$.

Usual strengths. 2 mg per 5 ml; 4 mg per 5 ml.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 90 volumes of *chloroform*, 50 volumes of *ethyl acetate*, 40 volumes of *methanol* and 1 volume of *ammonium hydroxide*.

Test solution. Dilute a measured volume of the oral solution containing about 20 mg of ondansetron to 100.0 ml in a mixture of 50 volumes of *methanol* and 50 volumes of *water*.

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Reference solution. A 0.025 per cent w/v solution of *ondansetron hydrochloride IPRS* in *methanol*.

Apply 10 µl of each solution. Allow the solvent front to rise 15 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. The chromatogram obtained with the test solution corresponds to that obtained with the reference solution.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 3.3 to 4.0.

Ondansetron impurity D. Not more than 0.1 per cent.

Determine by liquid chromatography (2.4.14).

Test solution. Dilute a measured volume of oral solution to obtain a solution having a concentration of 0.02 per cent w/v of ondansetron in the mobile phase.

Reference solution (a). A 0.000025 per cent w/v solution of *1,2,3,9-tetrahydro-9-methyl-3-methylene-4H-carbazol-4-one IPRS* (*ondansetron impurity D IPRS*) in the mobile phase.

Reference solution (b). A solution containing 0.000025 per cent w/v of *ondansetron impurity D IPRS* and 0.0001 per cent w/v of *N-methyl[2-(2-methylbenzhydryloxy)ethyl]amine hydrochloride IPRS (ondansetron impurity C IPRS)* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with porous silica bonded to nitrile groups (5 µm),
- mobile phase: a mixture of 80 volumes of *0.02 M monobasic potassium phosphate*, previously adjusted to pH 5.4 and 20 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 328 nm,
- injection volume: 40 µl.

Inject reference solution (b). The test is not valid unless the resolution between ondansetron impurity D and ondansetron impurity C is not less than 2.0, the tailing factor for ondansetron impurity D is not more than 2.0 and the relative standard deviation for replicate injections is not more than 4.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of ondansetron impurity D in the oral solution.

Related substances. Determined by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Dilute a volume of oral solution containing 4 mg of Ondansetron to 50.0 ml with the mobile phase and mix.

The relative retention time with reference to ondansetron for ondansetron impurity D is about 0.34, for imidazole is about 0.4, for 2-methyl imidazole is about 0.53, for des-C-methyl ondansetron hydrochloride is about 0.62, for *N*-desmethyl ondansetron maleate is about 0.83, for ondansetron impurity A is about 1.2.

Inject reference solution (b) and (c). The test is not valid unless the resolution between ondansetron impurity A and ondansetron is not less than 1.5 in the chromatogram obtained with reference solution (c) and in the chromatogram obatined with reference solution (b), the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to imidazole, 2-methyl imidazole, des-(-methyl) ondansetron hydrochloride, N- desmethyl ondansetron maleate, ondansetron impurity A is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent). The area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and sum of all the secondary peaks is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Determine by liquid chromatography (2.4.14).

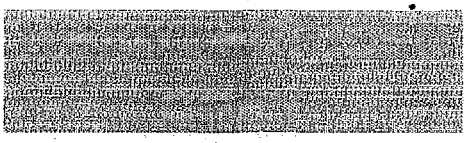
Test solution. Dilute a volume of the oral solution containing 8 mg of ondansetron to 100.0 ml with the mobile phase.

Reference solution (a). A 0.01 per cent w/v solution of *ondansetron hydrochloride IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). A solution containing 0.01 per cent w/v of *ondansetron hydrochloride IPRS* and 0.002 per cent w/v of *3[Dimethylaminomethyl]-1, 2, 3, 9-tetrahydro-9-methyl-4H-carbozol-4-one IPRS* (*ondansetron impurity A IPRS*) in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with porous silica (5 µm) with chemically bonded nitrile groups (Such as Supelco LC-CN),
- mobile phase: a mixture of 50 volumes of *0.02 M monobasic potassium phosphate* with the pH previously adjusted to pH 5.4 with *1 M sodium hydroxide* and 50 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 216 nm,
- injection volume: 10 µl.



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Inject reference solution (c). The test is not valid unless the relative retention time for ondansetron is about 1.0 and for ondansetron impurity A is about 1.1. The resolution between ondansetron and ondansetron impurity A is not less than 1.5.

Inject reference solution (a). The tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{18}H_{19}N_3O$ in the oral solution.

Microbial contamination (2.2.9). The total viable count is not more than 100 CFU per g, *Enterobacteriaceae* count is not more than 10 CFU per g and the total combined molds and yeasts count is not more than 50 CFU per g.

Storage. Store protected from light, at a temperature not exceeding 30°.

Ondansetron Tablets

Ondansetron Hydrochloride Tablets

Ondansetron Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of ondansetron, $C_{18}H_{19}N_3O$.

Usual strengths. 2 mg; 4 mg; 8 mg

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obt d with the reference solution (a).

Tests

Dissolution (2.5.2)

Apparatus No. 2 (Paddle),

Medium. 500 ml of *water,*

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter having an average pore size not more than 0.45 µm. Measure the absorbance of the filtered solution, suitably diluted with the medium if necessary, at the maximum at about 310 nm (2.4.7). Calculate the content of $C_{18}H_{19}N_3O$ in the medium from the absorbance obtained from a solution of known concentration of *ondansetron hydrochloride IPRS* in the same medium.

1 mg of $C_{18}H_{19}N_3O$ is equivalent to 1.25 mg of $C_{18}H_{19}N_3O,HCl,2H_2O$.

Q. Not less than 70 per cent of the stated amount of $C_{18}H_{19}N_3O$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of Ondansetron in 70 ml of the mobile phase, with the aid of ultrasound for 20 minutes and dilute to 100.0 ml with the mobile phase, centrifuge.

Reference solution (a). A 0.0005 per cent w/v solution of *ondansetron hydrochloride IPRS* in the mobile phase.

Reference solution (b). A solution containing 0.005 per cent w/v of ondansetron impurity A RS and 0.01 per cent w/v of *ondansetron hydrochloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with nitrile group bonded to porous silica (5 µm),
- mobile phase: a mixture of 20 volumes of *acetonitrile* and 80 volumes of buffer solution prepared by dissolving 2.7 g of *monobasic potassium phosphate* in 1000 ml of *water,* adjusting to pH 5.4 with *1M sodium hydroxide,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 216 nm,
- injection volume: 10 µl.

Name	Relative retention time	Correction factor
2-methyl-imidazole	0.22	1.89
Ondansetron impurity C[1]	0.40	0.83
Ondansetron impurity D[2]	0.47	0.77
Ondansetron impurity A[3]	0.87	1.11
Desmethylondansetron[4]	0.90	1.1
Ondansetron	1.0	---

[1]1,2,3,9-tetrahydro-9-methyl-4*H*-carbazol-4-one,

[2]1,2,3,9-tetrahydro-9-methyl-3-methylene-4*H*-carbazol-4-one,

[3]3[(dimethylamino)methyl]- 1,2,3,9-tetrahydro-9-methyl-4*H*-carbazol-4-one,

[4]1,2,3,9-tetrahydro-9-methyl-3-[(1*H*-imidazol-yl-)methyl]-4*H*-carbazol-4-one.

Inject reference solution (b). The test is not valid unless the resolution between the ondansetron impurity A and ondansetron is not less than 2.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak due to 2-methyl imidazole, desmethyl-ondansetron, ondansetron impurity A and ondansetron impurity C is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). The area of any secondary peak due to ondansetron impurity D is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent). The area of any other secondary peak is not more than 0.2 times the area of the principal peak in

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the chromatogram obtained with reference solution (a) (0.2 per cent). The sum of areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), using the chromatographic conditions and the reference solution described under Assay.

Test solution. Disperse one tablet in the solvent mixture and dilute to obtain a solution containing 0.005 per cent w/v of Ondanseteron Hydrochloride and filter.

Calculate the content of $C_{18}H_{19}N_3O$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *acetonitrile* and buffer solution prepared by dissolving 2.7 g of *monobasic potassium phosphate* in 1000 ml of *water* adjusted to pH 5.4 with *1M sodium hydroxide.*

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of ondansetron in 100.0 ml with the solvent mixture and dilute to filter. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution. A 0.005 per cent w/v solution of *ondansetron hydrochloride IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with nitrile group bonded to porous silica (5 µm),
- mobile phase: a mixture of 20 volumes of *acetonitrile* and 80 volumes of buffer solution prepared by dissolving 2.7 g of *monobasic potassium phosphate* in 1000 ml of *water,* adjusted to pH 5.4 with *1M sodium hydroxide,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 216 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{19}N_3O$ in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of ondansetron.

Oral Rehydration Salts

ORS Powder

Oral Rehydration Salts are dry, homogeneously mixed powders containing Dextrose, Sodium Chloride, Potassium Chloride and either Sodium Bicarbonate or Sodium Citrate for use in oral rehydration therapy after being dissolved in the requisite amount of water.

They may contain suitable flavouring agents and, where necessary, suitable flow agents in the minimum quantity required to achieve a satisfactory product but may not contain artificial sweetening agents like mono- and/or poly-saccharides. If saccharin/saccharin sodium or aspartame is used in preparations meant for paediatric use, the concentration of saccharin should be such that its daily intake is not more than 5 mg/kg of body weight and that of aspartame should be such that its daily intake is not more than 40 mg/kg of body weight.

Category: Replacement solution for diarrhoeal rehydration.

Usual strength. A formulation of reduced osmolarity (given below) recommended by the World Health Organization (WHO) for the Diarrhoeal Diseases Control Programme, and of the United Nations Children's Emergency Fund (UNICEF).

Composition of the formulation in terms of the amount, in g, to be dissolved in sufficient water to produce 1000 ml.

Sodium Chloride	2.6
Dextrose (anhydrous)	13.5
or	
Dextrose Monohydrate	14.85
Potassium Chloride	1.5
Sodium Citrate	2.9

The molar concentrations of sodium, potassium, chloride and citrate ions in terms of millimoles per litre are given below:

	mmol/l
Sodium	75
Potassium	20
Chloride	65
Citrate	10
Dextrose	75

The total osmolar concentration of the solution in terms of mOsmol per litre is 245.

Oral Rehydration Salts contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of Dextrose (anhydrous) or Dextrose Monohydrate (as appropriate) and of the requisite amounts of sodium, Na, potassium, K, chloride, Cl, citrate, $C_6H_5O_7$ and bicarbonate, HCO_3, calculated from the stated amounts of the relevant constituents.

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Description. A white to creamy-white, amorphous or crystalline powder.

Identification

A. When heated, melting and charring occurs and an odour of burnt sugar is produced.

B. Add a few drops of the solution prepared as directed in the label to 5 ml of *potassium cupri-tartrate solution*; a copious red precipitate is produced on boiling.

C. Gives reaction A of sodium salts, reaction (A) of potassium salts and reaction (A) of chlorides (2.3.1).

D. A quantity containing about 50 mg of *citric acid* gives the reactions of citrates (2.3.1).

Tests

Uniformity of weight. Complies with the test for contents of packaged dosage forms (2.5.6).

Seal test (*only for sachets*). Loosely bundle 10 sachets with a rubber band and submerge the bundle under water in a vacuum desiccator maintained at a pressure not exceeding 18 kPa for one minute. Examine the bundle for any fine stream of bubbles. Re-establish normal pressure and open the bundle. No penetration of water is observed in any sachet.

Other tests. Comply with the tests stated under Oral Powders.

Assay. Carry out the following assays on the well-mixed contents of an individual sachet or on a suitable sample from the well-mixed contents of a bulk container. Where the amount in an individual sachet is insufficient to carry out all the assays, take a separate sachet for the Assay for citrate and for the Assay for dextrose. For the Assays for total sodium, for potassium, for bicarbonate and for total chloride weigh about 8.0 g and dissolve in sufficient *water* to produce 500.0 ml (solution A).

For total sodium — Dilute a suitable volume of solution A with a sufficient volume of a solution of *strontium chloride* such that the final solution contains a 1500 to 2000-fold excess of strontium ions and determine by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 589 nm and using *sodium solution AAS*, suitably diluted with the *strontium chloride solution*, for the standard solutions or, alternately by Method A for flame photometry (2.4.4).

1 g of Sodium Chloride, and of Sodium Citrate is equivalent to 0.3934 and 0.2345 g of Na respectively.

For potassium — Dilute a suitable volume of solution A with a sufficient volume of solution of *strontium chloride* such that the final solution contains a 1500 to 2000-fold excess of strontium ions and determine by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 767 nm and using *potassium solution AAS*, suitably diluted with the *strontium chloride solution*, for the standard solutions or, alternatively by Method A for flame photometry (2.4.4).

1 g of Potassium Chloride is equivalent to 0.5245 g of K.

For total chloride — Titrate 50 ml of solution A with *0.1 M silver nitrate* using *potassium chromate solution* as indicator.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of Cl.

1 g of Sodium Chloride, and of Potassium Chloride is equivalent to 0.6066 and 0.4756 g of Cl respectively.

For citrate — Dissolve 2.0 g in 50 ml of *anhydrous glacial acetic acid* by heating at about 50°. Cool, allow to stand for 10 minutes. Titrate with *0.1 M perchloric acid*, using *1-naphtholbenzein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.006303 g of $C_6H_5O_7$.

1 g of Sodium Citrate is equivalent to 0.6430 g of $C_6H_5O_7$.

For bicarbonate — Titrate 200.0 ml of solution A with *0.1M hydrochloric acid* using *methyl orange solution* as indicator.

1 ml of *0.1M hydrochloric acid* is equivalent to 0.006101 g of HCO_3.

1 g of Sodium Bicarbonate is equivalent to 0.7263 g of HCO_3.

For dextrose — Dissolve 7.5 g in 40 ml of *water*, add 0.5 ml of *dilute ammonia solution*, and dilute to 100.0 ml with *water*. Mix well, allow to stand for 30 minutes, filter the solution, if turbid, and measure the optical rotation in a 2-dm tube (2.4.22). The observed rotation, in degrees, multiplied by 0.9477 or 1.0424 represents the weight in g of $C_6H_{12}O_6$ or $C_6H_{12}O_6,H_2O$ respectively, as appropriate, in the weight taken for the Assay.

Storage. Store protected from moisture in sachets, preferably made of aluminum foil, containing sufficient powder for a single dose or for a day's treatment or for use in hospitals, in bulk containers containing sufficient quantity to produce a volume of solution appropriate to the daily requirements of the hospital concerned.

Labelling. The label states (1) for sachets, the total weights, in g, of each constituent; (2) for bulk containers, the weights, in g, of each constituent in a stated quantity, in g, of the oral powder; (3) the molar concentration in millimoles per litre of sodium, potassium, chloride and citrate ions, and of dextrose as well as the total osmolar concentration in mOsmol per litre of the solution prepared from the oral powder; (4) the total weight of the contents of the container; (5) the directions for use; (6) that any portion of the solution prepared from the oral powder that remains unused for 24 hours after preparation should be discarded; (7) the storage conditions. g, of the oral powder.

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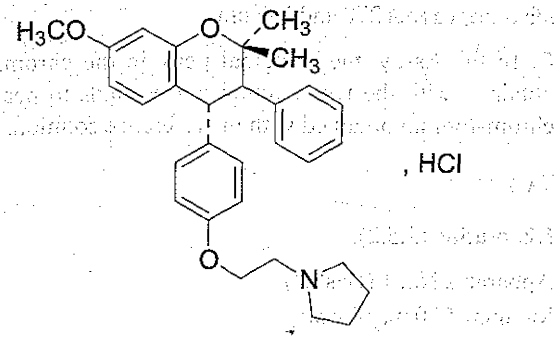
Ormeloxifene Hydrochloride

Centchroman Hydrochloride; Centchroman



$C_{30}H_{35}NO_3,HCl$ Mol. Wt. 493.5

Ormeloxifene Hydrochloride is *trans*-7-methoxy-2,2-dimethyl-3-phenyl-4[4-(2-pyrrolidino-thoxy)phenyl]chroman hydrochloride.

Ormeloxifene Hydrochloride contains not less than 98.5 per cent and not more than 101.5 per cent of $C_{30}H_{35}NO_3,HCl$, calculated on the dried basis.

Category. Antifertility agent (female oral contraceptive).

Description. A white to off-white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ormeloxifene hydrochloride IPRS* or with the reference spectrum of ormeloxifene hydrochloride.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.01 per cent w/v solution in *methanol* shows absorption maxima at about 278 and 282 nm.

C. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 30 volumes of mobile phase A and 70 volumes of mobile phase B.

Test solution. Dissolve 40 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the same solvent.

Reference solution. A 0.0004 per cent w/v solution of *ormeloxifene hydrochloride IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS 3V),
- mobile phase: A. a buffer solution pH 2.5 prepared by dissolving 0.63 g of *ammonium formate* in 1000 ml of *water*, add 2 ml of *triethylamine*, adjusted to pH 2.5 with *formic acid*,

  B. a mixture of 20 volumes of *acetonitrile* and 80 volumes of *methanol*,
- a gradient programme using the conditions given blow,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	40	60
20	30	70
40	30	70
42	40	60
55	40	60

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.6 times the area of the principal peak in the chromatogram obtained with the reference solution (0.3 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore the peak due to cis-isomer at relative retention time of about 0.9 and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

***cis*-Isomer.** Not more than 1.5 per cent of the total content of hydrochlorides of *trans*-and *cis*-isomers determined in the Assay.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 3.5 per cent, determined on 1.0 g by drying in an oven at 105° for 4 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. A 0.004 per cent w/v solution of the substance under examination in the mobile phase.

Reference solution. A solution containing 0.004 per cent w/v of *trans-ormeloxifene hydrochloride IPRS* and 0.0006 per cent w/v of *cis-ormeloxifene hydrochloride IPRS* in the mobile phase.

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Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: 80 volumes of *acetonitrile* and 20 volumes of *water* containing 0.04 per cent w/v solution of *tetramethylammonium hydroxide*, adjusted to pH 7.6 with *orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject the reference solution and use an attenuation such that the response for the principal peak due to *trans*-ormeloxifene hydrochloride is more than 50 per cent of the full-scale deflection of the recorder. Make a total of six injections. When the chromatograms are recorded under the conditions described above, trans-ormeloxifene hydrochloride is eluted before *cis*-ormeloxifene hydrochloride.The test is not valid unless the relative standard deviation of six replicate injections is not more than 6 per cent and the resolution between the peaks due to *cis*-ormeloxifene hydrochloride and *trans*-ormeloxifene hydrochloride is more than 1. If necessary, adjust the proportions and the flow rate of the mobile phase to obtain proper resolution. Inject a suitable volume of test solution. From the average peak areas of the six replicate analyses calculate the content of *trans*-ormeloxifene hydrochloride and *cis*-ormeloxifene hydrochloride in the substance under examination by comparing with the peak responses for *trans*-ormeloxifene hydrochloride and *cis*-ormeloxifene hydrochloride respectively obtained with reference solution.

Storage. Store protected from moisture.

Ormeloxifene Tablets

Ormeloxifene Hydrochloride Tablets; Centchroman Hydrochloride Tablets; Centchroman Tablets

Ormeloxifene Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of Ormeloxifene hydrochloride, $C_{30}H_{35}NO_3$,HCl.

Usual strength. 30 mg.

Identification

Shake a quantity of the powdered tablets containing 0.1 g of Ormeloxifene Hydrochloride with 10 ml of *chloroform*, filter and evaporate the filtrate to dryness at a pressure not exceeding 0.7 kPa. The residue complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ormeloxifene hydrochloride IPRS* or with the reference spectrum of ormeloxifene hydrochloride.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.01 per cent w/v solution in *methanol* shows absorption maxima at about 278 and 282 nm.

C. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of *water*,
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted if necessary, at the maximum at about 278 nm (2.4.7). Calculate the content of $C_{30}H_{35}NO_3$,HCl in the medium from the absorbance obtained from a solution of known concentration of *Ormeloxifene hydrochloride IPRS* in *water*.

Q. Not less than 70 per cent of the stated amount of $C_{30}H_{35}NO_3$,HCl in the medium.

***cis-Isomer**. Not more than 1.5 per cent of the total content of ormeloxifene hydrochlorides of trans- and cis-isomers determined under Assay.*

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.17).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.1 g of Ormeloxifene Hydrochloride with three quantities, each of 5 ml, of *methanol*, centrifuge each extract, dilute the combined extracts to 25 ml with *methanol* and then dilute 250 µl of the resulting solution to 25 ml with the mobile phase.

Reference solution. A solution containing 0.004 per cent w/v of *trans-ormeloxifene hydrochloride IPRS* and 0.0006 per cent w/v of *cis-ormeloxifene hydrochloride IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: 80 volumes of *acetonitrile* and 20 volumes of *water* containing 0.04 per cent w/v solution of *tetramethylammonium hydroxide*, adjusted to pH 7.6 with *phosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

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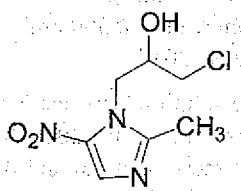
Inject the reference solution. The test is not valid unless the relative standard deviation of six replicate injections is not more than 6 per cent and the resolution between the peaks due to *cis*-ormeloxifene hydrochloride and *trans*-ormeloxifene hydrochloride is more than 1. If necessary, adjust the proportions and the flow rate of the mobile phase to obtain proper resolution. Inject a suitable volume of test solution and record the response. From the average peak areas of the six replicate analyses calculate the content of *trans*-ormeloxifene hydrochloride and *cis*-ormeloxifene hydrochloride in the substance under examination.

Storage. Store protected from moisture.

Ornidazole



$C_7H_{10}ClN_3O_3$ Mol. Wt. 219.6

Ornidazole is (*RS*)-1-chloro-3-(2-methyl-5-nitroimidazol-1-yl)propan-2-ol.

Ornidazole contains not less than 98.0 per cent and not more than 101.0 per cent of $C_7H_{10}ClN_3O_3$, calculated on the anhydrous basis.

Category. Antiamoebic.

Description. A white to yellowish white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ornidazole IPRS* or with the reference spectrum of ornidazole.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution in *0.1 M hydrochloric acid* shows an absorption maximum at about 277 nm; absorbance at 277 nm, 0.580 to 0.630.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in 25.0 ml of the mobile phase.

Reference solution (a). A 0.001 per cent w/v solution of *2-methyl-5-nitroimidazole IPRS* in the mobile phase.

Reference solution (b). A 0.001 per cent w/v solution of *ornidazole IPRS* in the mobile phase.

Reference solution (c). Dilute 20.0 ml of reference solution (a) and 10.0 ml of reference solution (b) to 200.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 70 volumes of *0.01M potassium dihydrogen orthophosphate* and 30 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 318 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the resolution between the peaks corresponding to 2-methyl-5-nitroimidazole and ornidazole is at least 1.5.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to 2-methyl-5-nitroimidazole is not more than 0.2 times the area of the principal peak obtained with reference solution (a) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 0.5 g.

Assay. Dissolve 0.15 g in 50 ml of *glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent of 0.02196 g of $C_7H_{10}ClN_3O_3$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Ornidazole Injection

Ornidazole Injection is a sterile solution of Ornidazole in propylene glycol or other suitable glycol, in a suitable mixture of these. It contains suitable alcohols.

Ornidazole Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ornidazole, $C_7H_{10}ClN_3O_3$.

Usual strength. 500 mg per 3 ml.

Description. A clear, light yellow coloured solution.

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Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to peak in the chromatogram obtained with reference solution.

Tests

Appearance of solution. A solution containing 0.5 g Ornidazole per 3 ml in a mixture of equal volumes of *propylene glycol* and *ethanol* is clear (2.4.1) and not more than intensely coloured than YS5 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Use freshly prepared solutions and carry out the test protected from light.

Test solution. Dilute a volume of injection with the mobile phase to obtain a solution containing 0.1 per cent w/v of Ornidazole.

Reference solution (a). A 0.5 per cent w/v solution of *2-methyl-5-nitroimidazole IPRS* with the mobile phase.

Reference solution (b). A 0.001 per cent w/v solution of *ornidazole IPRS* with the mobile phase.

Reference solution (c). Dilute 20.0 ml of reference solution (a) and 10.0 ml of reference solution (b) to 200.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 70 volumes of *0.01 M potassium dihydrogen phosphate* and 30 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 318 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the resolution between the peaks corresponding to 2-methyl-5-nitroimidazole and ornidazole is not less than 1.5.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to 2-methyl-5-nitroimidazole is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

Bacterial endotoxins (2.2.3). Not more than 0.7 Endotoxin Unit per mg of ornidazole.

Other tests. Comply with the tests stated under Parenteral Preparation (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 50 volumes of *methanol* and 50 volumes of *water*.

Test solution. Dilute a volume of injection with the solvent mixture to obtain a solution containing 0.0033 per cent w/v of Ornidazole.

Reference solution. A 0.0033 per cent w/v solution of *ornidazole IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 90 volumes of a 0.136 per cent w/v solution of *potassium dihydrogen orthophosphate* in *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 10 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 300 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_7H_{10}ClN_3O_3$ in the injection.

Storage. Store protected from light and moisture.

Labelling. The label states (1) the nature and composition of the solvent; (2) that it is meant for intramuscular injection only.

Ornidazole Tablets

Ornidazole Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of ornidazole, $C_7H_{10}ClN_3O_3$.

Usual strength. 500 mg.

Identification

Extract a quantity of the powdered tablets containing 0.1 g of Ornidazole with 40 ml of *chloroform*, filter and evaporate the filtrate to dryness. The residue complies with the following test.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ornidazole IPRS* or with the reference spectrum of ornidazole.

B. Heat a quantity of the powdered tablets containing 0.1 g of Ornidazole with 10 mg of *zinc powder*, 1 ml of *water* and

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0.25 ml *hydrochloric acid* for 5 minutes, cool in ice, add 0.5 ml of *sodium nitrite solution* and remove the excess of nitrite with *sulphuric acid*. Add 0.5 ml of *2-naphthol solution* and 2 ml of *5 M sodium hydroxide solution*. An orange-red colour is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 75 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter promptly, rejecting the first few ml of the filtrate and dilute a suitable volume of the filtrate with *0.1 M hydrochloric acid*. Measure the absorbance of the resulting solution at the maximum at about 277 nm (2.4.7). Calculate the content of ornidazole, $C_7H_{10}ClN_3O_3$ in the medium from the absorbance obtained from a solution of known concentration of *ornidazole IPRS*.

Q. Not less than 80 per cent of the stated amount of $C_7H_{10}ClN_3O_3$ in the medium.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 25 mg of ornidazole in 25.0 ml of the mobile phase.

Reference solution (a). A 0.001 per cent w/v solution of *2-methyl-5-nitroimidazole IPRS* in the mobile phase.

Reference solution (b). A 0.001 per cent w/v solution of *ornidazole IPRS* in the mobile phase.

Reference solution (c). Dilute 20.0 ml of reference solution (a) and 10.0 ml of reference solution (b) to 200.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 70 volumes of *0.01M potassium dihydrogen orthophosphate* and 30 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 318 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the resolution between the peaks corresponding to 2-methyl-5-nitroimidazole and ornidazole is at least 2.0.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to 2-methyl-5-nitroimidazole is not more than 0.2 times the area of the principal peak obtained with reference solution (a) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than the area of the peak in the chromatogram obtained with reference solution (b) (1.0 per cent).

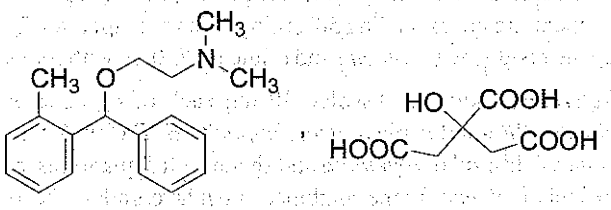
Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.2 g of Ornidazole, transfer to a sintered-glass crucible and extract with 10 ml of hot *acetone*. Repeat the extraction 6 times with hot *acetone*. Cool, add to the combined extracts 50 ml of *acetic anhydride*. Titrate with *0.1 M perchloric acid*, determining the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02196 g of $C_7H_{10}ClN_3O_3$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Orphenadrine Citrate



$C_{18}H_{23}NO,C_6H_8O_7$ Mol. Wt. 461.5

Orphenadrine Citrate is (*RS*)-dimethyl[2-(2-methylbenzhydryloxy)ethyl]amine dihydrogen citrate.

Orphenadrine Citrate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{18}H_{23}NO$, $C_6H_8O_7$, calculated on the dried basis.

Category. Skeletal muscle relaxant.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests B, C and D may be omitted if tests A and E are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *orphenadrine citrate IPRS*.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.06 per cent w/v solution in *ethanol (95 per cent)* shows absorption maxima at 258 nm, 264 nm and 271 nm; absorbances at the maxima are about 0.68, 0.72 and 0.47 respectively.

C. Dissolve 50 mg in 10 ml of *ethanol (50 per cent)*, add 10 ml of *picric acid solution* and allow to stand. The precipitate

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after recrystallisation from *ethanol (95 per cent)*, melts at about 89° or at about 107° (2.4.21).

D. Dissolve 5 mg in 2 ml of *sulphuric acid*; an orange-red colour is produced.

E. To 1 g add 10 ml *water* and 2 ml of *5 M sodium hydroxide*, shake with two quantities, each of 10 ml, of *chloroform* and discard the chloroform. Heat the aqueous solution to boiling with an excess of *mercuric sulphate solution*, filter if necessary and boil the resulting solution with 0.2 ml of *dilute potassium permanganate solution*; the solution is decolorised and a white precipitate is produced.

Tests

Related substances. Determine by gas chromatography (2.4.13).

Test solution. Dissolve 0.5 g of the substance under examination in *water* and dilute to 50 ml with *water*. Add 2 ml of *ammonia* and shake with 3 quantities, each of 10 ml of *toluene*. To the combined upper layers add *anhydrous sodium sulphate*, shake, filter and evaporate the filtrate, at a temperature not exceeding 50°, using a rotary evaporator. Take up the residue with *toluene* and dilute to 20.0 ml with *toulene*.

Reference solution. Dissolve 30 mg each of *orphenadrine citrate IPRS* and *orphenadrine impurity E IPRS* in 20 ml of *water*. Add 1 ml of *ammonia* and shake with 3 quantities, each of 5 ml of *toluene*. To the combined upper layers add *anhydrous sodium sulphate*, shake, filter and evaporate the filtrate, at a temperature not exceeding 50°, using a rotary evaporator. Take up the residue with *toluene* and dilute to 20.0 ml with *toluene*.

Chromatographic system
- a capillary column 60 m x 0.32 mm packed with poly(dimethyl)(diphenyl)siloxane (film thickness 1.0 µm),
- temperature:
  column. 240°,
  inlet port and detector at 290°,
- flame ionization detector,
- split ratio. 1: 25,
- flow rate: 1 ml per minute using nitrogen as the carrier gas.

Name	Relative retention time
Orphenadrine impurity B[1]	0.5
Orphenadrine impurity A[2]	0.6
Orphenadrine impurity D[3]	0.8
Orphenadrine impurity C[4]	0.9
Orphenadrine impurity E[5]	0.98
Orphenadrine (Retention time: about 13 minutes)	1.0
Orphenadrine impurity F[6]	1.1

[1]2-methylbenzophenone,
[2]2-methylbenzhydrol,
[3]diphenhydramine,
[4](*RS*)-2-[(2-methylphenyl)phenylmethoxy]ethanamine,
[5]meta-methylbenzyl isomer,
[6]para-methylbenzyl isomer.

Inject 2 µl of the reference solution. The test is not valid unless the resolution between the peaks due to orphenadrine impurity E and orphenadrine is not less than 1.5.

Inject 2 µl of the reference solution and the test solution. Run the chromatogram 1.3 times the retention time of the principal peak, the area of any peak corresponding to orphenadrine impurities A, B, C, D, E, F, each of, is not more than 0.3 per cent. The area of any other secondary peak is not more than 0.1 per cent and the sum of areas of all the secondary peaks is not more than 1.0 per cent. Ignore any peak with an area less than 0.05 per cent, calculated by area normalization.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm). Use 2 ml of lead standard solution (*10 ppm Pb*) to prepare the standard.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

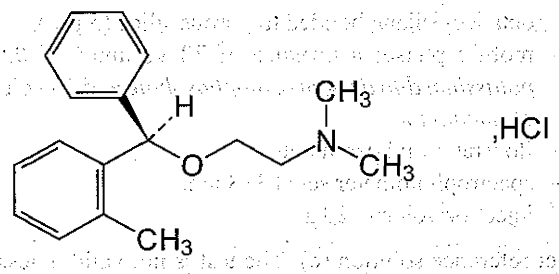
Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 1.0 g in 30 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.04615 g of $C_{18}H_{23}NO$, $C_6H_8O_7$.

Storage. Store protected from light.

Orphenadrine Hydrochloride



$C_{18}H_{23}NO,HCl$ Mol. Wt. 305.8

Orphenadrine Hydrochloride is (*RS*)-dimethyl [2-(2-methyl-benzhydryloxy) ethyl]amine hydrochloride.

Orphenadrine Hydrochloride contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{18}H_{23}NO,HCl$, calculated on the dried basis.

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Category. Antiparkinsonian.

Description. A white or almost white, crystalline powder.

Identification

Tests A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if tests A is carried out.

A. Determine by infrared absorption spectrophotometry,(2.4.6). Compare the spectrum with that obtained with *orphenadrine hydrochloride IPRS.*

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.06 per cent *w/v* solution in *ethanol (95 per cent)* shows three absorption maxima at about 258 nm,264 nm and 271 nm; absorbances at the maxima, about 1.07, 1:13 and 0.73 respectively.

C. Dissolve about 5 mg in 2 ml of *sulphuric acid;* an orange-red colour is produced.

D. Dissolve about 50 mg in 10 ml of *ethanol (95per cent),* add 10 ml of *picric acid solution* and allow to stand. The precipitate, after recrystallisation from *ethanol (95 per cent)* melts at about 89° or at about 107°(2.4.21).

Tests

Related substances. Determine by gas chromatography (2.4.13).

Test solution. Dissolve 0.3 g of the substance under examination in *water* and dilute to 50 ml with *water.* Add 2 ml of *ammonia* and shake with 3 quantities, each of 10 ml of *toluene.* To the combined upper layers add *anhydrous sodium sulphate,* shake, filter and evaporate the filtrate, at a temperature not exceeding 50°, using a rotary evaporator. Take up the residue with *toluene* and dilute to 20.0 ml with *toulene.*

Reference solution. Dissolve 20 mg each of *orphenadrine hydrochloride IPRS* and *orphenadrine impurity E IPRS* in 20 ml of *water.* Add 1 ml of *ammonia* and shake with 3 quantities, each of 5 ml of *toluene.* To the combined upper layers add *anhydrous sodium sulphate,* shake, filter and evaporate the filtrate, at a temperature not exceeding 50°, using a rotary evaporator. Take up the residue with *toluene* and dilute to 20.0 ml with *toluene.*

Chromatographic system

- a capillary column 60 m x 0.32 mm packed with poly(dimethyl)(diphenyl)siloxane (film thickness 1.0 µm),
- temperature:
  column. 240°,
  inlet port and detector at 290°,
- flame ionization detector,
- split ratio. 1:25,
- flow rate: 1 ml per minute using nitrogen as thecarrier gas.

Name	Relative retention time
Orphenadrine impurity B[1]	0.5
Orphenadrine impurity A[2]	0.6
Orphenadrine impurity D[3]	0.8
Orphenadrine impurity C[4]	0.9
Orphenadrine impurity E[5]	0.98
Orphenadrine (Retention time: about 13 minutes)	1.0
Orphenadrine impurity F[6]	1.1

[1]2-methylbenzophenone,
[2]2-methylbenzhydrol,
[3]diphenhydramine,
[4](*RS*)-2-[(2-methylphenyl)phenylmethoxy]ethanamine,
[5]meta-methylbenzyl isomer,
[6]para-methylbenzyl isomer.

Inject 2 µl of the reference solution. The test is not valid unless the resolution between the peaks due to orphenadrine impurity E and orphenadrine is not less than 1.5.

Inject 2 µl of the reference solution and the test solution. Run the chromatogram 1.3 times the retention time of the principal peak, the area of any peak corresponding to orphenadrine impurities A, B, C, D, E, F, each of, is not more than 0.3 per cent. The area of any other secondary peak is not more than 0.1 per cent and the sum of areas of all the secondary peaks is not more than 1.0 per cent. Ignore any peak with an area less than 0.05 per cent, calculated by area normalization.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm). Use 2 ml of lead standard solution (*10 ppm Pb*) to prepare the standard.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 1.0 g in 20 ml of *anhydrous glacial acetic acid,* add 20 ml of *mercuric acetate solution* and titrate with *0.1 M perchloric acid* using *1-naphtholbenzene solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03058 g of $C_{18}H_{23}NO,HCl$.

Storage. Store protected from light.

Orphenadrine Tablets

Orphenadrine Hydrochloride Tablets

Orphenadrine Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of orphenadrine hydrochloride, $C_{18}H_{23}NO,HCl$.

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Usual strength. 50 mg.

Identification

Extract a quantity of the powdered tablets containing about 0.15 g of Orphenadrine Hydrochloride with *chloroform*, filter and evaporate the filtrate to dryness. The residue complies with the following tests.

A. Dissolve 5 mg in 2 ml of *sulphuric acid;* an orange-red colour is produced.

B. Dissolve 50 mg in 10 ml of *ethanol (50 per cent)*, add 10 ml of *picric acid solution* and allow to stand. The precipitate after recrystallisation from *ethanol (95 per cent)*, melts at about 89° or at about 107° (2.4.21).

C. A 5.0 per cent w/v solution gives reaction (A) of chlorides (2.3.1).

Tests

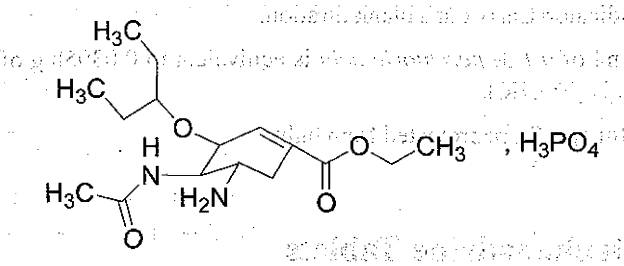
Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 70 mg Orphenadrine Hydrochloride and dissolve as completely as possible in a mixture of 5 ml of *water* and 5 ml of *2 M hydrochloric acid.* Without delay extract with four quantities, each 15 ml, of *chloroform*, filter the combined extracts and evaporate to about 20 ml. Add 30 ml of *anhydrous glacial acetic* and 2 ml of *mercuric acetate solution* and titrate with *0.02 M perchloric acid* determining the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.02 M perchloric acid* is equivalent to 0.006117 g of $C_{18}H_{23}NO,HCl$.

Storage. Store protected from light and moisture.

Oseltamivir Phosphate



$C_{16}H_{28}N_2O_4,H_3PO_4$ Mol. Wt. 410.4

Oseltamivir Phosphate is phosphoric acid salt of ethyl (3*R*,4*R*,5*S*)-4-(acetylamino)-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate.

Oseltamivir Phosphate contain not less than 98.0 per cent and not more than 102.0 per cent of $C_{16}H_{28}N_2O_4,H_3PO_4$, calculated on the anhydrous basis.

Category. Antiviral.

Description. A white or off-white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oseltamivir phosphate IPRS* or with the reference spectrum of oseltamivir phosphate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). −48.0° to −42.0°, determined in a 1.0 per cent w/v solution in *methanol*.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Dissolve 25 mg of the substance under examination in 25 ml of the mobile phase.

Reference solution (a). A 0.1 per cent w/v solution of *oseltamivir phosphate IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as YMC pack PRO C8),
- column temperature 50°,
- mobile phase: a mixture of 66 volumes of a buffer solution prepared by dissolving 6.8 g of *anhydrous monobasic potassium phosphate* in 1000 ml of *water* and adjusted to pH 6.0 with *dilute sodium hydroxide*, 24.5 volumes of *methanol* and 23.5 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 207 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of all the secondary peaks is not more

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than the area of the peak in the chromatogram obtained with reference solution (b) (1.0 per cent).

Phosphoric acid. 23.4 to 24.4 per cent, calculated on a anhydrous basis.

Dissolve 0.2 g in 40 ml of distilled *water*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.0049 g of phosphoric acid.

Heavy metals (2.3.13). 1 g complies with the limit test for heavy metals, Method A (20 ppm).

Water (2.3.43). Not more than 0.5 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of the mobile phase. Dilute 10.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.02 per cent w/v solution of *oseltamivir phosphate IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as YMC pack PRO C8),
- column temperature: 50°,
- mobile phase: a mixture of 66 volumes of *buffer solution* prepared by dissolving 6.8 g of *anhydrous monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 6.0 with *dilute sodium hydroxide*, 24.5 volumes of *methanol* and 23.5 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 207 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency in not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{28}N_2O_4,H_3PO_4$.

Storage. Store protected from light and moisture.

Oseltamivir Capsules

Oseltamivir Phosphate Capsules

Oseltamivir Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of oseltamivir, $C_{16}H_{28}N_2O_4$.

Usual strength. 75 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with dissolution medium.

Reference solution. Dissolve 50 mg of *oseltamivir phosphate IPRS* in 20 ml of the mobile phase and dilute to 100 ml with the dissolution medium. Dilute 5.0 ml of the solution to 25.0 ml with the dissolution medium.

Use the chromatographic system described under Assay.

Q. Not less than 80 per cent of the stated amount of $C_{16}H_{28}N_2O_4$ in the medium.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

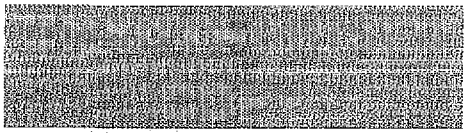
Test solution. Weigh a quantity of the mixed contents of 20 capsules containing about 50 mg of oseltamivir, disperse in 50 ml of mobile phase and filter.

Reference solution (a). Dissolve a quantity of *oseltamivir phosphate IPRS* in the mobile phase to obtain a solution equivalent to 0.1per cent w/v of oseltamivir.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as YMC pack PRO C8),
- mobile phase: a mixture of 66 volumes of a buffer solution prepared by dissolving 6.8 g of *anhydrous monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 6.0 with *dilute sodium hydroxide*, 24.5 volumes of *methanol* and 23.5 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 207 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.



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Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Weigh a quantity of the mixed contents of 20 capsules containing about 200 mg of oseltamivir, disperse in 100.0 ml of the mobile phase and filter. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. Dissolve a quantity of *oseltamivir phosphate IPRS* in the mobile phase to obtain a solution equivalent to 0.02 per cent w/v of oseltamivir.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 66 volumes of a buffer solution prepared by dissolving 6.8 g of *anhydrous monobasic potassium phosphate* in 1000 ml of *water* and adjusted to pH 6.0 with *dilute sodium hydroxide*, 24.5 volumes of *methanol* and 23.5 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 207 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency is not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{28}N_2O_4$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of oseltamivir.

Oseltamivir Oral Suspension

Oseltamivir Phosphate Oral Suspension

Oseltamivir Oral Suspension is a mixture consisting of Oseltamivir Phosphate with buffering agents and other excipients. It contains a suitable flavouring agent. It is filled in a sealed container.

The suspension is constituted by dispersing the contents of the sealed container in the specified volume of water just before use.

Oseltamivir Oral Suspension contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of oseltamivir $C_{16}H_{28}N_2O_4$.

The contents of the sealed container comply with the following requirement.

Usual strength. 15 mg per ml.

Water (2.3.43). Not more than 2.5 per cent, determined on 1.0 g.

The constituted suspension complies with the requirements stated under Oral liquids and with the following requirements.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (b).

B. To 2 ml of the reconstituted suspension, add 2 ml of *dilute nitric acid* and 4 ml of a 10 per cent w/v solution of *ammonium molybdate* and warm the solution. A bright yellow precipitate is formed.

Tests

pH (2.4.24). 3.0 to 5.0.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Weigh a quantity of the suspension containing 25 mg of oseltamivir, dissolve in 25.0 ml of the mobile phase and filter.

Reference solution (a). Dissolve a quantity of *oseltamivir phosphate IPRS* in the mobile phase to obtain a solution equivalent to 0.1 per cent w/v of oseltamivir.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: 70 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.0 with *dilute sodium hydroxide*, 15 volumes of *methanol* and 15 volumes of *acetonitrile*,

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- flow rate: 1.5 ml per minute,
- spectrophotometer set at 207 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogra tained with reference solution (b) (1.0 per cent) and the sum of areas of all the secondary peaks is not more than 3 times the area of the peak in the chromatogram obtained with reference solution (b) (3.0 per cent).

Other tests. Comply with the tests stated under Oral liquids.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Weigh a quantity of the suspension containing 60 mg of oseltamivir, dissolve in 250.0 ml of the mobile phase and filter. Dilute 10.0 ml of the solution to 25.0 ml with the mobile phase and filter.

Reference solution. A 0.0125 per cent w/v solution of *oseltamivir phosphate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.0 with *dilute sodium hydroxide*, 24.5 volumes of *methanol* and 23.5 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 207 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency in not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

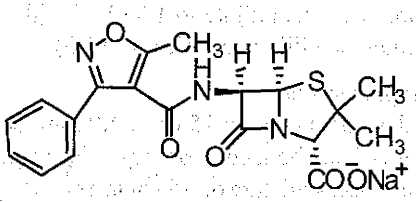
Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{28}N_2O_4$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states (1) the quantity of active ingredient in terms of the equivalent amount of oseltamivir; (2) the temperature of storage and the period during which the constituted suspension may be expected to be satisfactory for use.

Oxacillin Sodium



$C_{19}H_{18}N_3NaO_5S,H_2O$ Mol. Wt. 441.4

Oxacillin Sodium is Sodium 6-(5-methyl-3-phenyl-1,2-oxazole-4-carboxamido)penicillanate.

Oxacillin Sodium contains not less than 95.0 per cent and not more than 102.0 per cent of $C_{19}H_{18}N_3NaO_5S$, calculated on the anhydrous basis.

Category. Antibacterial.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oxacillin sodium monohydrate IPRS* or with the reference spectrum of oxacillin sodium monohydrate.

B. It gives reaction (A) of sodium salt (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution is clear (2.4.1) and its absorbance at 430 nm (2.4.7) is not more than 0.1, determined on 10.0 per cent w/v solution in *water*.

pH (2.4.24). 4.5 to 7.5, determined on 3.0 per cent w/v solution.

Specific optical rotation (2.4.22). +196° to +212°, determined in a 1.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution (a). Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase.

Test solution (b). Dilute 5.0 ml of test solution (a) to 50.0 ml with the mobile phase.

Reference solution (a). A 0.01 per cent w/v solution of *oxacillin sodium monohydrate IPRS* in the mobile phase.

Reference solution (b). Dilute 5.0 ml of test solution (b) to 50.0 ml with the mobile phase.

Reference solution (c). A solution containing 0.01 per cent w/v each of *oxacillin impurity E IPRS* (*cloxacillin sodium IPRS*) and *oxacillin sodium monohydrate IPRS* in the mobile phase.

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Reference solution (d). Dissolve 25 mg of the substance under examination in 1 ml of *0.05 M sodium hydroxide,* allow to stand for 3 minutes, then dilute to 100.0 ml with the mobile phase (generates oxacillin impurities B and D).

Chromatographic system
- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 75 volumes of a 0.27 per cent w/v solution of *potassium dihydrogen phosphate*, adjusted to pH 5.0 with *sodium hydroxide solution* and 25 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Name	Relative retention time
Oxacillin impurity A[1]	0.3
Oxacillin impurity B[2]	0.4
Oxacillin impurity B[3]	0.5
Oxacillin impurity C[4]	0.65
Oxacillin impurity D[5]	0.9
Oxacillin (Retention time: about 5 minutes)	1.0
Oxacillin impurity E[6]	1.5
Oxacillin impurity F[7]	1.9
Oxacillin impurity G[8]	2.1
Oxacillin impurity H[9]	3.5
Oxacillin impurity I[10]	3.8
Oxacillin impurity J[11]	5.8

[1]6-aminopenicillanic acid,
[2]isomer 1: penicilloic acid of oxacillin,
[3]isomer 2: penicilloic acid of oxacillin,
[4]5-methyl-3-phenylisoxazole-4-carboxylic acid,
[5]2 isomers: Penilloic acids of oxacillin,
[6]cloxacillin,
[7]thiooxacillin,
[8]cloxacillin isomer,
[9]penillic acid of oxacillin,
[10]6-APA oxacillin amide,
[11]ozolamide of 6-APA dimer.

Inject reference solution (c). The test is not valid unless the resolution between the peaks corresponding to oxacillin and oxacillin impurity E is not less than 2.5.

Inject reference solution (d). The two principal peaks eluting before oxacillin peak are due to oxacillin impurities B and D.

Inject reference solution (b) and test solution (a). Run the chromatogram 7 times the retention time of principal peak.In the chromatogram obtained with test solution (a), the sum of the areas of 2 isomer peaks of oxacillin impurity B is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent). The area of the peak corresponding to oxacillin impurity E is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and the sum of the areas of 2 isomer peaks of oxacillin impurity D, oxacillin impurities F, G, I and J, each of, is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). The area of any other secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Ethyl acetate and butyl acetate. Not more than 1.0 per cent of ethyl acetate and not more than 1.0 per cent of butyl acetate.

Determine by gas chromatography (2.4.13).

Test solution. Dissolve 0.2 g of the substance under examination in 6.0 ml of *water*.

Reference solution. Dissolve 83 mg of *ethyl acetate* and 83 mg of *butyl acetate* in *water* and dilute to 250.0 ml with *water*. Use 6.0 ml of the solution.

NOTE — Close the vials immediately with a rubber membrane stopper coated with polytetrafluoroethylene and secured with an aluminium crimped cap. Mix to obtain a homogeneous solution.

Chromatographic system
- a fused silica column 50 m x 0.32 mm, packed with poly(dimethyl)siloxane (film thickness 5 µm),
- temperature:

column	time (min)	temperature (°)
	0-6	70
	6-16	70 → 220
	16-18	220

- inlet port at 140° and detector at 250°,
- flow rate: 2 ml per minute using nitrogen as carrier gas.

Head-space conditions:
- equilibration temperature: 80°,
- equilibration time. 60 minutes,
- transfer line temperature. 140°,
- pressurization time. 30 seconds.
- flame ionization detector.

The retention time of ethyl acetate is about 10 minutes and of butyl acetate is about 15.5 minutes.

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Inject the reference solution and the test solution.

Calculate the content of ethyl acetate and butyl acetate.

***N,N*-Dimethylaniline** (2.3.21). Not more than 20 ppm, determined by method B.

2-Ethylhexanoic acid (2.3.51). Not more than 0.8 per cent.

Water (2.3.43). 3.0 per cent to 5.0 per cent, determined on 0.3 g.

Oxacillin Sodium intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.20 Endotoxin Unit per mg of oxacillin sodium.

Assay. Determine by liquid chromatography (2.4.14) as described under Related substances with the following modification.

Inject reference solution (a) and test solution (b).

Calculate the content of $C_{19}H_{18}N_3NaO_5S$.

Oxacillin Capsules

Oxacillin Capsules contain not less than 90.0 per cent and not more than 120.0 per cent of the stated amount of oxacillin, $C_{19}H_{19}N_3O_5S$.

Usual strengths. 250 mg; 500 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of *water*,
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Dilute the filtrate, if necessary, with the same solvent. Measure the absorbance of the solution at the maxima at about 225 nm (2.4.7). Calculate the content of $C_{19}H_{19}N_3O_5S$ in the medium from the absorbance obtained from a solution of known concentration of *oxacillin sodium IPRS*.

Q. Not less than 75 per cent of the stated amount of $C_{19}H_{19}N_3O_5S$ in the medium.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Dissolve a quantity of the contents of 20 capsules containing 100 mg of Oxacillin in *water* and dilute to 100.0 ml with *water*.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with *water*.

Chromatographic system
- a stainless steel column 30 cm x 4 mm, packed with phenyl groups bonded to porous silica (5 µm),
- mobile phase: dissolve 1.9 g of *monobasic potassium phosphate* in 700 ml of *water*, add 300 ml of *acetonitrile* and 100 ml of *methanol*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Disperse a quantity of the mixed contents of 20 capsules containing 100 mg of Oxacillin with *water* with the aid of ultrasound for 10 minutes and dilute to 100.0 ml with *water*, filter. Dilute 10.0 ml of the filtrate to 100.0 ml with *water*.

Reference solution. A 0.011 per cent w/v solution of *oxacillin sodium IPRS* in *water*.

Use chromatographic system as described under Related substances.

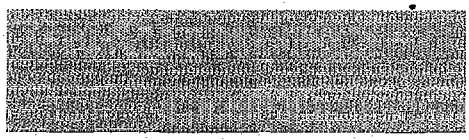
Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{19}N_3O_5S$ in the capsules.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the quantity of the active ingredient in terms of the equivalent amount of oxacillin.



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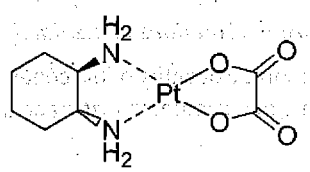
Oxaliplatin



$C_8H_{14}N_2O_4Pt$ Mol. Wt. 397.3

Oxaliplatin is *cis*-[(1*R*, 2*R*)-1,2-cyclohexanediamine-*N*,*N*']][oxalato(2-)-*O*,*O*']platinum.

Oxaliplatin contains not less than 98.0 per cent and not more than 102.0 per cent of $C_8H_{14}N_2O_4Pt$, calculated on the dried basis.

Category. Anticancer

Description. A white to off white, crystalline powder.

CAUTION — Oxaliplatin is potentially cytotoxic; Great care should be taken in handling the powder and preparing solutions.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oxaliplatin IPRS* or with the reference spectrum of oxaliplatin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (f).

Tests

Acidity. Dissolve 100.0 mg in 50 ml of *carbon dioxide-free water* and add 0.5 ml of *phenolphthalein solution*. The solution is colourless. Not more than 0.6 ml of *0.01M sodium hydroxide* is required to change the colour of the solution to pink.

Specific optical rotation (2.4.22). +74.5° to +78.0°, determined in a 0.5 per cent w/v solution in *water*, measured at 20°.

Related substances. A. Determine by liquid chromatography (2.4.14).

NOTE — Use vigorous shaking and very brief sonication to dissolve the substance to be examined. Inject the test solution within 20 minutes of preparation. Polypropylene autosampler vials should be used.

Test solution. Dissolve 50.0 mg of the substance under examination in 25.0 ml of *water*.

Reference solution (a). 0.006 per cent solution of *oxaliplatin related compound A IPRS* (oxalic acid dihydrate) in *water*.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 20.0 ml with *water*.

Reference solution (c). Transfer 25.0 ml of reference solution (a) into 100-ml volumetric flask and add 2.0 ml of 0.005 per cent w/v solution of *sodium nitrate* and dilute to volume with *water*.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 10.0 ml with *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: 20 volumes of *acetonitrile*, and 80 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in about 100.0 ml of *water*, add 10.0 ml of 10 per cent *tetrabutylammonium hydroxide* in *water* and dilute with *water* to 1000 ml, adjusted to pH 6.0 with *orthophosphoric acid*.
- flow rate: 2 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

Inject reference solution (b), (c) and (d). The test is not valid unless the resolution between the peaks due to oxalic acid dihydrate and sodium nitrate in the chromatogram obtained with reference solution (c) is not less than 2.0. In the chromatogram obtained with reference solution (b), the relative standard deviation for replicate injections is not more than 3.0 per cent. The signal-to-noise ratio for the oxaliplatin related compound A peak in the chromatogram obtained with reference solution (d) is not less than 10.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of the peak due to oxalic acid dihydrate is not more than 0.133 times the area of the peak due to oxalic acid dihydrate in the chromatogram obtained with reference solution (b) (0.1 per cent).

B. Determine by liquid chromatography (2.4.14).

NOTE — Use vigorous shaking and very brief sonication to dissolve the substance to be examined. Inject the test solution within 20 minutes of preparation. Polypropylene glassware should be used.

Buffer Solution. Dissolve 2.72 g of *monobasic potassium phosphate* (anhydrous) and 1.80 g of *1-pentanesulfonic acid sodium salt* in 2000 ml of *water*. Add 0.5 ml of *triethylamine* to the solution, and mix thoroughly. Adjusted to pH 4.3 with *orthophosphoric acid*.

Test solution. Dissolve 50.0 mg of the substance under examination in 25.0 ml of *water*.

Reference solution (a). 0.05 per cent w/v solution of *oxaliplatin IPRS* in *water*.

Reference solution (b). Dissolve 12.5 mg of *oxaliplatin related compound B IPRS* [(*SP*-4-2-(1*R*-*trans*)-(1,2-Cyclohexan-

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ediamine-*N,N'*) dinitratoplatinum(II)] in 25.0 ml *methanol* and sonicate for 2 minutes. Add 65.0 ml of *0.001 M nitric acid* and sonicate for additional 30 minutes. Cool and dilute to 100.0 ml.

NOTE — Oxaliplatin Related Compound B IPRS is converted to (SP-4-2)-diaqua[(1R ,2R)-cyclohexane-1,2-diamine-N,N'] platinum during preparation of the solution.

Reference solution (c). A 0.01 per cent w/v solution of *oxaliplatin related compound C IPRS* in *water.*

Reference solution (d). A 0.2 per cent w/v solution of *oxaliplatin IPRS* in *0.005 M sodium hydroxide solution.* Allow it to stand at room temperature for at least 5 days. Transfer 10.0 ml of the solution, 10.0 ml of reference solution (b) and 5.0 ml of reference solution (c) into a 100-ml volumetric flask and dilute with *water* to the volume.

NOTE— The preparation of the reference solution (d) forms diaquodiaminocyclohexaneplatinum dimer.

Reference solution (e). Transfer 1.0 ml of reference solution (a), 4.0 ml of reference solution (b) and 2.0 ml of reference solution (c) into a 50-ml volumetric flask and dilute with *water* to the volume.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A mixture of 15 volumes of *methanol* and 85 volumes of a buffer solution,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 50 µl.

Name	Relative retention time	Correction factor
Oxaliplatin related compound C[1]	0.8	—
Oxaliplatin	1.0	—
(*SP*-4-2)-Diaqua[(1*R* ,2*R*)-cyclohexane-1,2-diamine-*N,N* ']platinum	2.7	—
Diaquodiaminocyclohexaneplatinum dimer	6.0	0.4

[1][1*R-trans*-(1,2-Cyclohexanediamine-*N,N'*)]-*trans*-dihydroxido-[oxalato(2-)-*O,O'*]platinum(IV).

Inject reference solution (d) and (e). The test is not valid unless the tailing factor is between 0.8 and 2.0 for oxaliplatin and the resolution between the peaks due to oxaliplatin and oxaliplatin related compound C is not less than 2.0 in the chromatogram obtained with reference solution (d). The relative standard deviation for replicate injections is not more than 3.0 per cent for oxaliplatin, oxaliplatin related compound B, and oxaliplatin related compound C in the chromatogram obtained with reference solution (e).

Inject reference solution (e) and the test solution. In the chromatogram obtained with the test solution the area of (*SP*-4-2)-diaqua[(1*R* ,2*R*)-cyclohexane-1,2-diamine-*N,N'*]platinum is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.1 per cent) and oxaliplatin related compound C is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.1 per cent). The area of diaquodiaminocyclohexaneplatinum dimer, multiplied with correction factor 0.4 is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.1 per cent). The area of any secondary peak, multiplied by correction factor 0.25 is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.2 per cent) and the sum of the areas of all the secondary peaks including oxalic acid is not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.3 per cent).

C. *Oxaliplatin related compound D.* Not more than 0.1 per cent, determine by liquid chromatography (2.4.14).

NOTE — Use vigorous shaking and very brief sonication to dissolve the substance to be examined. Inject the test solution within 20 minutes of preparation. Polypropylene auto sampler vials should be used.

Test solution. Dissolve 15.0 mg of the substance under examination in 25.0 ml of *methanol.*

Reference solution (a). A 0.005 per cent w/v solution of *oxaliplatin related compound D IPRS (cis-[(1S, 2S)-1,2-Cyclohexanediamine-N,N'][oxalato(2-)-O,O']platinum)* in *methanol.*

Reference solution (b). Dilute reference solution (a) with *methanol* to obtain a 0.0015 per cent w/v solution of oxaliplatin related compound D.

Reference solution (c). A 0.075 per cent w/v solution of *oxaliplatin IPRS* in *methanol.*

Reference solution (d). Dilute 1.0 ml of reference solution (c) to 20.0 ml with *methanol.*

Reference solution (e). Transfer 40 ml of reference solution (c) to a 50-ml volumetric flask, and dilute with *methanol* to volume.

Reference solution (f). Transfer 40 ml of reference solution (c) to separate 50-ml volumetric flasks. Add 1.0, 3.0, and 5.0 ml of reference solution (b) to each flask, and dilute with *methanol* to volume. The concentration of oxaliplatin in these solutions is 0.6 mg per ml. The concentrations of oxaliplatin related compound D in these solutions are 0.3, 0.9, and 1.5 µg per ml, respectively.

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Reference solution (g). Transfer 5.0 ml of reference solution (d) and 4.0 ml of reference solution (a) to a 50-ml volumetric flask, and dilute with *methanol* to volume.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with cellulose tris (phenylcarbamate) (5µm),
- column temperature: 40°,
- mobile phase: 30 volumes of *ethanol,* and 70 volumes of *methanol,*
- flow rate: 0.3 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (f) containing 0.9 µg per ml of oxaliplatin related compound D, and reference solution (g). The test is not valid unless the resolution between the peaks due to oxaliplatin and oxaliplatin related compound D is not less than 1.5 in the chromatogram obtained with reference solution (g) and relative standard deviation is not more than 3.0 per cent for the peak height ratio of oxaliplatin related compound D to the sum of oxaliplatin and oxaliplatin related compound D in the chromatogram obtained with reference solution (f).

Inject reference solution (f), and the test solution. Subtract the oxaliplatin related compound D peak height obtained in reference solution (e) from the oxaliplatin related compound D peak height obtained in reference solution (f). Plot a calibration curve for reference solution (f) with the peak height ratios of oxaliplatin related compound D to the sum of oxaliplatin and oxaliplatin related compound D on the *y*-axis and the concentration ratios of oxaliplatin related compound D, in µg per ml, to the sum of oxaliplatin and oxaliplatin related compound D concentrations, in mg per ml, on the *x*-axis. Read the concentration ratio of oxaliplatin related compound D, in µg per ml, to the sum of oxaliplatin and oxaliplatin related compound D, in mg per ml, in the test solution from the calibration curve.

Calculate the percentage of oxaliplatin related compound D by

$$\frac{R}{10}$$

where, R = concentration ratio of oxaliplatin related compound D, in µg per ml, to the sum of oxaliplatin and oxaliplatin related compound D, in mg per ml, in the test solution from the calibration curve

NOTE— Oxaliplatin IPRS may contain a small amount of oxaliplatin related compound D.

Silver. Not more than 5 ppm, determine by atomic absorption spectrophotometry (2.4.2), measuring at 328 nm using a silver hollow cathode light as a radiation source, a fuel-lean air-acetylene flame, preferably a spectral width of 0.5 nm.

Test solution. Dissolve 0.5 g of the substance under examination in 15 ml of *nitric acid* by heating up to 80°. Cool and dilute to 25.0 ml with *water.*

Reference solutions. Add 50 ml of *nitric acid* to suitable volumes (1 to 3 ml) of *silver solution AAS (5 ppm)* and dilute to 100.0 ml with *water.*

Carry out a blank determination.

Platinum. Not less than 48 per cent and not more than 50.0 per cent, calculated on dried basis.

Weigh 200 mg of the oxaliplatin in a porcelain crucible and ignite in a furnace by stepwise increments as follows. Introduce the sample into the furnace, and increase the temperature to 200° within 15 minutes,then to 400° within 15 minutes, then to 600° within 15 minutes, then finally to 800° within 15 minutes. Allow to remain in the furnace at 800° for further 30 minutes. Remove, cool in a desiccator and weigh.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Bacterial endotoxins (2.2.3). Not more than 1.0 Endotoxin Units per mg of oxaliplatin.

Assay. Determine by liquid chromatography (2.4.14). As described under Related Substances B with following modifications.

Test solution. Dissolve 10 mg of the substance under examination in 100.0 ml of *water.*

Reference solution (f). Dilute 10.0 ml of reference solution (a) to 50.0 ml with *water.*

The relative retention times, with reference to oxaliplatin are 0.8 for oxaliplatin related compound C, 2.7 for oxaliplatin related compound B and 6 for diaquodiaminocyclohexaneplatinum dimer respectively.

Inject reference solution (d) and (f). The test is not valid unless the tailing factor is not between 0.8 and 2.0 for oxaliplatin and the resolution between the peaks due to oxaliplatin and oxaliplatin related compound C is not less than 2.0 in the chromatogram obtained with reference solution (d). The relative standard deviation for replicate injections is not more than 2.0 per cent for oxaliplatin peak obtained with reference solution (f).

Inject reference solution (f) and the test solution.

Calculate the content of $C_8H_{14}N_2O_4Pt$.

Storage. Store protected from light and moisture.

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Oxaliplatin Injection

Oxaliplatin Injection is a sterile solution of Oxaliplatin in Water for Injections.

Oxaliplatin Injection contains not less than 90.0 per cent and not more than 110.0 per cent of $C_8H_{14}N_2O_4Pt$.

Usual strength. 5 mg per ml.

Identification

A. When examined in the range 200 nm to 400 nm (2.4.7), a 0.01 per cent w/v solution in *water* shows an absorption maxima and minima at the same wavelength as that of *oxaliplatin IPRS* solution prepared in the same manner.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (b).

Tests

pH. (2.4.24). 4.0 to 7.0.

Oxalic acid. Not more than 0.6 per cent, determine by liquid chromatography (2.4.14).

NOTE— Use auto sampler vials made of polypropylene.

Test solution. A 0.2 per cent w/v solution of oxaliplatin.

Reference solution (a). A 0.0035 per cent w/v solution of *oxaliplatin related compound A IPRS* (Oxalic acid dihydrate) in *water.*

Reference solution (b). A 0.01 per cent w/v solution of *succinic acid* in reference solution (a).

Reference solution (c). Dilute 10.0 ml of reference solution (a) to 100.0 ml with *water.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: 20 volumes of *acetonitrile,* and 80 volumes of a buffer solution prepared by dissolving 1.36 g of *monobasic potassium phosphate* in 10 ml of 10 per cent *tetrabutylammonium hydroxide* in *water* and dilute with *water* to 1000 ml, ajusted to pH 6.0 with *orthophosphoric acid.*
- flow rate: 2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

The relative retention time with respect to oxalic acid for succinic acid is 0.8.

Inject reference solution (a), (b) and (c). The test is not valid unless the resolution between the peaks due to oxalic acid and succinic acid is not less than 2.0 and tailing factor is between 0.5 to 2.0 with reference solution (b). In the chromatogram obtained with reference solution (c), the peak for oxaliplatin related compound A, the signal to noise ratio is not less than 10. The relative standard deviation for replicate injections is not more than 3.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any peak due to oxaliplatin related compound A is not more than 0.34 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Use auto sampler vials made of polypropylene.

Buffer Solution. Dissolve 1.36 g of *monobasic potassium phosphate* and 0.55 g of *sodium heptanesulfonate* in 1000 ml of *water.* Adjusted to pH 3.0 with *orthophosphoric acid.*

Test solution. A 0.2 per cent w/v solution of oxaliplatin.

Reference solution (a). A 0.2 per cent w/v solution of *oxaliplatin IPRS* in *0.005 M sodium hydroxide.* Allow this solution to stand at room temperature for 5 days. Transfer 5.0 ml of the solution into a 50-ml volumetric flask, and dilute with *water* to volume.

NOTE— The preparation of the reference solution (a) forms (SP-4-2)-diaqua[(1R ,2R)-cyclohexane-1,2-diamine-N,N']platinum and diaquodiaminocyclohexaneplatinum dimer.

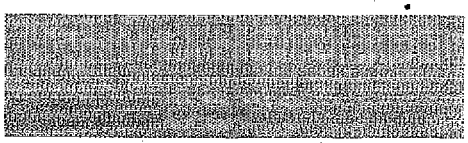
Reference solution (b). Transfer 12.5 mg of *oxaliplatin related compound B IPRS* ([*SP*-4-2-(1*R-trans*)]-(1,2-Cyclohexanediamine-*N,N'*) dinitratoplatinum(II)) in to a 100-ml volumetric flask, add 25.0 ml *methanol* and sonicate for 2 minutes. Add 65.0 ml of *0.01 M nitric acid* and sonicate for additional 30 minutes. Cool and dilute to 100.0 ml with *0.01 M nitric acid.*

Reference solution (c). dilute 5.0 ml of reference solution (b) to 20.0 ml with *0.01 M nitric acid.*

NOTE — Oxaliplatin Related Compound B IPRS is converted to (SP-4-2)-diaqua[(1R ,2R)-cyclohexane-1,2-diamine-N,N']platinum during preparation of the solution.

Chromatographic system

- a stainless steel column 7.5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 10°,
- mobile phase: A. a mixture of 19 volumes of *methanol* and 81 volumes of a buffer solution,
  B. a mixture of 50.5 volumes of *methanol* and 49.5 volumes of a buffer solution,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.



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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
45	0	100
45.5	100	0
53.0	100	0

Name	Relative retention time	Correction factor
(*SP*-4-2)-Diaqua[(1*R* ,2*R*)-cyclohexane-1,2-diamine-*N*,*N* ']platinum	1.0	---
Diaquodiaminocyclohexaneplatinum dimer[1]	1.4	0.4

[1](*SP*-4-2)-diaqua[(1*R* ,2*R*)-cyclohexane-1,2-diamine-*N*,*N* ']platinum and diaquodiaminocyclohexaneplatinum dimer.

Inject reference solution (a) and (c). The test is not valid unless the tailing factor is not more than 2.0 for (*SP*-4-2)-diaqua[(1*R* ,2*R*)-cyclohexane-1,2-diamine-*N*,*N*']platinum peak and resolution between the peaks due to (*SP*-4-2)-diaqua[(1*R* ,2*R*)-cyclohexane-1,2-diamine-*N*,*N*']platinum and (*SP*-4-2)-diaqua[(1*R* ,2*R*)-cyclohexane-1,2-diamine-*N*,*N*'] platinum and diaquodiaminocyclohexaneplatinum dimer is not less than 8.0 with reference solution (a). The relative standard deviation for replicate injections is not more than 3.0 per cent with reference solution (c).

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution the area of *SP*-4-2)-diaqua[(1*R* ,2*R*)-cyclohexane-1,2-diamine-*N*,*N*']platinum is not more than 0.42 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.65 per cent) and Diaquodiaminocyclohexaneplatinum dimer, multiplied with correction factor is not more than 0.32 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent). Any secondary peak, multiplied with correction factor 0.25 is not more than 0.13 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent) and the sum of the areas of all the secondary peaks including oxalic acid and (*SP*-4-2)-diaqua[(1*R* ,2*R*)-cyclohexane-1,2-diamine-*N*,*N*']platinum is not more than 1.57 times the area of the principal peak in the chromatogram obtained with reference solution (c) (2.45 per cent).

Bacterial endotoxins (2.2.3). Not more than 1.0 Endotoxin Units per mg of oxaliplatin.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Use auto sampler vials made of polypropylene.

Acidified water. adjust to pH 3.0 with *orthophosphoric acid.*

Test solution. Dilute a measured volume of the injection to obtain a solution containing 0.01 per cent w/v oxaliplatin in *water.*

Reference solution (a). A 0.01 per cent w/v solution of *oxaliplatin IPRS* and *[SP-4-2-(1R -trans)]-(1,2-cyclohexanediamine-N, N') dichloridoplatinum(II)* in *water.*

Reference solution (b). A 0.01 per cent w/v solution of *oxaliplatin IPRS in* water.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: 1 volume of *acetonitrile* and 99 volumes of *acidified water,*
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

The relative retention time with reference to oxaliplatin peak for *[SP-4-2-(1R -trans)]-(1,2-cyclohexanediamine-N,N') dichloridoplatinum(II)* is 0.9.

Inject reference solution (a). The test is not valid unless the tailing factor is not more than 2.0 for oxaliplatin peak and the resolution between the peaks due to oxaliplatin and [SP-4-2-(1R -trans)]-(1,2-cyclohexanediamine-*N*,*N'*) dichloridoplatinum(II) is not less than 2.0 obtained with reference solution (a). The relative standard deviation for replicate injections is not more than 1.0 per cent for oxaliplatin peak obtained with reference solution (b).

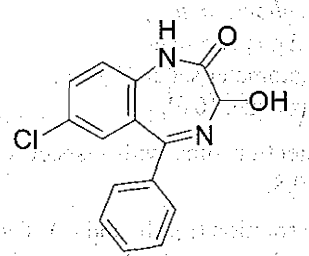
Inject reference solution (b) and the test solution.

Calculate the content of $C_8H_{14}N_2O_4Pt$.

Storage. Store in single-dose or multiple-dose containers, preferably of Type I glass, protected from light, at a temperature below 30°.

Labelling. Label it to state that it is to be diluted with a 5 per cent dextrose solution. Oxaliplatin Injection must not be diluted in sodium chloride solutions or in chloride-containing solutions.

Oxazepam



$C_{15}H_{11}ClN_2O_2$ Mol. Wt 286.7

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Oxazepam is 7-chloro-3-hydroxy-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

Oxazepam contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{15}H_{11}ClN_2O_2$, calculated on the dried basis.

Category. Anxiolytic.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oxazepam IPRS* or with the reference spectrum of oxazepam.

B. Dissolve about 20 mg in sufficient *ethanol (95 per cent)* to produce 100 ml. Dilute 10.0 ml of the solution to 50.0 ml with *ethanol (95 per cent)* (solution A). Dilute 5.0 ml of solution A to 20.0 ml with *ethanol (95 per cent)* (solution B). (*NOTE — Prepare the solutions immediately before use, protected from light).*

When examined in the range 230 nm to 360 nm (2.4.7), solution A shows an absorption maximum at about 316 nm. When examined in the range 220 nm to 250 nm, solution B shows an absorption maximum at about 229 nm, is 1.220 to 1.300.

C. Dissolve about 20 mg in a mixture of 5 ml of *hydrochloric acid* and 10 ml of *water*. Heat to boiling for 5 minutes and cool. Add 2 ml of a 0.1 per cent *w/v* solution of *sodium nitrite* and allow to stand for 1 minute. Add 1 ml of a 0.5 per cent w/v solution of *sulphamic acid*, mix and allow to stand for 1 minute. Add 1 ml of 0.1 per cent w/v solution of a *N-(1-naphthyl) ethylenediamine dihydrochloride*; a red colour is produced.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Prepare the solutions immediately before use.

Solvent mixture. A mixture of equal volumes of *acetonitrile* and *water*.

Test solution. Dissolve 40 mg of the substance under examination in 50.0 ml of the solvent mixture.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Further dilute 2.0 ml of the solution to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with end-capped octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. dissolve 3.48 g of *dipotassium hydrogen phosphate* in 900 ml of *water*, adjusted to pH 10.5 with a 4.0 per cent w/v *dilute sodium hydroxide solution*, diluted to 1000 ml with *water*,

  B. *acetonitrile*,
- flow rate: 1ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 235 nm,
- injection volume:10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	75	25
4	75	25
34	25	75
45	25	75
50	75	25
60	75	25

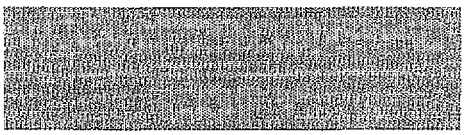
The relative retention time with reference to oxazepam for oxazepam impurity E (7-chloro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one 4-oxide) is about 0.7, for oxazepam impurity A ((5*RS*)-7-chloro-5-phenyl-4,5-dihydro-1*H*-1,4-benzodiazepine-2,3-dione) is about 0.8, for oxazepam impurity B (7-[(2-aminoethyl)amino]-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid) is about 1.2, for oxazepam impurity C (1-ethyl-4-oxo-6,7-bis(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid) is about 1.4 and for oxazepam impurity D (1-ethyl-6-fluoro-7-(piperazin-1-yl)quinolin-4(1*H*)-one) is about 2.0.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Correction factor for oxazepam impurity A is 4.0 and for oxazepam impurity B is 1.1.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to oxazepam impurity A, B, C, D, E, each of, is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent), the area of any other secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.



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Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° at a pressure not exceeding 0.7 kPa.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in *methanol* and dilute to 100.0 ml with *methanol*. Dilute 5.0 ml of the solution to 50.0 ml with *methanol*.

Reference solution. A 0.002 per cent w/v solution of *oxazepam IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of *methanol*, 40 volumes of *water* and 0.1 volume of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 232 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 1000 theoretical plates, tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{11}ClN_2O_2$.

Storage. Store protected from light and moisture.

Oxazepam Tablets

Oxazepam Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of oxazepam, $C_{15}H_{11}ClN_2O_2$.

Usual strengths. 10 mg; 15 mg; 30 mg.

Identification

A. Extract a quantity of the powdered tablets containing 20 mg of Oxazepam with 25 ml of *chloroform*, filter, evaporate to dryness and dry the residue at 60° at a pressure not exceeding 0.7 kPa.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oxazepam IPRS or* with the *reference spectrum* of oxazepam.

B. When examined in the range 210 nm to 360 nm (2.4.7), the solution obtained in the Assay shows absorption maxima at about 230 nm and 316 nm.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.01 M hydrochloric acid*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the medium, if necessary, at the maximum at about 236 nm (2.4.7). Calculate the content of $C_{15}H_{11}ClN_2O_2$ in the medium taking 1080 as the specific absorbance at 236 nm.

Q. Not less than 70 per cent of the stated amount of $C_{15}H_{11}ClN_2O_2$.

Related substances. *Carry out the test protected from light.*

Determine by thin-layer chromatography (2.4.17) coating the plate with *silica gel GF 254*.

Wash the plate with *methanol* before use.

Mobile phase. A mixture of 100 volumes of *dichloromethane* and 10 volumes of *methanol*.

Test solution. Shake a quantity of powdered tablets containing 30 mg of Oxazepam with 6 ml of *acetone* and centrifuge.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *acetone*.

Reference solution (b). Dilute 1.0 ml of the test solution to 500.0 ml with *acetone*.

Reference solution (c). A solution containing 0.1 per cent w/v each of *oxazepam IPRS* and *bromazepam IPRS*.

Apply to the plate 20 µl of each of the solutions. Allow the mobile phase to rise 17 cm in the same direction as in the washing with methanol. Dry in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 25 mg of Oxazepam and shake with 150 ml of *ethanol (95 per cent)* for 30 minutes. Add sufficient *ethanol (95 per cent)* to produce 250.0 ml and centrifuge. Dilute 5.0 ml of the supernatant liquid to 100.0 ml with the same solvent and measure the absorbance of the resulting solution at the maximum at about 230 nm (2.4.7). Calculate the content of $C_{15}H_{11}ClN_2O_2$ taking 1250 as the specific absorbance at 230 nm.

Storage. Store protected from light at a temperature not exceeding 30°.

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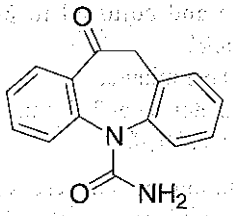
Oxcarbazepine



$C_{15}H_{12}N_2O_2$ Mol. Wt. 252.3

Oxcarbazepine is 10,11-dihydro-10-oxo-5*H*-dibenz[*b*,*f*]azepine-5-carboxamide.

Oxcarbazepine contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{15}H_{12}N_2O_2$, calculated on the dried basis.

Category. Anticonvulsants.

Description. An off-white to yellow crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oxcarbazepine IPRS* or with the reference spectrum of oxcarbazepine.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Use freshly prepared solutions.

Test solution. Dissolve 50 mg of the substance under examination in 100.0 ml of the mobile phase.

Reference solution (a). A 0.05 per cent w/v solution of *oxcarbazepine IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil C18),
- column temperature 50°,
- mobile phase: a mixture of 22 volumes of *methanol*, 16 volumes of *acetonitrile* and 62 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adding 2 ml of *triethylamine* and adjusted to pH 6.0 with *dilute orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatograms for about 80 minutes. The relative retention times with reference to oxcarbazepine are 1.7 for dibenz[b,f] azepine-5-carboxylic acid amide (oxcarbazepine impurity A), 7.9 for 10-methoxy-5H-dibenz[b,f]azepine (oxcarbazepine impurity B), and 2.5 for 10-methoxy-5H-dibenz [b,f]azepine-5-carboxylic acid amide (oxcarbazepine impurity C).

Calculate the contents of the impurities in the test solution using the correction factors, 0.68 for oxcarbazepine impurity A, 0.76 for oxcarbazepine impurity B and 0.80 for oxcarbazepine impurity C. Not more than 1.0 per cent of oxcarbazepine impurity A and not more than 0.1 per cent, each of, oxcarbazepine impurity B and oxcarbazepine impurity C and not more than 0.1 per cent of any other impurity is found and the sum of all the impurities is not more than 1.5 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Determine by liquid chromatography (2.4.14) as described under related substances using the following solution.

Test solution. Dissolve 10 mg of the substance under examination in 100.0 ml of the mobile phase.

Reference solution. A 0.01 per cent w/v solution of *oxcarbazepine IPRS* in the mobile phase.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency is not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{12}N_2O_2$.

Storage. Store protected from moisture.

Oxcarbazepine Tablets

Oxcarbazepine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of oxcarbazepine, $C_{15}H_{12}N_2O_2$.

Usual strengths. 150 mg; 300 mg; 450 mg; 600 mg.

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Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution (b) corresponds to the peak in the chromatogram obtained with reference solution (b).

B. Extract a quantity of the powdered tablets containing about 50 mg of oxcarbazepine with 100 ml with *methanol* and filter. Dilute 2.0 ml of the solution to 100.0 ml with *methanol*. When examined in the range 200 nm to 350 nm (2.4.7), the solution shows an absorption maximum at about 254 nm.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of 0.75 per cent w/v solution of *sodium lauryl sulphate* in *water*,

Speed and time. 75 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtered solution, suitably diluted with the medium if necessary, at the maximum at about 305 nm (2.4.7). Calculate the content of $C_{15}H_{12}N_2O_2$ in the medium from the absorbance obtained from a solution of known concentration of *oxcarbazepine IPRS* prepared by dissolving in minimum volume of *methanol* and then diluting with the dissolution medium.

Q. Not less than 75 per cent of the stated amount of $C_{15}H_{12}N_2O_2$ in the medium.

Related substances. Determine by liquid chromatography (2.4.14).

Note — Use freshly prepared solutions.

Test solution (a). Weigh a quantity of the powdered tablets containing 50 mg of Oxcarbazepine add 15 ml *methanol*, mix with the aid of ultrasound for 15 minutes and dilute to 100.0 ml with *methanol*. Mix and centrifuge.

Test solution (b). Dilute 5.0 ml of test solution (a) to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *oxcarbazepine IPRS* in *methanol* with the aid of ultrasound and dilute to 50.0 ml with *methanol*.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 25.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil BDS C18),
- column temperature 50°,
- mobile phase: a mixture of 66 volumes of *0.05M potassium dihydrogen phosphate*, 14 volumes of *acetonitrile* and 20 volumes of *methanol*, add 0.1 ml of *triethylamine* and adjusted to pH 6.0 with *ortho-phosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject test solution (a) and reference solution (c). Run the chromatograms for three times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the peak in the chromatogram obtained with reference solution (c) (1.0 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the peak in the chromatogram obtained with reference solution (c) (2.0 per cent).

Other tests. Comply with the tests stated under Tablets.

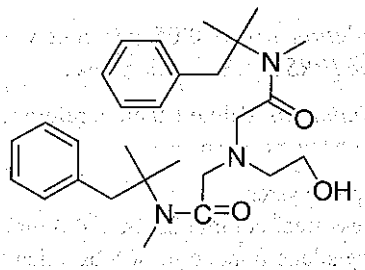
Assay. Determine by liquid chromatography (2.4.14) as described under Related substances.

Inject test solution (b) and reference solution (b).

Calculate the content of $C_{15}H_{12}N_2O_2$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Oxetacaine



$C_{28}H_{41}N_3O_3$ Mol. Wt. 467.6

Oxetacaine is 2,2′-(2-hydroxyethylimino)bis[*N*-(α,α-dimethylphenethyl)-*N*-methylacetamide].

Oxetacaine contains not less than 99.0 per cent and not more than 100.5 per cent of $C_{28}H_{41}N_3O_3$ calculated on the dried basis.

Category. Local Anaesthetic.

Description. A white or almost white powder.

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Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oxetacaine IPRS* or with the reference spectrum of oxetacaine.

Tests

Melting point (2.4.21). 100° to 104°.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 1 volume of *18 M ammonia*, 20 volumes of *ethanol* and 79 volumes of *toluene*.

Test solution. Dissolve 1.0 g of the substance under examination in 10.0 ml of *ethyl acetate*.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with *ethyl acetate*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 5.0 ml with *ethyl acetate*.

Apply to the plate 5 µl of each solution. After development, dry the plate in warm air and spray with a solution containing 6 per cent w/v of *ammonium thiocyanate* and 2 per cent w/v of *cobalt (II) chloride*, allow to dry in air for 10 minutes. In the chromatogram obtained with the test solution, any secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent) and not more than one secondary spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

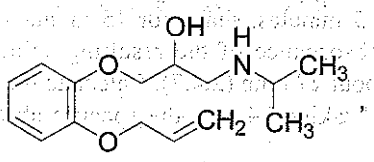
Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 60°, at a pressure not exceeding 0.7 kPa for 4 hours.

Assay. Dissolve 1.0 g in 50 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.04676 g $C_{28}H_{41}N_3O_3$.

Oxprenolol Hydrochloride



$C_{15}H_{23}NO_3,HCl$ Mol. Wt. 301.8

Oxprenolol Hydrochloride is (*RS*)-1-(2-allyloxyphenoxy)-3-isopropylaminopropan-2-ol hydrochloride.

Oxprenolol Hydrochloride contains not less than 98.5 per cent and not more than 101.5 per cent of $C_{15}H_{23}NO_3,HCl$, calculated on the dried basis.

Category. Beta-adrenoceptor antagonist (antihypertensive; antianginal; antiarrhythmic).

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Test B may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oxprenolol hydrochloride IPRS* or with the reference spectrum of oxprenolol hydrochloride.

B. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to spot in the chromatogram obtained with reference solution (c).

C. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution GYS6 (2.4.1).

pH (2.4.24). 4.5 to 6.0, determined in a freshly prepared 10.0 per cent w/v solution.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. 90 volumes of *chloroform* and 10 volumes of *methanol*.

Mobile phase. A mixture of 88 volumes of *chloroform*, 12 volumes of *methanol* and 2 volumes of *strong ammonia solution*.

Test solution (a). Dissolve 0.5 g of the substance under examination in 10 ml of the solvent mixture.

Test solution (b). Dissolve 0.5 g of the substance under examination in 100 ml of the solvent mixture.

Reference solution (a). A 0.02 per cent w/v solution of the substance under examination in the solvent mixture.

Reference solution (b). A 0.01 per cent w/v solution of the substance under examination in the solvent mixture.

Reference solution (c). A 0.5 per cent w/v solution of *oxprenolol hydrochloride IPRS* in the solvent mixture.

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Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 13 cm. Dry the plate in warm air for 10 minutes, allow to cool, spray with *anisaldehyde solution*, heat at 105° for 10 minutes and examine in daylight. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 60° over *phosphorus pentoxide* at a pressure of 1.5 to 2.5 kPa for 6 hours.

Assay. Dissolve 0.25 g in 60 ml of *anhydrous glacial acetic acid*, add 5 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25).Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03018 g of $C_{15}H_{23}NO_3$,HCl.

Storage. Store protected from light and moisture.

Oxprenolol Tablets

Oxprenolol Hydrochloride Tablets

Oxprenolol Hydrochloride Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of oxprenolol hydrochloride, $C_{15}H_{23}NO_3$,HCl. The tablets are coated.

Usual strengths. 40 mg; 80 mg.

Identification

A.To a quantity of the powdered tablets containing 50 mg of Oxprenolol Hydrochloride add 10 ml of *water* and 2 ml of *dilute sodium hydroxide solution*, extract with 10 ml of *chloroform* and reserve the aqueous layer for test C. Dry the chloroform extract over *anhydrous sodium sulphate*, filter and evaporate the filtrate to dryness.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oxprenolol hydrochloride IPRS* treated in the same manner or with the reference spectrum of oxprenolol.

B. When examined in the range 230 nm to 360 nm (2.4.7), the solution obtained in the Assay shows an absorption maximum only at about 273 nm.

C. The aqueous layer obtained in test A gives reaction (A) of chlorides (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 100 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the medium, if necessary, at the maximum at about 272 nm (2.4.7). Calculate the content of $C_{15}H_{23}NO_3$,HCl in the medium from the absorbance obtained from a solution of known concentration of *oxprenolol hydrochloride IPRS* in dissolution medium.

Q. Not less than 80 per cent of the stated amount of $C_{15}H_{23}NO_3$,HCl.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 88 volumes of *chloroform*, 12 volumes of *methanol* and 2 volumes of *strong ammonia solution*.

Test solution. Extract a quantity of the powdered tablets containing 0.25 g of Oxprenolol Hydrochloride with 5 ml of *water*, centrifuge and use the supernatant liquid.

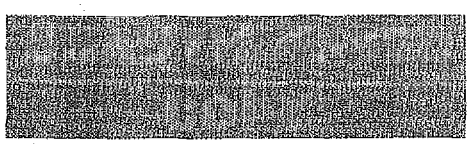
Reference solution. Dilute 1.0 volume of the test solution to 200.0 volumes with *water*.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 13 cm. Dry the plate in warm air for 10 minutes, allow to cool, spray with *anisaldehyde solution*, heat at 105° for 10 minutes and examine in daylight. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 20 mg of Oxprenolol Hydrochloride, add 25 ml of *0.1 M hydrochloric acid* and sufficient *water* to produce 250.0 ml. Mix with the aid of ultrasound for 5 minutes, shake for 15 minutes and filter. Measure the absorbance of the resulting solution at the maximum at about 273 nm (2.4.7). Calculate the content of $C_{15}H_{23}NO_3$,HCl taking 74.5 as the specific absorbance at 273 nm.

Storage. Store protected from moisture.



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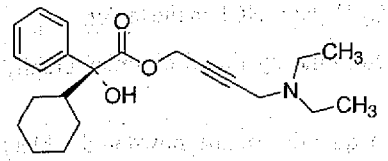
Oxybutynin Hydrochloride

Oxybutynin Chloride



, HCl and enantiomer

$C_{22}H_{31}NO_3,HCl$ Mol. Wt. 394.0

Oxybutynin is 4-(Diethylamino)but-2-ynyl (*RS*)-2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride.

Oxybutynin Hydrochloride contains not less than 99.0 per cent and not more than 102.0 per cent of $C_{22}H_{31}NO_3,HCl$, calculated on the dried basis.

Category. Anticholinergic.

Identification

Test B and C may be omitted if tests A and D are carried out. Tests A may be omitted if tests C, B and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oxybutynin hydrochloride IPRS* or with reference spectrum of oxybutynin hydrochloride.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. *Methanol*.

Test solution. Dissolve 50 mg of the substance under examination in *ethanol (95 per cent)* and dilute to 10.0 ml with the *ethanol (95 per cent)*.

Reference solution. A 0.5 per cent w/v solution of *oxybutynin hydrochloride IPRS* in *ethanol (95 per cent)*.

Apply to the plate 5 µl of each solution. After development, dry the plate in warm air. Expose the plate to the vapour of iodine for 30 minutes and examine the plate again. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution.

C. Melting point (2.4.21). 124° to 129°.

D. It gives reaction (A) of chlorides (2.3.1).

Tests

Solution A. A 5.0 per cent w/v solution in *water*.

Appearance of solution. Solution A is clear (2.4.1) and not more intensely colored than reference solution BYS5 (2.4.1).

Optical rotation (2.4.22). - 0.10° to + 0.10°, determined on *Solution A*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of *oxybutynin hydrochloride IPRS* and 5.0 mg of *oxybutynin impurity A IPRS (4-(diethylamino)but-2-ynyl (RS)-2-(cyclohex-3-enyl)-2-cyclohexyl-2- hydroxyacetate)* in the mobile phase and dilute to 10.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b) Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Chromatographic system

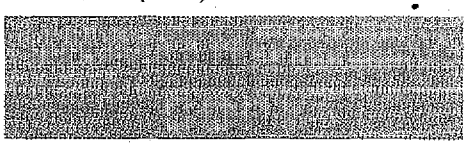
- a stainless steel column 15 cm x 3.9 mm, packed with octylsilane bonded to silica gel (5 µm),
- mobile phase: a mixture of 49 volumes of a buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen phosphate* and 4.36 g of *di potassium hydrogen phosphate* in 1000 ml of *water* and 51 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

The retention time of oxybutynin peak is about 15 minutes and impurity A peak is about 24 minutes.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to oxybutynin and oxybutynin impurity A is not less than 11.0.

Inject reference solution (a), (b) and the test solution. Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution the area of the peak corresponding to impurity A is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent), the area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all secondary peaks other than impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). 12 ml of solution A complies with the limit test for heavy metals, Method D (20 ppm), using 10.0 ml of *lead standard solution (1 ppm Pb)*.



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Loss on drying (2.4.19). Not more than 3.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Sulphated ash (2.3.18). Not more than 0.1 per cent

Assay. Dissolve 0.300 g in a mixture of 5.0 ml of *0.01 M hydrochloric acid* and 50.0 ml of *ethanol (95 per cent)* and and titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25). Read the volume added between the two points of inflexion.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.0394 g of $C_{22}H_{31}NO_3$,HCl.

Storage. Store protected from moisture.

Oxybutynin Prolonged-release Tablets

Oxybutynin Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Oxybutynin Prolonged-release Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of oxybutynin hydrochloride, $C_{22}H_{31}NO_3$,HCl.

Usual strengths. 5 mg; 10 mg.

Identification

Disperse a quantity of the powdered tablets containing 25 mg of oxybutynin hydrochloride in 20 ml of *water* add sufficient *2M sodium hydroxide* to adjusted to pH 12.0 and extract with four quantities, each of 20 ml *hexane*. Filter the collected hexane layers through *anhydrous sodium sulphate* (Whatman GF/C is suitable). Evaporate the filtrate to dryness under a current of nitrogen, to yield a clear, sticky liquid residue. Prepare a thin film from the residue to be held between two plates or in cell of suitable path length constructed of material transparent to infrared radiation. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oxybutynin hydrochloride IPRS* treated in the same manner or with the reference spectrum of oxybutynin.

Tests

Dissolution (2.5.2). Complies with the test stated under Tablets.

Uniformity of content. Complies the test stated under tablets.

Determine by liquid chromatography (2.4.14), using the chromatographic condition as described under Assay.

To one tablet, add 50 ml of *0.01 M hydrochloric acid*, disperse with the aid of ultrasound for 20 minutes and dilute to volume with *0.01 M hydrochloric acid* to produce a solution containing 0.005 per cent w/v of oxybutynin hydrochloride.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{31}NO_3$,HCl. in the tablet.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 50 mg of oxybutynin hydrochloride in 40 ml of *0.01 M hydrochloric acid* with the aid of ultrasound for 15 minutes, add sufficient *0.01 M hydrochloric acid* to produce 50.0 ml, mix and filter.

Reference solution (a). A 0.0015 per cent w/v solution of *oxybutynin impurity A (4-(diethylamino)but-2-ynyl (RS)-2-(cyclohex-3-enyl)-2-cyclohexyl-2- hydroxyacetate)* in *0.01 M hydrochloric acid.*

Reference solution (b). A 0.0014 per cent w/v solution of *phenylcyclohexylglycolic acid IPRS* in *0.01M hydrochloric acid.*

Reference solution (c). Dilute 1.0 ml of test solution to 200.0 ml with *0.01 M hydrochloric acid.*

Reference solution (d). A 0.001 per cent w/v solution of *oxybutynin impurity A (4-(diethylamino)but-2-ynyl (RS)-2-(cyclohex-3-enyl)-2-cyclohexyl-2- hydroxyacetate)* in reference solution (c).

Chromatographic system

- a stainless steel column, 15 cm x 4.6 mm, packed with octadecylsilyl silica gel (5 µm),
- mobile phase: A. a solution contaning 0.34 per cent w/v solution of *potassium dihydrogen orthophosphate* and 0.436 per cent w/v solution of *dipotassium hydrogen orthophosphate* in *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
5	70	30
6	45	55
50	45	55
51	70	30
60	70	30

Inject reference solution (d). The test is not valid unless the resolution between the peaks corresponding to oxybutynin

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hydrochloride and oxybutynin impurity A is not less than 10.0.

Inject reference solution (a), (b), (c) and the test solution. In the chromatogram obtained with test solution, the area of any peak corresponding to oxybutynin impurity A is not more than the area of the area of principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent). The area of any peak corresponding to phenylcyclohexylglycolic acid is not more than 0.5 times the area of principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent), the area of any other secondary peak, other than the peak corresponding to oxybutynin impurity A and phenylcyclo-hexylglycolic acid is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.5 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Disperse 10 tablets in 400 ml of *0.01 M hydrochloric acid* for 20 minutes with the aid of ultrasound, cool, add sufficient *0.01 M hydrochloric acid* to produce 500 ml, mix and filter (Whatman GF/C is suitable). Dilute a volume of the solution with sufficient *0.01M hydrochloric acid* to produce a solution containing 0.005 per cent w/v of oxybutynin hydrochloride.

Reference solution. A 0.005 per cent w/v solution of *oxybutynin hydrochloride IPRS* in *0.01M hydrochloric acid.*

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with nitrile silica gel (5 µm),
- column temperature 40°,
- mobile phase: a mixture of 70 volumes of a buffer solution prepared by dissolving 4.8 g of anhydrous *potassium dihydrogen orthophosphate* in 1000 ml of *water,* adjusted to pH between 3.0 to 3.5 with *orthophosphoric acid* and 30 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 200 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{31}NO_3$,HCl in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Oxybutynin Tablets

Oxybutynin Hydrochloride Tablets; Oxybutynin Chloride Tablets

Oxybutynin Tablets contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of oxybutynin hydrochloride, $C_{22}H_{31}NO_3$, HCl.

Usual strengths. 2.5 mg; 5.0 mg.

Identification

To a quantity of the powdered tablets containing 25 mg of Oxybutynin Hydrochloride, add sufficient *2 M sodium hydroxide* adjust to pH 12.0 and extract with four 20-ml quantities of *hexane.* Filter the collected hexane layers through *anhydrous sodium sulphate* and evaporate to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum obtained with *oxybutynin hydrochloride IPRS* treated in the same manner or with the reference spectrum of oxybutynin.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water,*
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, diluted if necessary, with the dissolution medium, discard the first few ml of filtrate.

Reference solution. A 0.0005 per cent w/v solution of *oxybutynin hydrochloride IPRS* in the dissolution medium, diluted further if necessary.

Use the chromatographic system as described under Assay.

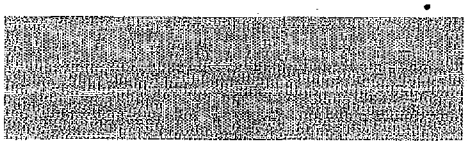
Calculate the content of $C_{22}H_{31}NO_3$,HCl.

Q. Not less than 75 per cent of the stated amount of $C_{22}H_{31}NO_3$, HCl.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing about 50 mg of Oxybutynin Hydrochloride in 40 ml of *0.01 M hydrochloric acid* with the aid of ultrasound for 15 minutes and dilute to 50.0 ml with same solvent and filter.

Reference solution (a). A 0.0015 per cent w/v solution of *oxybutynin impurity A IPRS [4-(diethylamino)but-2-ynyl (RS)-2-(cyclohex-3-enyl)-2-cyclohexyl-2-hydroxyacetate]* in *0.01 M hydrochloric acid.*



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Reference solution (b). A 0.001 per cent w/v solution of *phenylcyclohexylglycolic acid IPRS [(RS)-2-cyclohexyl-2-hydroxy-2-phenylacetic acid (phenylcyclohexylglycolic acid)]* in *0.01 M hydrochloric acid.*

Reference solution (c). Dilute 1.0 ml of the test solution to 200.0 ml with *0.01 M hydrochloric acid.*

Reference solution (d). A 0.001 per cent w/v solution of *oxybutynin impurity A IPRS* in reference solution (c).

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm)
- mobile phase: A. a mixture of 0.34 per cent w/v solution of *potassium dihydrogen orthophosphate* and 0.436 per cent w/v solution of *dipotassium hydrogen orthophosphate*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
5	70	30
6	45	55
50	45	55
51	70	30
60	70	30

The retention time are about 31 minutes for the peaks due to oxybutynin hydrochloride and about 47 minutes for oxybutynin impurity A.

Inject reference solution (d). The test is not valid unless the resolution between the peaks due to oxybutynin hydrochloride and oxybutynin impurity A is not less than 10.0

Inject reference solution (a), (b), (c) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to oxybutynin impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent) and the area of any peak corresponding to phenylcyclohexylglycolic acid is not more than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). The area of any other secondary peak is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent) and the sum of areas of all other secondary peaks is not more than the area of the peak in the chromatogram obtained with reference solution (c) (0.5 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay, using the following solutions.

Test solution. Disperse one tablet in *0.01 M hydrochloric acid,* sonicate and dilute if necessary to obtain a solution containing 0.005 per cent w/v of Oxybutynin Hydrochloride in *0.01 M hydrochloric acid.*

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 25 mg of Oxybutynin Hydrochloride with 400 ml of *0.01M hydrochloric acid,* with the aid of ultrasound for 20 minutes and dilute to 500.0 ml with *0.01 M hydrochloric acid* mix and filter.

Reference solution. A 0.005 per cent w/v solution of *oxybutynin hydrochloride IPRS* in *0.01 M hydrochloric acid.*

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with nitrile groups chemically bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 300 volumes of *acetonitrile* and 700 volumes of 0.48 per cent w/v solution of *anhydrous potassium dihydrogen orthophosphate* previously adjusted to pH 3.0 to 3.5 with *orthophosphoric acid.*
- flow rate: 2 ml per minute,
- spectrophotometer set at 200 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{31}NO_3,HCl$ in the tablets.

Oxygen

O_2 Mol. Wt. 32.0

Oxygen contains not less than 99.0 per cent v/v of O_2.

This monograph applies to oxygen for medicinal use only.

Oxygen should be stored in approved metal cylinders, the shoulder of the metal cylinder should be painted white and the remainder black. The cylinder should carry a label stating "Oxygen". In addition, "Oxygen" or the symbol "O_2" should be stencilled in paint on the shoulder of the cylinder.

When Oxygen is intended for use in a room in which magnetic resonance imaging (MRI) is being performed, the cylinder and fittings should be made from suitable non- ferromagnetic materials and labelled accordingly.

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Description. A colourless gas.

Identification

A. Shake with *alkaline pyrogallol solution;* the gas under examination is absorbed and the solution becomes dark brown.

B. It complies with the limits of the Assay.

Tests

Carbon dioxide. Not more than 300 ppm v/v, determined by using a carbon dioxide detector tube (2.1.1).

Carbon monoxide. Not more than 5 ppm v/v, determined by using a carbon monoxide detector tube (2.1.1).

Water vapour. Not more than 67 ppm v/v, determined by using a water vapour detector tube (2.1.1).

Assay. Determine the concentration of oxygen using a paramagnetic analyser (2.3.33).

Storage. Storeas a compressed gas or store in liquid form in appropriate containers, complying with the legal regulations. Oils and grease are not to be used unless they are oxygen-compatible.

Labelling. The label states 1) Oxygen containers should carry a label stating "Oxygen" and "for medicinal use"; 2) "liquid" if oxygen was produced by the air liquefaction process 3). Where it is piped directly from the cylinder or storage tank to the patient point of use, label each outlet "Oxygen".

Oxygen 93 Per Cent

Oxygen 93 Per Cent contains not less than 90.0 per cent and not more than 96.0 per cent, v/v of O_2, the remainder consisting mostly of argon and nitrogen. It is produced from air by the molecular sieve process.

This monograph applies to oxygen 93 Per Cent for medicinal use. It does not apply to gas produced using individual concentrators for domiciliary use.

Description. A colourless gas.

Identification

A. Shake with *alkaline pyrogallol solution;* the gas under examination is absorbed and the solution becomes dark brown.

B. It complies with the limits of the Assay.

Tests

Carbon dioxide. Not more than 300 ppm v/v, determined by using a carbon dioxide detector tube (2.1.1).

Carbon monoxide. Not more than 5 ppm v/v, determined by using a carbon monoxide detector tube (2.1.1).

Nitrogen monoxide and nitrogen dioxide. Not more than 2 ppm v/v in total, determined by using a nitrogen monoxide and nitrogen dioxide detector tube (2.1.1).

Sulfur dioxide. Not more than 1 ppm v/v, determined by using a sulfur dioxide detector tube (2.1.1).

Oil. Not more than 0.1 mg per m^3, determined by using an oil detector tube (2.1.1).

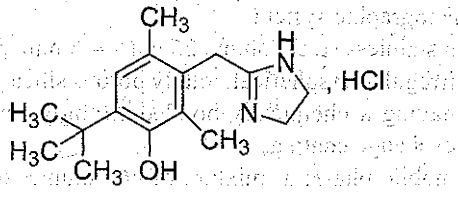
Water vapour. Not more than 67 ppm v/v, determined by using a water vapour detector tube (2.1.1).

Assay. Determine the concentration of oxygen using a paramagnetic analyser (2.3.33).

Storage. Oxygen 93 per cent obtained from an oxygen concentrator is normally used on site, where it is produced. It is fed directly into a medicinal gas pipeline or administration system. Where authorised by the competent authority, it may be stored in appropriate containers complying with the legal regulations. Oils and grease are not to be used unless theyare oxygen-compatible.

Labelling. The label state 1) The cylinder should carry a label stating"Oxygen 93 Per Cent" and "For medicinal use"; 2) Where it is piped directly from the cylinder or storage tank to the patient point of use, label each outlet "Oxygen 93 Per Cent"; 3) If it is stored in cylinders, the shoulder of the cylinder should be painted white and the remainder black.

Oxymetazoline Hydrochloride



$C_{16}H_{24}N_2O,HCl$ Mol. Wt. 296.8

Oxymetazoline Hydrochloride is Phenol, 3-[(4,5-dihydro-1H -imidazol-2-yl)methyl]-6-(1,1-dimethylethyl)-2,4-dimethyl-, monohydrochloride. 6-tert-Butyl-3-(2-imidazolin-2-ylmethyl)-2,4-dimethylphenol monohydrochloride.

Oxymetazoline Hydrochloride contains not less than 98.5 per cent and not more than 101.5 per cent of $C_{16}H_{24}N_2O,HCl$, calculated on the dried basis.

Description. A white or almost white powder.

Category. Sympathomimetic amine.

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Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *oxymetazoline hydrochloride IPRS* or with the reference spectrum of oxymetazoline hydrochloride.

B. When examined in the range of 220 nm to 360 nm (2.4.7). A 0.01 per cent w/v solution of the substance under examination in *water*, shows absorption maxima at about 279 nm. The specific absorbance of the substance under examination, calculated on the dried basis, does not differ by more than 3.0 per cent from the specific absorbance of *oxymetazoline hydrochloride IPRS* prepared in the same manner.

C. To a solution of about 50 mg in 3 ml of *water*, add 1 ml of *6 M ammonium hydroxide*, filter and acidify the filtrate with *nitric acid*, the filtrate gives to the test for chloride (2.3.1)

Test

pH (2.4.24). 4.0 to 6.5, determined in a 5.0 per cent w/v solution.

Heavy metals (2.3.13). 2 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution. A 0.05 per cent w/v solution of *oxymetazoline hydrochloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with irregular or spherical, totally porous silica gel (10 µm) having a chemically bonded strongly acidic cation-exchange coating,
- mobile phase: a mixture of 46 volumes of *water*, 40 volumes of *methanol*, 10 volumes of *1 M sodium acetate* and 4 volumes of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{24}N_2O,HCl$.

Storage. Store in tightly closed containers.

Oxymetazoline Hydrochloride Nasal Solution

Oxymetazoline Hydrochloride Nasal Solution is a solution of Oxymetazoline Hydrochloride in *water* adjusted to a suitable tonicity.

Oxymetazoline Hydrocloride Nasal Solution contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of the oxymetazoline hydrochloride, $C_{16}H_{24}N_2O,HCl$.

Usual strength. 0.05 per cent.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.0 to 6.5.

Other tests. Comply with the tests stated under Nasal Preparations.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Use the Nasal Solution under examination.

Reference solution. Dissolve a suitable quantity of *oxymetazoline hydrochloride IPRS* in mobile phase to obtain a solution containing the same concentration of oxymetazoline hydrochloride as that stated on the label of the nasal solution.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with irregular or spherical, totally porous silica gel (10 µm) having a chemically bonded strongly acidic cation-exchange coating,
- mobile phase: a mixture of 46 volumes of *water*, 40 volumes of *methanol*, 10 volumes of *1 M sodium acetate* and 4 volumes of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{24}N_2O,HCl$ in the nasal solution.

Storage. Store in tightly closed containers.

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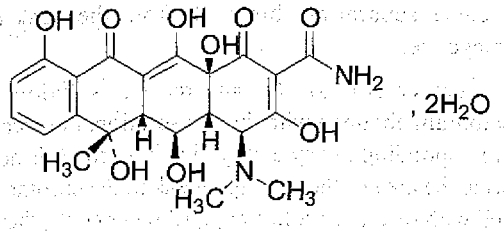
Oxytetracycline

Oxytetracycline Dihydrate



$C_{22}H_{24}N_2O_9,2H_2O$ Mol. Wt. 496.5

Oxytetracycline is (4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-dimethyl amino-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxonaphthacene-2-carboxamide dihydrate, a substance produced by the growth of certain strains of *Streptomyces rimosus* or obtained by any other means. It contains a variable quantity of water.

Oxytetracycline contains not less than 94.5 per cent and not more than 102.0 per cent of, $C_{22}H_{24}N_2O_9$, calculated on the anhydrous basis.

Category. Antibacterial.

Description. A tan yellow or light yellow (with or without a greenish tinge), crystalline powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with the substance prepared by mixing 25 g of *silica gel G* with 50 ml of a mixture of 2.5 ml of *glycerin* and 47.5 ml of *0.1 M disodium edetate* previously adjusted to pH 7.0 with *dilute ammonia solution*. After spreading the plate, allow it to stand at room temperature till it is dry (70 to 90 minutes).

Mobile phase. The lower layer formed after shaking 200 ml of a mixture of 2 volumes of *ethyl acetate*, 2 volumes of *chloroform* and 1 volume of *acetone* with 25 ml of *0.1 M disodium edetate* previously adjusted to pH 7.0 with *dilute ammonia solution*.

Test solution. Dissolve 0.05 g of the substance under examination in 100 ml of *methanol*.

Reference solution (a). A 0.05 per cent w/v solution of *oxytetracycline IPRS* in *methanol*.

Reference solution (b). A solution containing 0.05 per cent w/v each of *demethylchlortetracycline hydrochloride IPRS*, *oxytetracycline hydrochloride IPRS* and *tetracycline hydrochloride IPRS* in *methanol*.

Apply to the plate 1 µl of each solution, freshly prepared. After development, dry the plate in air, expose to the vapours of ammonia and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

B. To about 0.5 mg add 2 ml of *sulphuric acid*; a red colour is produced. Add the solution to 1 ml of *water*; the colour changes to yellow.

C. Dissolve about 2 mg in 5 ml of a 1 per cent w/v solution of *sodium carbonate* and add 2 ml of *diazotised sulphanilic acid solution*; an orange-red to brownish-red colour is produced.

Tests

pH (2.4.24). 4.5 to 7.5, determined in a 1.0 per cent w/v suspension in freshly boiled and cooled *water*.

Specific optical rotation (2.4.22). −216° to −203°, determined at 20° in a 1.0 per cent w/v solution in *0.1 M hydrochloric acid*, after allowing the solution to stand protected from light for 30 minutes before measurement.

Light absorption. Absorbance of a 0.002 per cent w/v solution in *buffer solution pH 2.0* at the maximum at about 353 nm, 0.58 to 0.62 (2.4.7).

Light-absorbing impurities. A. Dissolve 20 mg in sufficient of a mixture of 1 volume of *1 M hydrochloric acid* and 99 volumes of *methanol* to produce 10 ml. Absorbance of the resulting solution at about 430 nm, when measured within 1 hour of preparing the solution, not more than 0.25, calculated on the anhydrous basis (2.4.7).

B. Dissolve 0.1 g in sufficient of a mixture of 1 volume of *1 M hydrochloric acid* and 99 volumes of *methanol* to produce 10 ml. Absorbance of the resulting solution at about 490 nm, when measured within 1 hour of preparing the solution, not more than 0.20, calculated on the anhydrous basis (2.4.7).

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Dissolve 20 mg of the substance under examination in *0.01 M hydrochloric acid* and dilute to 25.0 ml with *0.01 M hydrochloric acid*.

Reference solution (a). A 0.008 per cent w/v solution of *oxytetracycline IPRS* in *0.01 M hydrochloric acid*. Dilute 1.0 ml of the solution to 10.0 ml with *0.01 M hydrochloric acid*.

Reference solution (b). A 0.08 per cent w/v solution of *oxytetracycline for system suitability IPRS* (containing impurity A, B, C, D and E) in *0.01 M hydrochloric acid*.

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Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: A. a 0.05 per cent v/v solution of *trifluoroacetic acid*,
  B. a mixture of 80 volumes of *acetonitrile*, 15 volumes of *methanol* and 5 volumes of *tetrahydrofuran*,
- a gradient programme using the conditions given below,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
5	90	10
20	65	35
25	90	10
30	90	10

Name	Relative retention time	Correction factor
Oxytetracycline impurity A[1]	0.9	—
Oxytetracycline (Retention time: about 6.5 minute)	1.0	—
Oxytetracycline impurity B[2]	1.2	—
Oxytetracycline impurity C[3]	1.3	—
Oxytetracycline impurity D[4]	1.4	0.4
Oxytetracycline impurity E[5]	2.2	0.4

[1](4*R*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epioxytetracycline),

[2](4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (tetracycline),

[3](4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-2-acetyl-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-4a,5a,6,12a-tetrahydrotetracene-1,11(4*H*,5*H*)-dione (2-acetyl-2-decarbamoyloxy-tetracycline),

[4](3*S*,4*S*,5*S*)-4-[(1*R*)-4,5-dihydroxy-9-methyl-3-oxo-1,3-dihydronaphtho[2,3-*c*]furan-1-yl]-3-(dimethylamino)-2,5-dihydroxy-6-oxocyclohex-1-ene-1-carboxamide,

[5](3*S*,4*S*,5*R*)-4-[(1*R*)-4,5-dihydroxy-9-methyl-3-oxo-1,3-dihydronaphtho[2,3-*c*]furan-1-yl]-3-(dimethylamino)-2,5-dihydroxy-6-oxocyclohex-1-ene-1-carboxamide.

Inject reference solution (b). The test is not valid unless the peak-to-valley ratio is not less than 3.0, where *Hp* is the height above the baseline of the peak due to oxytetracycline impurity A and *Hv* is the height above the baseline of the lowest point of the curve separating this peak from the peak due to oxytetracycline and not less than 3.0, where *Hp* is the height above the baseline of the peak due to oxytetracycline impurity B and *Hv* is the height above the baseline of the lowest point of the curve separating this peak from the peak due to oxytetracycline.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to oxytetracycline impurity C is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent), the area of any peak corresponding to oxytetracycline impurity B is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent), the area of any peak corresponding to oxytetracycline impurity A is not more than 0.7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent), the area of any peak corresponding to oxytetracycline impurity D and oxytetracycline impurity E is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any other secondary peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.5 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 0.4 g complies with limit the test for heavy metals, Method B (50 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). 6.0 per cent to 9.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in *0.01M hydrochloric acid* and dilute to 25.0 ml with *0.01M hydrochloric acid*. Dilute 5.0 ml of the solution to 50.0 ml with *0.01 M hydrochloric acid*.

Reference solution. A 0.008 per cent w/v solution of *oxytetracycline IPRS* in *0.01 M hydrochloric acid*.

Use chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

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Calculate the content of $C_{22}H_{24}N_2O_9$.

Oxytetracycline intended for use in the manufacture of parenteral preparations without a further procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.4 Endotoxin Unit per mg of Oxytetracycline.

Oxytetracycline intended for use in the manufacture of parenteral preparations without a further sterilisation procedure complies with the following additional requirement.

Sterility. Complies with the test for sterility (2.2.11).

Storage. Store protected from light and moisture. If it is intended for use in the manufacture of parenteral preparations, the container should be sterile and sealed so as to exclude micro-organisms.

Labelling. The label states whether or not the contents are intended for use in the manufacture of parenteral preparations.

Oxytetracycline Injection

Oxytetracycline Dihydrate Injection

Oxytetracycline Injection is a sterile solution of oxytetracycline with or without one or more suitable buffering agents, anaesthetics, preservatives, antioxidants, complexing agents and solvents.

Oxytetracycline Injection contains not less than 90.0 per cent and not more than 120.0 per cent of the stated amount of anhydrous oxytetracycline, $C_{22}H_{24}N_2O_9$.

Usual strengths. 50 mg per ml; 125 mg per ml.

Description. A clear, yellow to tan yellow solution. It may have a greenish tinge.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with the substance prepared by mixing 25 g of *silica gel G* with 50 ml of a mixture of 2.5 ml of *glycerin* and 47.5 ml of *0.1 M disodium edetate* previously adjusted to pH 7.0 with *dilute ammonia solution*. After spreading the plate, allow it to stand at room temperature till it is dry (70 to 90 minutes).

Mobile phase. The lower layer formed after shaking 200 ml of a mixture of 2 volumes of *ethyl acetate*, 2 volumes of *chloroform* and 1 volume of *acetone* with 25 ml of *0.1 M disodium edetate* previously adjusted to pH 7.0 with *dilute ammonia solution*.

Test solution. Shake a quantity containing 10 mg of anhydrous oxytetracycline with 20 ml of *methanol*, centrifuge if necessary and use the clear supernatant liquid.

Reference solution (a). A 0.05 per cent w/v solution of *oxytetracycline hydrochloride IPRS* in *methanol*.

Reference solution (b). A solution containing 0.05 per cent w/v each of *demethylchlortetracycline hydrochloride IPRS*, *oxytetracycline hydrochloride IPRS* and *tetracycline hydrochloride IPRS* in *methanol*.

Apply to the plate 1 µl of each solution, freshly prepared. After development, dry the plate in air, expose to the vapours of ammonia and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

B. Add 0.1 ml to 2 ml of *sulphuric acid*; a red colour is produced. Add the solution to 1 ml of *water*; the colour changes to yellow.

Tests

pH (2.4.24). 8.0 to 9.0.

Bacterial endotoxins (2.2.3). Not more than 0.4 Endotoxin Unit per mg of Oxytetracycline.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Phosphate buffer pH 7.5. A mixture of 85 volumes of 0.33 M *dibasic potassium phosphate* and 15 volumes of 0.33 M *monobasic sodium phosphate*, adjusted to pH 7.5.

Test solution. Dilute a volume of the injection containing 100 mg of oxytetracycline to 500.0 ml with *0.01 M hydrochloric acid*.

Reference solution (a). A 0.02 per cent w/v solution of *oxytetracycline IPRS* in *0.01 M hydrochloric acid*.

Reference solution (b). A 0.02 per cent w/v solution of *tetracycline hydrochloride IPRS* in *0.01 M hydrochloric acid*. To 3.0 ml of the solution, add 1.5 ml of reference solution (a) and dilute to 25.0 ml with *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with styrene-divinylbenzene co-polymer (5 to10 µm),
- column temperature: 60°,
- mobile phase: a solution prepared by mixing 50 g of *tertiary butyl alcohol* with 200 ml of *water* in 1000-ml volumetric flask, add 60 ml of phosphate buffer pH 7.5, 50 ml of 1.0 per cent w/v solution of *tetrabutylammonium hydrogen sulphate*, adjusted to pH 7.5 with *1M sodium*

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hydroxide and 10 ml of a 0.04 per cent w/v solution of *disodium edetate*, adjusted to pH 7.5 with *2 M sodium hydroxide* and dilute to volume with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention time with reference to tetracycline for oxytetracycline is about 0.6.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peak due to oxytetracycline and tetracycline is not less than 5.0 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 1.25 and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (a).

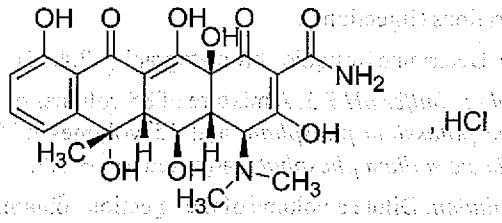
Inject reference solution (a) and the test solution.

Calculate the content of $C_{22}H_{24}N_2O_9$ in the injection.

Storage. Store protected from light.

Labelling. The label states (1) the strength in mg of anhydrous oxytetracycline per ml; (2) that the contents are to be used for intramuscular use only; (3) the names of any preservatives used.

Oxytetracycline Hydrochloride



$C_{22}H_{24}N_2O_9,HCl$ Mol. Wt. 496.9

Oxytetracycline Hydrochloride is (4*S*,4a*R*,5*S*,5a*R*,6*S*, 12a*S*)-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxonaphthacene-2-carboxamide hydrochloride, a substance produced by the growth of certain strains of *Streptomyces rimosus* or obtained by any other means.

Oxytetracycline Hydrochloride contains not less than 94.5 per cent and not more than 102.0 per cent of, $C_{22}H_{24}N_2O_9$, HCl, calculated on the anhydrous basis.

Category. Antibacterial.

Description. A pale yellow, crystalline powder; hygroscopic.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with the substance prepared by mixing 25 g of *silica gel G* with 50 ml of a mixture of 2.5 ml of *glycerin* and 47.5 ml of *0.1 M disodium edetate* previously adjusted to pH 7.0 with *dilute ammonia solution*. After spreading the plate, allow it to stand at room temperature till it is dry (70 to 90 minutes).

Mobile phase. The lower layer formed after shaking 200 ml of a mixture of 2 volumes of *ethyl acetate*, 2 volumes of *chloroform* and 1 volume of *acetone* with 25 ml of *0.1 M disodium edetate* previously adjusted to pH 7.0 with *dilute ammonia solution*.

Test solution. Dissolve 0.05 g of the substance under examination in 100 ml of *methanol*.

Reference solution (a). A 0.05 per cent w/v solution of *oxytetracycline hydrochloride IPRS* in *methanol*.

Reference solution (b). A solution containing 0.05 per cent w/v each of *demethylchlortetracycline hydrochloride IPRS*, *oxytetracycline hydrochloride IPRS* and *tetracycline hydrochloride IPRS* in *methanol*.

Apply to the plate 1 µl of each solution, freshly prepared. After development, dry the plate in air, expose to the vapours of ammonia and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

B. To about 0.5 mg add 2 ml of *sulphuric acid*; a red colour is produced. Add the solution to 1 ml of *water*; the colour changes to yellow.

C. Dissolve about 2 mg in 5 ml of a 1 per cent w/v solution of *sodium carbonate* and add 2 ml of *diazotised sulphanilic acid solution*; an orange-red to brownish-red colour is produced.

D. A 5 per cent w/v solution gives the reactions of chlorides (2.3.1).

Tests

pH (2.4.24). 2.0 to 3.0, determined in a 1.0 per cent w/v solution.

Specific optical rotation (2.4.22). – 200° to – 188°, determined at 20° in a 1 per cent w/v solution in *0.1 M hydrochloric acid*.

Light absorption (2.4.7). Absorbance of a 0.002 per cent w/v solution in *chloride buffer solution pH 2.0* at the maximum at about 353 nm, 0.54 to 0.58.

Light-absorbing impurities. A. Dissolve 20 mg in sufficient of a mixture of 1 volume of *1 M hydrochloric acid* and 99 volumes of *methanol* to produce 10 ml. Absorbance of the resulting solution at about 430 nm, when measured within 1 hour of preparing the solution, not more than 0.50, calculated on the anhydrous basis (2.4.7).

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B. Dissolve 0.1 g in sufficient of a mixture of 1 volume of *1 M hydrochloric acid* and 99 volumes of *methanol* to produce 10 ml. Absorbance of the resulting solution at about 490 nm, when measured within 1 hour of preparing the solution, not more than 0.20, calculated on the anhydrous basis (2.4.7).

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Dissolve 20 mg of the substance under examination in *0.01 M hydrochloric acid* and dilute to 25.0 ml with *0.01 M hydrochloric acid.*

Reference solution (a). A 0.008 per cent w/v solution of *oxytetracycline IPRS* in *0.01 M hydrochloric acid.* Dilute 1.0 ml of the solution to 10.0 ml with *0.01 M hydrochloric acid.*

Reference solution (b). A 0.08 per cent w/v solution of *oxytetracycline for system suitability IPRS* (containing impurity A, B, C, D and E) in *0.01 M hydrochloric acid.*

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: A. a 0.05 per cent v/v solution of *trifluoroacetic acid*,
  B. a mixture of 80 volumes of *acetonitrile*, 15 volumes of *methanol* and 5 volumes of *tetrahydrofuran*,
- a gradient programme using the conditions given below,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
5	90	10
20	65	35
25	90	10
30	90	10

Name	Relative retention time	Correction factor
Oxytetracycline impurity A[1]	0.9	—
Oxytetracycline (Retention time: about 6.5 minute)	1.0	—
Oxytetracycline impurity B[2]	1.2	—
Oxytetracycline impurity C[3]	1.3	—
Oxytetracycline impurity D[4]	1.4	0.4
Oxytetracycline impurity E[5]	2.2	0.4
Oxytetracycline Impurity F[6]	2.3	—

[1](4*R*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epioxytetracycline),

[2](4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (tetracycline),

[3](4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-2-acetyl-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-4a,5a,6,12a-tetrahydrotetracene-1,11(4*H*,5*H*)-dione(2-acetyl-2-decarbamoyloxytetracycline),

[4](3*S*,4*S*,5*S*)-4-[(1*R*)-4,5-dihydroxy-9-methyl-3-oxo-1,3-dihydronaphtho[2,3-*c*]furan-1-yl]-3-(dimethylamino)-2,5-dihydroxy-6-oxocyclohex-1-ene-1-carboxamide,

[5](3*S*,4*S*,5*R*)-4-[(1*R*)-4,5-dihydroxy-9-methyl-3-oxo-1,3-dihydronaphtho[2,3-*c*]furan-1-yl]-3-(dimethylamino)-2,5-dihydroxy-6-oxocyclohex-1-ene-1-carboxamide,

[6](4*S*,4a*R*,5*R*,12a*S*)-4-(dimethylamino)-3,5,10,11,12a-pentahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (anhydro-*oxytetracycline*).

Inject reference solution (b). The test is not valid unless the peak-to-valley ratio is not less than 3.0, where *Hp* is the height above the baseline of the peak due to oxytetracycline impurity A and *Hv* is the height above the baseline of the lowest point of the curve separating this peak from the peak due to oxytetracycline and not less than 3.0, where *Hp* is the height above the baseline of the peak due to oxytetracycline impurity B and *Hv* is the height above the baseline of the lowest point of the curve separating this peak from the peak due to oxytetracycline.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to oxytetracycline impurity C is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent), the area of any peak corresponding to oxytetracycline impurity B is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent), the area of any peak corresponding to oxytetracycline impurity A is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent), the area of any peak corresponding to oxytetracycline impurity D and oxytetracycline impurity E, each of, is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the sum of the areas of the peaks due to oxytetracycline impurity D, E and F is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent), the area of any other secondary peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.5 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

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Heavy metals (2.3.13). 0.4 g complies with the limit test for heavy metals, Method B (50 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). Not more than 2.0 per cent w/w, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in *0.01M hydrochloric acid* and dilute to 25.0 ml with *0.01M hydrochloric acid.* Dilute 5.0 ml of the solution to 50.0 ml with *0.01 M hydrochloric acid.*

Reference solution. A 0.008 per cent w/v solution of *oxytetracycline IPRS* in *0.01 M hydrochloric acid.*

Use chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{24}N_2O_9$,HCl. 1 mg of $C_{22}H_{24}N_2O_9$ is equivalent to 1.079 mg of $C_{22}H_{24}N_2O_9$,HCl.

Oxytetracycline Hydrochloride intended for use in the manufacture of parenteral preparations without a further procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.4 Endotoxin Unit per mg.

Oxytetracycline Hydrochloride intended for use in the manufacture of parenteral preparations without a further sterilisation procedure complies with the following additional requirement.

Sterility. Complies with test for sterility (2.2.11).

Storage. Store protected from light and moisture. If it is intended for use in the manufacture of parenteral preparations, the container should be sterile and sealed so as to exclude micro-organisms.

Labelling. The label states whether or not the contents are intended for use in the manufacture of parenteral preparations.

Oxytetracycline Capsules

Oxytetracycline Hydrochloride Capsules

Oxytetracycline Capsules contain not less than 95.0 per cent and not more than 110.0 per cent of the stated amount of oxytetracycline hydrochloride, $C_{22}H_{24}N_2O_9$,HCl.

Usual strengths. 250 mg; 500 mg.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with the substance prepared by mixing 25 g of *silica gel G* with 50 ml of a mixture of 2.5 ml of *glycerin* and 47.5 ml of *0.1 M disodium edetate* previously adjusted to pH 7.0 with *dilute ammonia solution.* After spreading the plate, allow it to stand at room temperature till it is dry (70 to 90 minutes).

Mobile phase. The lower layer formed after shaking 200 ml of a mixture of 2 volumes of *ethyl acetate,* 2 volumes of *chloroform* and 1 volume of *acetone* with 25 ml of *0.1 M disodium edetate* previously adjusted to pH 7.0 with *dilute ammonia solution.*

Test solution. Extract a quantity of the contents of the capsules containing 10 mg of Oxytetracycline Hydrochloride with 20 ml *methanol*, centrifuge and use the supernatant liquid.

Reference solution (a). A 0.05 per cent w/v solution of *oxytetracycline hydrochloride IPRS* in *methanol.*

Reference solution (b). A solution containing 0.05 per cent w/v each of *demethylchlortetracycline hydrochloride IPRS, oxytetracycline hydrochloride IPRS* and *tetracycline hydrochloride IPRS* in *methanol.*

Apply to the plate 1 µl of each solution, freshly prepared. After development, dry the plate in air, expose to the vapours of ammonia and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

B. To 0.5 mg of the contents of the capsules add 2 ml of *sulphuric acid*; a red colour is produced. Add the solution to 1 ml of *water*; the colour changes to yellow.

C. Dissolve about 2 mg of the contents of the capsules in 5 ml of a 1 per cent w/v solution of *sodium carbonate* and add 2 ml of *diazotised sulphanilic acid solution*; a light brown colour is produced.

D. The contents of the capsules give the reactions of chlorides (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid,*
Speed and time. 100 rpm and 45 minutes.

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Withdraw a suitable volume of the medium and filter through a membrane filter disc with an average pore diameter not greater than 1.0 µm. Measure the absorbance of the filtrate, suitably diluted if necessary, at the maximum at about 353 nm (2.4.7). Calculate the content of $C_{22}H_{24}N_2O_9$,HCl in the medium taking 282 as the specific absorbance at 353 nm.

Q. Not less than 75 per cent of the stated amount of $C_{22}H_{24}N_2O_9$,HCl in the medium.

Light-absorbing impurities. Dissolve a portion of the mixed contents of five capsules as completely as possible in sufficient of a mixture of 1 volume of *1 M hydrochloric acid* and 99 volumes of *methanol* to produce two solutions of Oxytetracycline Hydrochloride containing (1) 0.2 per cent w/v and (2) 1.0 per cent w/v and filter each solution. Absorbance of the filtrate obtained from solution (1) at about 430 nm, when measured within 1 hour of preparing the solution, not greater than 0.75 and of the filtrate obtained from solution (2) at about 490 nm, not more than 0.40 (2.4.7).

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g of the mixed contents of the capsules by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 3 hours.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Phosphate buffer pH 7.5. A mixture of 85 volumes of 0.33 M *dibasic potassium phosphate* and 15 volumes of 0.33 M *monobasic sodium phosphate*, adjusted to pH 7.5.

Test solution. Disperse a quantity of mixed content of 20 capsules containing 100 mg of oxytetracycline hydrochloride in *0.01 M hydrochloric acid* and dilute to 500.0 ml with *0.01 M hydrochloric acid*.

Reference solution (a). A 0.02 per cent w/v solution of *oxytetracycline hydrochloride IPRS* in *0.01 M hydrochloric acid*.

Reference solution (b). A 0.02 per cent w/v solution of *tetracycline hydrochloride IPRS* in *0.01 M hydrochloric acid*. To 3.0 ml of the solution, add 1.5 ml of reference solution (a) and dilute to 25.0 ml with *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with styrene-divinylbenzene co-polymer (5 to10 µm),
- column temperature: 60°,
- mobile phase: a solution prepared by mixing 50 g of *tertiary butyl alcohol* with 200 ml of *water* in 1000-ml volumetric flask, add 60 ml of phosphate buffer pH 7.5, 50 ml of 1.0 per cent w/v solution of *tetrabutylammonium hydrogen sulphate*, adjusted to pH 7.5 with *1 M sodium hydroxide* and 10 ml of a 0.04 per cent w/v solution of *disodium edetate*, adjusted to pH 7.5 with *2 M sodium hydroxide* and dilute to volume with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention time with reference to tetracycline for oxytetracycline is about 0.6.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peak due to oxytetracycline and tetracycline is not less than 5.0 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 1.25 and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{22}H_{24}N_2O_9$,HCl in the capsules.

Storage. Store protected from light and moisture.

Oxytetracycline Eye Ointment

Oxytetracycline Hydrochloride Eye Ointment

Oxytetracycline Eye Ointment contains not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of oxytetracycline hydrochloride, $C_{22}H_{24}N_2O_9$,HCl.

Usual strength. 1 per cent w/w.

Identification

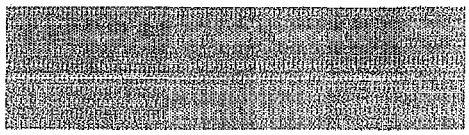
Determine by thin-layer chromatography (2.4.17), coating the plate with the substance prepared by mixing 25 g of *silica gel G* with 50 ml of a mixture of 2.5 ml of *glycerin* and 47.5 ml of *0.1 M disodium edetate* previously adjusted to pH 7.0 with *dilute ammonia solution*. After spreading the plate, allow it to stand at room temperature till it is dry (70 to 90 minutes).

Mobile phase. The lower layer formed after shaking 200 ml of a mixture of 2 volumes of *ethyl acetate*, 2 volumes of *chloroform* and 1 volume of *acetone* with 25 ml of *0.1 M disodium edetate* previously adjusted to pH 7.0 with *dilute ammonia solution*.

Test solution. A solution prepared by heating a quantity containing 20 mg of Oxytetracycline Hydrochloride with 20 ml of *methanol* for 20 minutes, cooling in ice, filtering, carefully evaporating the filtrate to dryness and dissolving the residue in 20 ml of *methanol*.

Reference solution (a). A 0.05 per cent w/v solution of *oxytetracycline hydrochloride IPRS* in *methanol*.

Reference solution (b). A solution containing 0.05 per cent w/v each of *demethylchlortetracycline hydrochloride IPRS*, *oxytetracycline hydrochloride IPRS* and *tetracycline hydrochloride IPRS* in *methanol*.



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Apply to the plate 1 µl of each solution, freshly prepared. After development, dry the plate in air, expose to the vapours of ammonia and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

Tests

Water (2.3.43). Not more than 1.0 per cent, determined on 0.5 g.

Other tests. Comply with the tests stated under Eye Ointments.

Assay. Weigh 1.0 g and transfer to a separating funnel. Add 25 ml of *peroxide-free ether*, shake well and extract with five quantities, each of 20 ml, of *0.1 M hydrochloric acid*. Combine the extracts and dilute to 200.0 ml with *0.1 M hydrochloric acid*. Dilute a suitable volume of the resulting solution with *buffer solution No 3* (2.2.10), to produce a solution containing 1 µg of oxytetracycline per ml.

Determine by the microbiological assay of antibiotics, Method B (2.2.10), and express the results as a percentage of oxytetracycline hydrochloride taking each mg of oxytetracycline to be equivalent to 1.079 mg of oxytetracycline hydrochloride, $C_{22}H_{24}N_2O_9,HCl$.

Storage. Store protected from light and moisture.

Oxytetracycline Hydrochloride Injection

Oxytetracycline Hydrochloride Injection is a sterile material consisting of Oxytetracycline Hydrochloride with or without buffering agents and other excipients. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution may be used within three days of preparation when stored in a refrigerator (2° to 8°).

Oxytetracycline Hydrochloride Injection contains not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of oxytetracycline, $C_{22}H_{24}N_2O_9$.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Usual strengths. The equivalent of 250 mg and 500 mg of oxytetracycline.

Description. A pale yellow, crystalline powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with the substance prepared by mixing 25 g of *silica gel G* with 50 ml of a mixture of 2.5 ml of *glycerin* and 47.5 ml of *0.1 M disodium edetate* previously adjusted to pH 7.0 with *dilute ammonia solution*. After spreading the plate, allow it to stand at room temperature till it is dry (70 to 90 minutes).

Mobile phase. The lower layer formed after shaking 200 ml of a mixture of 2 volumes of *ethyl acetate*, 2 volumes of *chloroform* and 1 volume of *acetone* with 25 ml of *0.1 M disodium edetate* previously adjusted to pH 7.0 with *dilute ammonia solution*.

Test solution. Dissolve 0.05 g of the substance under examination in 100 ml of *methanol*.

Reference solution (a). A 0.05 per cent w/v solution of *oxytetracycline hydrochloride IPRS* in *methanol*.

Reference solution (b). A solution containing 0.05 per cent w/v each of *demethylchlortetracycline hydrochloride IPRS*, *oxytetracycline hydrochloride IPRS* and *tetracycline hydrochloride IPRS* in *methanol*.

Apply to the plate 1 µl of each solution, freshly prepared. After development, dry the plate in air, expose to the vapours of ammonia and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

B. To about 0.5 mg add 2 ml of *sulphuric acid*; a red colour is produced. Add the solution to 1 ml of *water*; the colour changes to yellow.

C. Dissolve about 2 mg in 5 ml of a 1 per cent w/v solution of *sodium carbonate* and add 2 ml of *diazotised sulphanilic acid solution*; an orange-red to brownish-red colour is produced.

Tests

Appearance of solution. A 10.0 per cent w/v solution is clear (2.4.1) and yellow.

pH (2.4.24). 2.0 to 3.0, determined in a 1.0 per cent w/v solution.

Light-absorbing impurities. A. Dissolve 20 mg in sufficient of a mixture of 1 volume of *1 M hydrochloric acid* and 99 volumes of *methanol* to produce 10 ml. Absorbance of the resulting solution at about 430 nm, when measured within

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1 hour of preparing the solution, not more than 0.50, calculated on the anhydrous basis (2.4.7).

B. Dissolve 0.1 g in sufficient of a mixture of 1 volume of *1 M hydrochloric acid* and 99 volumes of *methanol* to produce 10 ml. Absorbance of the resulting solution at about 490 nm, when measured within 1 hour of preparing the solution, not more than 0.20, calculated on the anhydrous basis (2.4.7).

Bacterial endotoxins (2.2.3). Not more than 0.4 Endotoxin Unit per mg.

Assay. Determine by liquid chromatography (2.4.14).

Phosphate buffer pH 7.5. A mixture of 85 volumes of 0.33 M *dibasic potassium phosphate* and 15 volumes of 0.33 M *monobasic sodium phosphate*, adjusted to pH 7.5.

Test solution. Reconstitute 5 vials and pool the sample. Dilute a volume of the injection in *0.01 M hydrochloric acid* to obtain a solution containing 0.02 per cent w/v of oxytetracycline.

Reference solution (a). A 0.02 per cent w/v solution of *oxytetracycline IPRS* in *0.01 M hydrochloric acid*.

Reference solution (b). A 0.02 per cent w/v solution of *tetracycline hydrochloride IPRS* in *0.01 M hydrochloric acid*. To 3.0 ml of the solution, add 1.5 ml of reference solution (a) and dilute to 25.0 ml with *water*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with styrene-divinylbenzene co-polymer (5 to10 µm),
- column temperature: 60°,
- mobile phase: a solution prepared by mixing 50 g of *tertiary butyl alcohol* with 200 ml of *water* in 1000-ml volumetric flask, add 60 ml of phosphate buffer pH 7.5, 50 ml of 1.0 per cent w/v solution of *tetrabutylammonium hydrogen sulphate*, adjusted to pH 7.5 with *1 M sodium hydroxide* and 10 ml of a 0.04 per cent w/v solution of *disodium edetate*, adjusted to pH 7.5 with *2 M sodium hydroxide* and dilute to volume with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention time with reference to tetracycline for oxytetracycline is about 0.6.

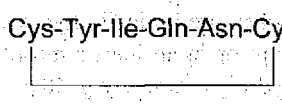
Inject reference solution (a) and (b). The test is not valid unless the resolution between the peak due to oxytetracycline and tetracycline is not less than 5.0 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 1.25 and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. Calculate the content of $C_{22}H_{24}N_2O_9$ in the injection.

Storage. Store protected from light and moisture.

Labelling. The label states (1) the quantity of Oxytetracycline Hydrochloride contained in it in terms of the equivalent amount of oxytetracycline; (2) that the contents are to be used for intravenous injection only; (3) the names of the buffering agents used.

Oxytocin



$C_{43}H_{66}N_{12}O_{12}S_2$ Mol. Wt.1007.2

Oxytocin is a cyclic nonapeptide hormone obtained by a process of fractionation from the posterior lobe of the pituitary gland of healthy oxen or other mammals or by synthesis that has the property of stimulating contraction of the uterus and milk ejection in receptive animals. It may be presented as a solid or as a solution in a solvent containing an appropriate antimicrobial preservative such as 0.2 per cent w/v solution of chlorbutol.

If it is derived from animal species, Oxytocin contains not less than 95.0 per cent and not more than 105.0 per cent of the stated number of Units of oxytocic activity. If it is a synthetic product presented as a solid, it contains not less than 560 Units per mg, calculated with reference to the peptide content and when presented as a liquid, it contains not less than 150 Units per ml.

By convention, for the purpose of labeling oxytocin preparations, 1 mg of oxytocin peptide ($C_{43}H_{66}O_{12}S_2$) is equivalent to 600 IU of biological activity.

Category. Uterine stimulant.

Description. When presented as a solid, a white or almost white powder. When presented as a liquid, a clear colourless liquid.

Identification

Test B may be omitted if tests A and C are carried out. Test C may be omitted if tests A and B are carried out.

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Amino acid analysis.

For hydrolysis—Acid hydrolysis using *hydrochloric acid* containing phenol is the most common procedure used for

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protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

Hydrolysis solution. 6 M hydrochloric acid containing 0.1 per cent to 1.0 per cent of *phenol*.

Procedure

Liquid phase hydrolysis. Place the protein or peptide sample in a hydrolysis tube, and dry (the sample is dried so that water in the sample will not dilute the acid used for the hydrolysis). Add 200 µl of hydrolysis solution per 500 µg of lyophilised protein. Freeze the sample tube in a dry ice-acetone bath, and flame seal in vacuum. Samples are typically hydrolysed at 110° for 24 hours in vacuum or in an inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g. 48 hours and 72 hours) are investigated if there is a concern that the protein is not completely hydrolysed.

Vapour phase hydrolysis. This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimised by using vapour phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of hydrolysis solution. The hydrolysis solution does not come in contact with the test sample. Apply an inert atmosphere or vacuum (less than 200 µm of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110° for a 24 hours hydrolysis time. Acid vapour hydrolysis the dried sample. Any condensation of the acid in the sample vials is to be minimised. After hydrolysis, dry the test sample in vacuum to remove any residual acid.

For analysis—Post-column ninhydrin derivatisation : Ion-exchange chromatography with post-column ninhydrin derivatisation is one of the most common methods employed for quantitative amino acid analysis. As a rule, a lithium-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster sodium-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

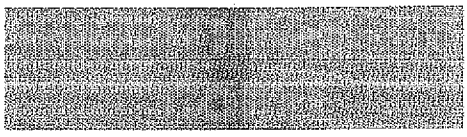
When the amino acid reacts with *ninhydrin*, the reactant has a characteristic purple or yellow colour. Amino acids, except imino acid, give a purple colour, and show an absorption maximum at 570 nm. The imino acids such as proline give a yellow colour, and show an absorption maximum at 440 nm. The post-column reaction between *ninhydrin* and amino acids eluted from the column is monitored at 440 nm and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

The detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for the proline derivative. Response linearity is obtained in the range of 20-500 pmol with correlation coefficients exceeding 0.999. To obtain good composition data, samples larger than 1 µg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking 1/6 of the sum of the number of moles of aspartic acid, glutamic acid, proline, glycine, isoleucine and leucine as equal to 1.

The values fall within the following limits: aspartic acid: 0.90 to 1.10; glutamic acid: 0.90 to 1.10; proline: 0.90 to 1.10; glycine: 0.90 to 1.10; leucine: 0.90 to 1.10; isoleucine: 0.90 to 1.10; tyrosine: 0.7 to 1.05; half-cystine: 1.4 to 2.1. Not more than traces of other amino acids are present.

C. For biological response, dissect out the uterus from a virgin female rat weighing between 120 to 200 g and in diestrus stage. Immediately before injection confirm the uterine stage of the rat by vaginal smear. Suspend one horn of the uterus in a organ bath containing 9.0 g of *sodium chloride*, 0.42 g of *potassium chloride*, 0.16 g of *calcium chloride*, 0.50 g of *sodium bicarbonate*, 0.25 g of *dextrose*, and 0.0053 g of *magnesium chloride* per litre of the solution. Maintain the bath temperature at 32° or any other suitable temperature at which spontaneous contractions of the uterus are abolished and the preparations maintain its sensitivity. Oxygenate the bath solution with a mixture of 95 per cent *oxygen* and 5 per cent *carbon dioxide*. Record the contractions of the uterine muscle on a recorder, using a suitable instrument producing linear response (for example an isotonic liver with a load of not exceeding 2 g or isotonic and linear transducer). Add to the bath two appropriate dilutions of the oxytocin reference solution, and record the contraction of the muscle following each dilution. The appropriate dilutions (doses) are those dilutions of the reference solution which produce clearly distinctive submaximal contractions. The required dilution normally lies between 0.1 to 5 micro units per ml of the bath solution. When maximal contraction is reached, replace the bath solution by a fresh solution and wait until the muscle is relaxed completely and the pointer of the recorder returns to the base line. The doses of the different reference solutions should be added at regular intervals depending upon the rate of the recovery of the uterine muscle. Dissolve or dilute the preparation to be tested in a suitable diluent (preferably using bath solution) to obtain responses on the addition of two dilutions similar to the one used with the oxytocin reference solution. The two selected dilutions of the reference solution and preparation under examination should be applied according to a randomised block or Latin square design and



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at least three responses to each dilution should be recorded. The magnitude of contractions obtained with the reference solution is comparable to the contractions obtained with the test solution.

Tests

Peptide. 90.0 to 110.0 per cent of the stated amount of oxytocin, $C_{43}H_{66}N_{12}O_{12}S_2$ expressed per mg for the solid, and in mg per ml for the liquid.

Determine by liquid chromatography (2.4.14).

Test solution. A 0.035 per cent w/v solution of the substance under examination in 1.56 per cent w/v solution of *sodium dihydrogen phosphate*.

Reference solution. A 0.035 per cent w/v solution of *oxytocin IPRS* in 1.56 per cent w/v solution of *sodium dihydrogen phosphate*.

Chromatographic system
- a stainless steel column 12 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: appropriate proportions of a 1.56 per cent w/v solution of *sodium dihydrogen phosphate* (mobile phase A) and a mixture of equal volumes of *acetonitrile* and *water* (mobile phase B),
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl

Equilibrate the column with a mixture of 70 volumes of mobile phase A and 30 volumes of mobile phase B and record the chromatograms as follows. Operate by gradient elution increasing continuously and linearly the proportion of mobile phase B by 1.0 per cent v/v per minute for 30 minutes. Finally elute using the same mixture for 15 minutes to re-equilibrate the column.

Calculate the content of the peptide, $C_{43}H_{66}N_{12}O_{12}S_2$.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. A 0.025 per cent w/v solution of the substance under examination in mobile phase A.

Reference solution. Dissolve the contents of one vial of *oxytocin IPRS* in mobile phase A to produce a solution containing the same concentration as in test solution.

Chromatographic system
- a stainless steel column 12.5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a 1.6 per cent w/v solution of *sodium dihydrogen phosphate*,
  B. a mixture of 50 volumes of *acetonitrile* and 50 volumes of *water*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 25 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
30	40	60
30.1	70	30
45	70	30

Inject the reference solution and the test solution.

Calculate the units of the oxytocic activity or the potency.

Oxytocin intended for use in the manufacture of parenteral preparations without a further procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per Unit of oxytocin.

Oxytocin intended for use in the manufacture of parenteral preparations without a further sterilisation procedure complies with the following additional requirement.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store protected from light and moisture, at a temperature between 2° to 8°. If the substance is sterile, store in sterile airtight, tamper-proof container.

Labelling. The label states (1) the number of Units of oxytocic activity per mg (for solid) or per ml (for liquid); (2) either the animal species from which it is obtained or whether it is synthetic, as appropriate; (3) whether or not the contents are intended for use in the manufacture of parenteral preparations.

Oxytocin Injection

Oxytocin Injection is a sterile solution of Oxytocin in Water for Injections.

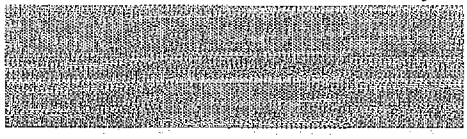
Oxytocin Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated number of Units of oxytocin activity.

Usual strengths. 5 Units per ml; 10 Units per ml.

Description. A clear colourless liquid.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.



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Tests

pH (2.4.24). 3.0 to 5.0

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per Unit of oxytocin.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Use the injection under examination.

Reference solution. Dissolve the contents of one vial of *oxytocin IPRS* in a 1.65 per cent w/v solution of *sodium dihydrogen orthophosphate* to produce a solution containing the same concentration in µg of oxytocin as that stated on the label of the injection.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm) (Such as Nucleosil C18),
- column temperature: 40°,
- mobile phase: a mixture of 85 volumes of a 0.2 per cent v/v solution of *orthophosphoric acid* and 15 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 100 µl.

Inject the reference solution. The test is not valid unless the theoretical plates are not less than 2,000.

Inject the reference solution and the test solution.

Calculate the content of $C_{43}H_{66}N_{12}O_{12}S_2$ in the injection.

Oxytocin Injection containing Oxytocin of natural origin obtained by extraction and purification complies with the following additional requirement.

Vasopressin impurity. Not more than 0.5 Unit per ml.

Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 5.0 g of chlorobutanol in 5.0 ml of *glacial acetic acid*, add 1.1 g of *sodium acetate*, 5.0 g of *ethanol*, and dilute to 1000 ml with *water* and mix.

Test solution. Dilute 2.0 ml of injection under examination to 25 ml with 0.25 per cent w/v of *glacial acetic acid* and mix.

Reference solution. Dissolve the contents of one vial of *vasopressin IPRS* in a known volume of solvent mixture. If necessary dilute the prepared solution to a working concentration range.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: a mixture of 87 volumes of 6.6 per cent v/v solution of *dibasic ammonium phosphate*, adjusted to pH 3.0 with *orthophosphoric acid* and 13 volumes of *acetonitrile*, filter through 0.45 µm nylon membrane,
- flow rate: 1 ml per minute,
- spectrophometer set at 220 nm,
- injection volume: 20 µl.

Equilibrate the column at least for one hour. Run the chromatogram minimum of 60 minutes.

Inject the reference solution. The test is not valid unless the resolution between vasopressin and the adjacent peak is not less than 1.5 and relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Storage. Store protected from light and unless otherwise indicated on the label, at a temperature between 2°to 8°.

Labelling. The label states (1) the number of Units of oxytocic activity per ml; (2) either the animal spieces from which it is obtained or whether it is synthetic, as appropriate.

Oxytocin Nasal Solution

Oxytocin Nasal Solution is a solution of Oxytocin in a suitable solvent containing an appropriate antimicrobial preservative.

Oxytocin Nasal Solution contains not less than 85.0 per cent and not more than 120.0 per cent of the stated number of Units of oxytocic activity.

Usual strength. 40 Units per ml.

Description. A clear, colourless solution.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 3.0 to 5.0.

Other tests. Comply with the tests stated under Nasal Preparations.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Use the nasal solution under examination.

Reference solution. Dissolve the contents of one vial of *oxytocin IPRS* in a 1.65 per cent w/v solution of *sodium dihydrogen orthophosphate* to produce a solution containing

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the same concentration in µg of oxytocin as that stated on the label of the nasal solution.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm) (Such as Nucleosil C18),
- column temperature: 40°,
- mobile phase: a mixture of 85 volumes of a 0.2 per cent v/v solution of *orthophosphoric acid* and 15 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 200 µl.

Inject the reference solution. The test is not valid unless the theoretical plates is not less than 50,000.

Inject the reference solution and the test solution.

Calculate the content of $C_{43}H_{66}N_{12}O_{12}S_2$ in the nasal solution.

Oxytocin Nasal Solution containing Oxytocin of natural origin obtained by extraction and purification complies with the following additional requirement.

Vasopressin impurity. Not more than 0.5 Unit per ml.

Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 5.0 g of *chlorobutanol* in 5.0 ml of *glacial acetic acid*, add 1.1 g of *sodium acetate*, 5.0 g of *ethanol*, and dilute to 1000 ml with *water* and mix.

Test solution. Dilute 2.0 ml of injection under examination to 25 ml with 0.25 per cent w/v of *glacial acetic acid* and mix.

Reference solution. Dissolve the contents of one vial of *vasopressin IPRS* in a known volume of solvent mixture. If necessary dilute the prepared solution to a working concentration range.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: a mixture of 87 volumes of 6.6 per cent v/v solution of *dibasic ammonium phosphate*, adjusted to pH 3.0 with *orthophosphoric acid* and 13 volumes of *acetonitrile*, filter through 0.45 µm nylon membrane,
- flow rate: 1 ml per minute,
- spectrophometer set at 220 nm,
- injection volume: 20 µl.

Equilibrate the column at least for one hour. Run the chromatogram minimum of 60 minutes.

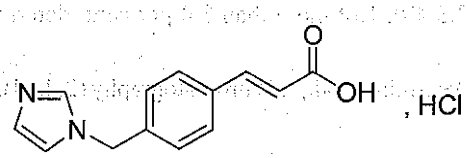
Inject the reference solution. The test is not valid unless the resolution between vasopressin and the adjacent peak is not less than 1.5 and relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Storage. Store protected from light and unless otherwise indicated on the label, at a temperature between 2°to 8°.

Labelling. The label states (1) the number of Units of oxytocic activity per ml; (2) either the animal species from which it is obtained or whether it is synthetic, as appropriate; (3) that the preparation is intended for intranasal administration only.

Ozagrel Hydrochloride



$C_{13}H_{13}ClN_2O_2$ Mol. Wt. 264.7

Ozagrel Hydrochloride is (2*E*)-3-[4-(1*H*-imidazol-1-ylmethyl)phenyl]-2-Propenoic acid hydrochloride.

Ozagrel Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{13}H_{13}ClN_2O_2$, calculated on the anhydrous basis.

Category. Antithrombotic.

Description. A white or almost white, crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ozagrel hydrochloride IPRS* or with the reference spectrum of ozagrel hydrochloride.

Tests

pH (2.4.24). 2.0 to 5.0, determined in a 0.1 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of *water* and 20 volumes of *acetonitrile*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),

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- mobile phase: a mixture of 10 volumes of *acetonitrile* and 90 volumes of buffer solution prepared by dissolving 0.77 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 4.0 with *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 10 µl.

Inject the test solution. The area of any secondary peak is not more than 0.5 per cent and the sum of the areas of all secondary peaks is not more than 1.0 per cent, calculated by area normalization.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 8.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of *water* and 20 volumes of *acetonitrile*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture. Dilute 2.0 ml of the solution to 25.0 ml with the solvent mixture.

Reference solution. A 0.004 per cent w/v solution of *ozagrel hydrochloride IPRS* in the solvent mixture.

Use chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{13}H_{13}ClN_2O_2$.

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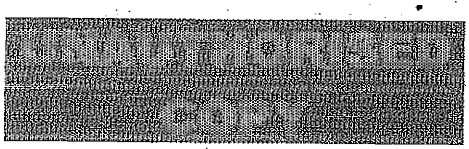

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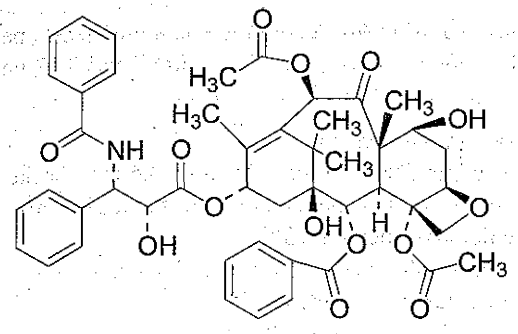
Paclitaxel

Taxol



$C_{47}H_{51}NO_{14}$ Mol. Wt. 853.9

Paclitaxel is 5β,20-epoxy-1,2α,4,7β,10β,13α-hexahydroxytax-11-en-9-one 4,10-diacetate 2-benzoate 13-ester with(2*R*,3*S*)-*N*-benzoyl-3-phenylisoserine.

A taxane derivative first isolated from the bark of the Pacific yew tree, *Taxus brevifolia*.

Paclitaxel contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{47}H_{51}NO_{14}$, calculated on the anhydrous basis.

Category. Anticancer.

Description. A white or almost white powder.

CAUTION — Paclitaxel is potentially cytotoxic. Great care should be taken in handling the powder and preparing solutions.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *paciltaxel IPRS* or with the reference spectrum of paclitaxel.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). – 55.0° to – 49.0° at 20°, determined in 1.0 per cent w/v solution in *methanol*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 10 mg of substance under examination in 10 ml of *acetonitrile*.

Reference solution (a). A 0.001 per cent w/v solution of *paclitaxel IPRS* in *acetonitrile*.

Reference solution (b). A solution containing 0.008 per cent w/v of 10-deacetyl-7-epipaclitaxel and 0.1 per cent w/v of *paclitaxel IPRS* in *acetonitrile*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 35°,
- mobile phase: A. a mixture of 60 volumes of *water* and 40 volumes of *acetonitrile*,
  B. *acetonitrile*,
- flow rate: 1.2 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 227nm,
- injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
20	100	0
60	10	90
62	100	0
70	100	0

The relative retention time for 10-deacetyl-7-epipaclitaxel is about 0.94 in respect to paclitaxel.

Inject reference solution (b). The test is not valid unless the resolution between the peak due to paclitaxel and 10-deacetyl-7-epipaclitaxel is not less than 1.2.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (a) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (a) (2.0 per cent).

Heavy metals (2.3.13). 1 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). Not more than 4.0 per cent, determined on 0.1 g by coloumetry method.

Bacterial endotoxins (2.2.3). Not more than 0.4 Endotoxin Unit per mg of paclitaxel.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 10^2 CFU per g. 1 g is free from *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. 10 g is free from *Salmonella* and *Shigella*.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 200 µl of *glacial acetic acid* in 1000 ml of *methanol*.

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Test solution. Dissolve 0.1 g of the substance under examination in 100.0 ml in solvent mixture.

Reference solution. A 0.1 per cent w/v solution of *paclitaxel IPRS* in solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with pentafluoro phenyl groups bonded to porous silica (5 µm),
- column temperature 35°,
- mobile phase: a mixture of 55 volumes of *water* and 45 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 227nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5. The relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{47}H_{51}NO_{14}$.

Storage. Store protected from light, at a temperature not exceeding 25°.

Paclitaxel Injection

Paclitaxel Injection is a sterile solution of Paclitaxel suitable for dilution, for intravenous use.

Paclitaxel Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of paclitaxel, $C_{47}H_{51}NO_{14}$.

Usual strengths. 30 mg; 100 mg; 260 mg; 300 mg.

Description. A clear colourless to slight yellow viscous solution.

Identification

In the Assay, the principal peak in the chromatogram obtained with test solution corresponds to the peak in the chromatogram obtained with reference solution.

Tests

pH (2.4.24). 3.0 to 7.0, determined in a 10 per cent v/v solution in *water.*

Light absorption (2.4.7). Absorbance of the injection at about 425 nm, not more than 0.1.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Accurately measure the volume of injection containing 12 mg of Paclitaxel, dilute to 10 ml with *acetonitrile.*

Reference solution (a). A 0.0012 per cent w/v solution of *paclitaxel IPRS* in *acetonitrile.*

Reference solution (b). A solution containing 0.006 per cent w/v of *10-deacetyl -7-epipaclitaxel IPRS* and 0.12 per cent w/v of *paclitaxel IPRS* in *acetonitrile.*

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 35°,
- mobile phase: A. a mixture of 60 volumes of *water* and 40 volumes of *acetonitrile*,
  B. *acetonitrile*,
- flow rate: 1.2 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 227 nm,
- injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
26	100	0
66	17	83
67	100	0

Inject reference solution (b). The test is not valid unless the resolution between the peak due to paclitaxel and 10-deacetyl-7-epipaclitaxel is not less than 1.2. The relative retention time for 10-deacetyl-7-epipaclitaxel is about 0.94 in respect to paclitaxel.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.8 times the area of the principal peak in the chromatogram obtained with the reference solution (a) (0.8 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (a) (2.0 per cent).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Bacterial endotoxins (2.2.3). Not more than 0.67 Endotoxin Unit per mg of paclitaxel.

Sterility (2.2.11). Complies with the test for sterility.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 200 µl of *glacial acetic acid* in 1000 ml of *methanol.*

Test solution. Accurately measure the volume of injection containing 6 mg of Paclitaxel and dissolve in 10 ml of solvent

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mixture.

Reference solution. A 0.06 per cent w/v solution of *paclitaxel IPRS* in solvent mixture.

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm, packed with penta fluro phenyl group chemically bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 55 volumes of *water* and 45 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 227nm,
- injection volume: 10 µl.

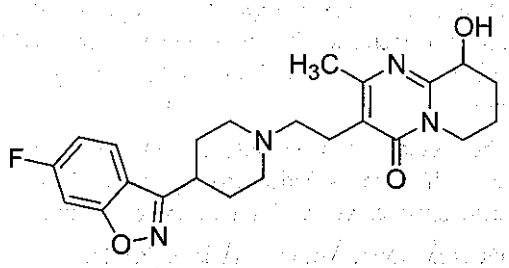
Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{47}H_{51}NO_{14}$.

Storage. Store protected from light, at a temperature not exceeding 25°.

Paliperidone



$C_{23}H_{27}FN_4O_3$ Mol. Wt. 426.5

Paliperidone is *(9RS)*-3-[2-[4-(6-Fluoro-1,2-benzisoxazol-3-yl)-piperidin-1-yl]ethyl]-9-hydroxy-2-methyl -6,7,8,9-tetrahydro-4*H*-pyrido[1,2-a]pyrimidin-4-one.

Paliperidone contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{23}H_{27}FN_4O_3$, calculated on the anhydrous basis.

Category. Antipsychotic.

Description. A white to yellow powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *paliperidone IPRS* or with the reference spectrum of paliperidone.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution (a).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Protect the solutions from light. And use freshly prepared solutions.

Solvent mixture. Dissolve 0.71 g of *disodium hydrogen phosphate* and 0.62 g *sodium dihydrogen phosphate* in 1000 ml of *water*.

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of the *methanol* and dilute to 100.0 ml with solvent mixture.

Reference solution (a). A 0.05 per cent w/v solution of *paliperidone IPRS* in equal volume of *methanol* and solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with solvent mixture. Further dilute 5.0 ml of the solution to 50.0 ml with solvent mixture.

Reference solution (c). A solution containing 0.05 per cent w/v of *paliperidone resolution mixture IPRS* (paliperidone is major, paliperidone related compound B, paliperidone related compound C, paliperidone hydroxybenzoyl analog and paliperidone ketone as minor components) in 50.0 ml of the *methanol* and dilute with solvent mixture.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 100 volumes of *methanol* and 900 volumes of a buffer solution prepared by dissolving 9.5 g of *tetrabutylammonium hydrogen sulphate* in 1000 ml of *water*,
  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 0.9 ml per minute,
- spectrophotometer set at 275 nm,
- injection volume. 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	100	0
35	85	15
37	100	0
45	100	0

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Name	Relative retention time	Correction factor
Paliperidone related compound C[1]	0.57	1.0
Paliperidone related compound B[2]	0.83	1.0
Paliperidone	1.0	—
Paliperidone hydroxybenzoyl analog[3]	1.1	1.0
Paliperidone ketone[4]	1.27	1.7

[1]3-(2-chloroethyl) -9-hydroxy-2-methyl -6,7,8,9-tetrahydro-4*H*-pyrido[1,2-a]pyrimidin-4-one,

[2]6-fluoro-3-(piperidin-4-yl)benzisoxazole,

[3]3-{2-[4-(4-fluoro-2-hydroxybenzoyl)-piperidin-1-yl]ethyl}-9-hydroxy-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2a]pyrimidin-4-one,

[4]3-{2-[4-(6-fluorobenzo[d] isoxazol-3-yl)-piperidin-1-yl]ethyl}-2-methyl-,7,8-dihydro-4*H*-pyrido [1,2-a]pyrimidine-4,9(6*H*)-dione.

Inject reference solution (c). The test is not valid unless the resolution between paliperidone impurities B and paliperidone is not less than 2.0 and resolution between paliperidone and paliperidone hydroxybenzyl analog peaks is not less than 2.0.

Inject reference solution (b). The test is not valid unless the signal-to-noise ratio of the principal peak is not less than 10 and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject reference solution (a) and the test solution. In the chromatogram obtained with test solution, the area of any peak due to paliperidone impurities B, C and paliperidone-hydroxy benzyl analog is not more than 0.1 per cent, the area of any peak due to paliperidone ketone is not more than 0.5 per cent, the area of any secondary peak is not more than 0.1 per cent and the sum of areas of all the secondary peaks is not more than 0.7 per cent, calculated by area nomalization procedure. Ignore any peak with an area less than 0.05 per cent.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.4.43). Not more than 0.5 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Use chromatography system, reference solution (a) and test solution as described under Related substances.

Inject reference solution (a). The test is not valid unless the tailing factor of the principal peak is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1 per cent.

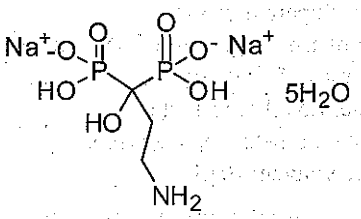
Inject the test solution and reference solution (a).

Calculate the content of $C_{23}H_{27}FN_4O_3$.

Storage. Store in tight containers protected from moisture at room temperature.

Pamidronate Disodium

Pamidronate Disodium Pentahydrate



$C_3H_9NNa_2O_7P_2, 5H_2O$ Mol. Wt. 369.1

Pamidronate Disodium is phosphonic acid, (3-amino-1 -hydroxypropylidene) bisdisodium salt, pentahydrate.

Pamidronate Disodium contains not less than 98.0 per cent and not more than 102.0 per cent of $C_3H_9NNa_2O_7P_2$, calculated on the anhydrous basis.

Category. Antiosteoporotic.

Description. A white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pamidronate disodium IPRS* or with the reference spectrum of pamidronate disodium.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

C. It gives reaction (A) of sodium salts (2.3.1).

Tests

Appearance of solution (2.4.1). Dissolve 1 g of the substance under examination in 50 ml of *water* with gentle warming (solution A) and 1 g in 25 ml of *2 M sodium hydroxide* with gentle warming (solution B) then cool to room temperature. The solutions are clear (2.4.1). Measure the absorbance of the solutions at 420 nm (2.4.7), using *water* as the blank for solution (A) and *2 M sodium hydroxide* for solution (B), the absorbance of each solution is not more than 0.10.

pH (2.4.24). 7.8 to 8.8, determined in 1.0 per cent w/v solution.

Related substances. A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 90 volumes of *methanol*, 80 volumes of *diisopropyl ether*, and 40 volumes of 25 per cent v/v *ammonia*.

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Spray reagent. Dissolve 0.2 g of *ninhydrin* in 100 ml of a mixture of 95 volumes of *butyl alcohol* and 5 volumes of *2 M acetic acid.*

Test solution. Dissolve 30 mg of the substance under examination in *water* and dilute to 10.0 ml with *water.*

Reference solution . A 0.0006 per cent w/v solution of *beta alanine IPRS* in *water.*

Apply to the plate 10 µl of each solution. After development, dry the plate at 100° until the *ammonia* disappears completely. Spray with spray reagent, and heat again at 100° for about 15 minutes and examine the plate under white light. In the chromatogram obtained with the test solution, the spot having RF value of about 0.5 is not more intense than the spot obtained with the reference solution (0.2 per cent).

B. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Reference solution (a). A 0.03 per cent w/v solution of *orthophosphoric acid* in *water.*

Reference solution (b). A 0.025 per cent w/v solution of *phosphorous acid* in *water.*

Reference solution (c). Dilute 2 ml each of reference solution (a) and reference solution (b) to 50.0 ml with *water.*

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to phosphate and phosphite is not less than 2.5 (phosphate peak elutes first), and the relative standard deviation for replicate injections for phosphate peak is not more than 10 per cent and for phosphite peak is not more 20 per cent.

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to phosphate (as phosphoric acid) is not more than 3.33 times the area of the phosphate peak in the chromatogram obtained with reference solution (c) (0.5 per cent). The area of any peak corresponding to phosphite (as phosphorous acid) is not more than 4 time the area of phosphite peak in the chromatogram obtained with reference solution (c) (0.5 per cent), and total phosphate and phosphite combined is not more than 0.5 per cent. The area of any other peak is not more than the area of the phosphate peak in the chromatogram obtained with reference solution (c) (0.5 per cent) and the sum of the areas of all the secondary peaks, other than beta alanine, phosphate as orthophosphoric acid and phosphite as phosphorous acid, is not more than the area of the phosphate peak in the chromatogram obtained with reference solution (c) (0.5 per cent).

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). 23.0 per cent to 25.5 per cent, determined on 0.1 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in *water* and dilute to 50.0 ml with *water.*

Reference solution. A 0.2 per cent w/v solution of *pamidronate disodium IPRS* in *water.*

Chromatographic system

- a stainless steel column 10 cm × 4.6 mm, packed with anion exchange resin made of porous polymethacrylate or polyacrylate gel with quaternary ammonium group (10 µm),
- column temperature: 35°,
- mobile phase: A 0.47 ml of anhydrous *formic acid* in 2500 ml of *water,* adjusted to pH 3.5 with *2 M sodium hydroxide*,
- flow rate: 1 ml per minute,
- refractive index detector,
- injection volume: 100 µl.

Inject the reference solution. The test is not valid unless the tailing factor is between 0.3 to 1.2 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_3H_9NNa_2O_7P_2$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Pamidronate Disodium Injection

Pamidronate Disodium Pentahydrate Injection

Pamidronate Disodium Injection is a freeze dried sterile mixture of Pamidronate Disodium and suitable excipients.

Pamidronate Disodium Injection contains not less than 93.0 per cent and not more than 108.0 per cent of the stated amount of pamidronate disodium, $C_3H_9NNa_2O_7P_2$.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Usual strengths. 30 mg per vial; 60 mg per vial; 90 mg per vial.

Description. A white or almost white powder.

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The content of the sealed container comply with the requirements stated under Parenteral Preparation (Powder for Injection) and with the following requirement.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 6.0 to 7.0, determined in a solution constituted as described in labelling.

Beta alanine. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G.*

Mobile phase. A mixture of 90 volumes of *methanol,* 80 volumes of *diisopropyl ether* and 40 volumes of 25 per cent v/v *ammonia.*

Spray reagent. Dissolve 0.2 g of *ninhydrin* in 100 ml of a mixture of 95 volumes of *butyl alcohol* and 5 volumes of *2 M acetic acid.*

Test solution. Dilute a suitable volume of reconstituted solution with *water*, to obtain a solution containing 0.3 per cent w/v of anhydrous pamidronate disodium.

Reference solution. A 0.00075 per cent w/v solution of *beta alanine IPRS* in *water.*

Apply to the plate 10 µl of each solution. After development, dry the plate at 100° until the odour of *ammonia* disappears completely. Spray with spray reagent and heat again at 100° for about 15 minutes and examine under day light. In the chromatogram obtained with test solution, the spot having RF value of about 0.5 is not more intense than the spot obtained with the reference solution (0.25 per cent).

Water (2.3.43). Not more than 5.0 per cent.

Sterility (2.2.11). Complies with the test for sterility.

Bacterial endotoxins (2.2.3). Not more than 3.88 Endotoxin Unit per mg of anhydrous Pamidronate Disodium.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Re-constitute 5 vials with *water,* and mix. Dilute a suitable volume with *water* to obtain a solution containing 0.2 per cent w/v of anhydrous Pamidronate Disodium.

Reference solution. A 0.25 per cent w/v solution of *pamidronate disodium IPRS* in *water.*

Chromatographic system

- a stainless steel column 10 cm × 4.6 mm, packed with anion exchange resin made up of porous polymethacrylate or polyacrylate gel with quaternary ammonium group (10 µm),
- column temperature: 35°,
- mobile phase: A 0.47 ml of anhydrous *formic acid* in 2500 ml of *water,* adjusted to pH 3.5 with *2 M sodium hydroxide,*
- flow rate: 1 ml per minute,
- refractive index detector,
- injection volume: 100 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_3H_9NNa_2O_7P_2$ in the injection.

Storage. Store at a temperature not exceeding 30°.

Labelling. The label states the quantity of pamidronate disodium pentahydrate in the terms of the equivalent amount of pamidronate disodium.

Pancreatin

Pancreatin is a preparation of mammalian pancreas containing protease, lipase and amylase activity. It may contain Sodium Chloride.

Pancreatin contains not less than the minimum protease activity, amylase activity and lipase activity determined under the conditions of the Assay.

Category. Digestive enzyme.

Description. A white or buff-coloured, amorphous powder.

Identification

A. Triturate 0.5 g with 10 ml of *water* and adjusted to pH 8.0 by the addition of *1 M sodium hydroxide* using *cresol red solution* as indicator. Divide the resulting solution into two equal portions. Boil one portion [solution (1)] and leave the other untreated [solution (2)]. To each add a few shreds of *congo red fibrin*, warm to 39° ± 1° and maintain at this temperature for 1 hour. Solution (2) is stained red and solution (1) is colourless or not more than slightly pink.

B. Triturate 0.25 g with 10 ml of *water* and adjusted to pH 8.0 by the addition of *1 M sodium hydroxide* using *cresol red solution* as indicator. Divide the resulting solution into two equal portions. Boil one portion [solution (1)] and leave the other untreated [solution (2)]. Dissolve 0.1 g of *soluble starch* in 100 ml of boiling *water*, boil for 2 minutes, cool and dilute to 150 ml with *water*. Add solution (1) to half the starch mucilage and solution (2) to the remainder and maintain the mixtures at 39° ± 1° for 5 minutes. To 1 ml of each mixture add 10 ml of *iodinated potassium iodide solution*. The liquid containing solution (2) retains the colour of the solution of iodine and the liquid containing solution (1) acquires an intense blue colour.

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Tests

Fat. Not more than 5.0 per cent, determined by the following method. Extract 1 g with *light petroleum (40° to 60°)* for 3 hours in an apparatus for the continuous extraction of drugs (2.1.8), evaporate the extract and dry the residue at 105° for 2 hours.

Microbial contamination (2.2.9). 1 g is free from *Escherichia coli*; 10 g is free from *Salmonellae* and *Shigella*.

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 0.5 g by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 4 hours.

Assay. *For protease activity* — Weigh 4.0 g of purified casein and dissolve in 90 ml of *water* containing 3 ml of *1 M sodium hydroxide*, adjusted to pH 8.7 and add sufficient *water* to produce 100.0 ml. Weigh 0.5 g of the substance under examination, triturate with *water* and add sufficient *water* to produce 300.0 ml to give the test solution. Dilute 15.0 ml of the *casein solution* with 30 ml of *water*, warm to 55° and add 10.0 ml of the unfiltered test solution. Heat rapidly to 55° and keep at this temperature for 20 minutes. Cool rapidly to room temperature. Dilute a further portion of 15.0 ml of the casein solution with 30 ml of *water*, add 10.0 ml of the unfiltered test solution, previously boiled and cooled, heat rapidly to 55° and keep at this temperature for 20 minutes. Cool to room temperature. To each solution add 0.75 ml of *phenolphthalein solution* and 10 ml of *formaldehyde solution*. Titrate each solution with *0.1 M sodium hydroxide* until the colour of the solution matches that produced by mixing 10 ml of *buffer solution pH 8.7* and 0.15 ml of *phenolphthalein solution*. The difference between the two titrations is not less than 4.5 ml.

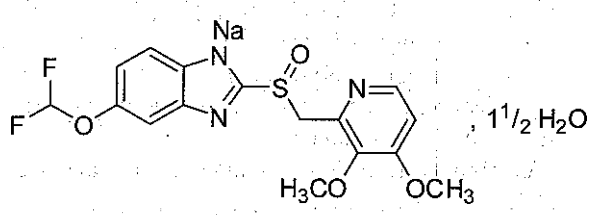
For lipase activity — To 95 ml of *water*, add 6.5 ml of *triacetin* and 0.2 ml of a 0.1 per cent w/v solution of *bromocresol purple*, neutralise with *0.5 M sodium hydroxide* and add sufficient *water* to produce 110 ml. Place 50 ml of the solution in each of two large tubes 3 cm x 20 cm A and B contained in a thermostat at 30°. Insert in each tube a rubber stopper having two holes, one for the tip of a burette and the other for a short glass tube through which passes a thread operating a glass stirring coil. Stir the contents of the tube until they attain the temperature of the thermostat. Prepare a solution of 0.1 g of the substance under examination in 10.0 ml of *water*. To tube A add 1.0 ml of the solution, to tube B add 1.0 ml of the solution previously boiled. Adjust and maintain the pH of the solutions in the two tubes to 6.2 to 6.4 by the addition of *0.05 M sodium hydroxide* dropwise, stirring frequently. After 30 minutes, the difference between the volumes of *0.05 M sodium hydroxide* added to the two tubes is not less than 1.0 ml.

For amylase activity — Not less than 100 Units per g. Dissolve 0.1 g or a quantity containing 10 Units, weighed, in sufficient *buffer solution pH 6.8* to produce 1000.0 ml. Filter if necessary (1 ml of the test solution should be capable of digesting about 10 mg of dry soluble maize or corn starch). Into each of six stoppered test-tubes add 5.0 ml of *starch substrate* without touching the sides of the test-tube. Place the test-tubes in a *water*-bath maintained at 40° ± 0.1°. When the temperature of the solution in the tubes has reached 40°, add 0.35 ml, 0.4 ml, 0.45 ml, 0.5 ml, 0.55 ml and 0.6 ml of the test solution to each of the test-tubes marked 1 to 6 respectively and record the time of addition. Mix thoroughly and replace the tubes in the *water*-bath. After exactly 60 minutes remove the tubes and cool rapidly in cold water. Add to each tube 0.05 ml of *0.02 M iodine* and mix well. Note the tube containing the lowest volume of test solution, which does not show a bluish or violet tinge (if there is doubt, warm the solution slightly, when the colour distinction is prominent). From this volume calculate the number of grams of dry soluble maize or corn starch digested by 1.0 g of the substance under examination. This represents the number of Units of amylase activity per g.

Storage. Store protected from moisture.

Labelling. The label states the name of any added substance.

Pantoprazole Sodium



$C_{16}H_{14}F_2N_3NaO_4S, 1½\,H_2O$ Mol. Wt. 432.4

Pantoprazole Sodium is sodium 5-(difluromethoxy)-2[[(3,4 dimethoxy-pyridin-2-yl)methyl]sulphinyl]-benzimidazol-1-ide, sesquihydrate.

Pantoprazole Sodium contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{16}H_{14}F_2N_3NaO_4S$, calculated on the anhydrous basis.

Category. Antiulcer.

Description. A white to off-white powder.

Identification

Test A may be omitted if tests B and C are carried out. Test B may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pantoprazole sodium IPRS* or with the reference spectrum of pantoprazole sodium.

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B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

C. It gives the reaction (A) of sodium salts (2.3.1).

Tests

Optical rotation (2.4.22). -0.40° to +0.40°, determined on 1.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *acetonitrile* and *0.001 M sodium hydroxide*.

Test solution. Dissolve about 23 mg of the substance under examination in 50.0 ml of the solvent mixture.

Reference solution. A 0.06 per cent w/v solution of *pantoprazole sodium IPRS* in the solvent mixture. Dilute 5.0 ml of the solution to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 12.5 cm x 4.0 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS-3),
- column temperature: 40°,
- mobile phase: A. dissolve 1.74 g of *dibasic potassium phosphate* to 1000 ml with *water*, adjusted to pH 7.0 with *orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 290 nm and for impurity C, at 305 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
40	20	80
45	20	80
55	80	20

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution the area of the peak due to pantoprazole impurity C determined at wavelength 305 nm at relative retention time about 0.6 is not more than 0.077 times the area of the principal peak at 290 nm in the chromatogram obtained with the reference solution (0.5 per cent), the area of any other secondary peak is not more than 0.077 times the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of areas of all the secondary peaks is not more than 0.15 times the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not less than 5.0 per cent and not more than 8.0 per cent, determined on 0.15 g.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture (a). Dilute 25.0 ml of *ammonium hydroxide* to 500.0 ml with *water*.

Solvent mixture (b). Equal volumes of *acetonitrile* and *water*.

Test solution. Dissolve about 20 mg of the substance under examination 10.0 ml of solvent mixture (b) and dilute to 50.0 ml with solvent mixture (a). Dilute 3.0 ml of the solution to 20.0 ml with solvent mixture (a).

Reference solution. Dissolve 20 mg of *pantoprazole sodium IPRS* in 10.0 ml of solvent mixture (b) and dilute to 50.0 ml with solvent mixture (a). Dilute 3.0 ml of the solution to 20.0 ml with solvent mixture (a).

Chromatographic system

- a stainless steel column 15 cm x 3.9 mm packed with octadecylsilane bonded to porous silica (4 µm) (Such as Inertsil ODS-3),
- column temperature: 30°,
- sample temperature: 4°,
- mobile phase: A. a mixture of 85 volumes of buffer solution prepared by dissolving 1.32 g of *dibasic ammonium phosphate* to 1000 ml with *water*, adjusted to pH 7.5 with *orthophosphoric acid* and 15 volumes of *acetonitrile*,
  B. a mixture of 70 volumes of *acetonitrile* and 30 volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 285 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	86	14
10	86	14
35	42	58
36	86	14
46	86	14

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

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Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{14}F_2N_3NaO_4S$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Pantoprazole Gastro-resistant Tablets

Pantoprazole Tablets; Pantoprazole Sodium Gastro-resistant Tablets; Pantoprazole Sodium Tablets

Pantoprazole Gastro-resistant Tablets contain an amount of Pantoprazole sodium equivalent to not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pantoprazole, $C_{16}H_{15}F_2N_3O_4S$. They are made gastro-resistant by enteric-coating or by other means.

Usual strenth. 20 mg; 40 mg.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

B. When examined in the range 230 nm to 350 nm (2.4.7), a 0.001 per cent w/v solution in *methanol* shows absorption maxima at about 289 nm.

Tests

Dissolution (2.5.2).

NOTE — Prepare the solutions immediately before use. Protect the solutions from light.

A. Apparatus No. 2 (Paddle),
Medium. 1000 ml of *0.1 M hydrochloric acid*,
Speed and time. 100 rpm and 2 hours.

Determine by liquid chromatography (2.4.14).

Test solution. Withdraw the medium completely and disperse the intact tablet in 100 ml of the mobile phase and filter.

Reference solution. Dissolve a weighed quantity of *pantoprazole sodium IPRS* in the mobile phase and dilute with the mobile phase to obtain a solution having a known concentration similar to the test solution.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{15}F_2N_3O_4S$ released in the acid medium by subtracting the content of $C_{16}H_{15}F_2N_3O_4S$ in the test solution from the total content of Pantoprazole $C_{16}H_{15}F_2N_3O_4S$ determined in the Assay.

Complies with the acceptance criteria given under acid stage.

B. Apparatus No. 2 (Paddle),
Medium. 1000 ml of *phosphate buffer pH 6.8*,
Speed and time. 75 rpm and 45 minutes.

Transfer another 6 tablets and run the apparatus for 2 hours in *0.1 M hydrochloric acid*. Decant the medium without losing the tablets, add phosphate buffer pH 6.8 and run the apparatus for 45 minutes. Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *phosphate buffer 6.8* and *0.5 M sodium hydroxide*.

Test solution. Dilute 5.0 ml of the filtrate to 10.0 ml with *0.5M sodium hydroxide*.

Reference solution. Dissolve 20 mg of *pantoprazole sodium IPRS* in 30 ml of *0.02M sodium hydroxide*, with the aid of ultrasound, add 2 ml of *acetonitrile* and dilute to 50.0 ml with *0.02M sodium hydroxide*. Dilute a portion of the solution with the solvent mixture to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{15}F_2N_3O_4S$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{16}H_{15}F_2N_3O_4S$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

NOTE—Prepare the solutions immediately before use. Protect the solutions from light.

Test solution. Weigh and powder 20 tablets. Weigh a quantity of powder containing about 20 mg of Pantoprazole Sodium, disperse in 100 ml of the mobile phase and filter.

Reference solution. A 0.02 per cent w/v solution of *pantoprazole sodium IPRS* in the mobile phase.

Chromatographic system
- stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* and 1 g of *hexane sulphonic acid sodium salt* in 1000 ml of *water*, adjusted to pH 7.3 with *1 M sodium hydroxide* and 50 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 290 nm,
- injection volume: 10 µl.

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Inject the reference solution. The test is not valid unless the theoretical plates are not less than 2000, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{15}F_2N_3O_4S$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of equivalent amount of Pantoprazole.

Pantoprazole Gastro-resistant and Domperidone Prolonged-release Capsules

Pantoprazole Sodium Gastro-resistant and Domperidone Prolonged-release Capsules

Pantoprazole Sodium Gastro-resistant and Domperidone maleate Prolonged release Capsules manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Pantoprazole Gastro-resistant and Domperidone Prolonged-release Capsules contains an amount of pantoprazole sodium equivalent to pantoprazole and domperidone not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pantoprazole, $C_{16}H_{15}F_2N_3O_4S$ and of domperidone, $C_{22}H_{24}ClN_5O_2$.

Usual strength. Pantoprazole 40 mg and Domperidone 30 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

For Pantoprazole Sodium —

NOTE — Prepare the solutions immediately before use. Protect the solutions from light.

A. Apparatus No. 2 (Paddle),
Medium. 1000 ml of *0.1 M hydrochloric acid,*
Speed and time. 100 rpm and 2 hours.

Determine by liquid chromatography (2.4.14).

Test solution. Withdraw the medium completely without any loss of residue. Transfer the residue into 50-ml volumetric flask, dissolve in 20 ml of *0.1M sodium hydroxide*, with the aid of ultrasound and make up the volume with *0.1M sodium hydroxide*. Centrifuge at about 3000 rpm for 10 minutes. Dilute 2.0 ml of the solution to 25.0 ml with the mobile phase.

Reference solution. A 0.08 per cent w/v solution of *pantoprazole sodium IPRS* in *0.1M sodium hydroxide*. Dilute 2.0 ml of the solution to 25.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- sample temperature: 15°,
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 2.72 g *potassium dihydrogen orthophosphate* and 0.525 g of *dipotassium hydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 7.4 with *dilute potassium hydroxide solution* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 289 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{15}F_2N_3O_4S$ released in the acid medium by subtracting the content of $C_{16}H_{15}F_2N_3O_4S$ in the test solution from the total content of Pantoprazole $C_{16}H_{15}F_2N_3O_4S$ determined in the Assay.

Complies with the acceptance criteria given under acid stage.

B. Apparatus No. 2 (Paddle),
Medium. 1000 ml of a buffer solution prepared by dissolving 19.0 g of *trisodium orthophosphate* in 1000 ml of *water*, adjusted to pH 6.8 with *hydrochloric acid*,
Speed and time. 100 rpm and 45 minutes.

Determine by liquid chromatography (2.4.14).

Test solution. Transfer another 6 capsules and run the apparatus for 2 hours in *0.1 M hydrochloric acid*. Decant the medium without losing the residue, add buffer solution pH 6.8 and run the apparatus for 45 minutes. Withdraw a suitable volume of the medium and filter.

Reference solution. A 0.1 per cent w/v solution of *pantoprazole sodium IPRS* in *0.1M sodium hydroxide*. Dilute 1.0 ml of the solution to 50.0 ml with the dissolution medium.

Use chromatographic system as described under Acid stage.

Inject the reference solution and the test solution.

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Calculate the content of $C_{16}H_{15}F_2N_3O_4S$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{16}H_{15}F_2N_3O_4S$.

For Domperidone — Complies with the test stated under Capsules.

Related substances. *For Pantoprazole* — Determine by liquid chromatography (2.4.14).

NOTE — Reference solution and test solution is stable up to 48 hours at 2° to 8°.

Solvent mixture. Equal volumes of *0.001M sodium hydroxide* and *acetonitrile*.

Test solution. Weigh a quantity of the mixed content of 20 capsules containing about 100 mg of pantoprazole into 250-ml volumetric flask. Add 150.0 ml of solvent mixture and sonicate and make up the volume with same solvent.

Reference solution. A 0.00004 per cent w/v solution of *pantoprazole sodium IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 12.5 cm x 4 mm packed with octadecylsilane bonded to porous silica (5 µm), (Such as Purosphere® STAR RP- 18e),
- column temperature: 40°,
- sample temperature: 8°,
- mobile phase: A. a mixture of 95 volumes of a buffer solution prepared by dissolving 1.74 g *anhydrous dipotassium hydrogen phosphate* in 1000 ml of *water*, adjusted to pH 7.0 with *orthophosphoric acid* and 5 volumes of *acetonitrile*,

  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 290 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
40	25	75
45	85	15
50	85	15

Name	Relative retention time	Correction factor
Related Compound A[1]	0.89	1.12
Pantoprazole	1.00	---
Related Compound D and F[2, 3]	1.17	0.85
Related Compound B[4]	1.56	0.64

[1]5-(Difluoromethoxy)-2-[[(3,4-dimethoxy- 2-pyridyl) methyl] sulfonyl]-1H- benzimidazole,

[2]5-(Difluoromethoxy)-2-[(RS)-[(3, 4-dimethoxypyridin-2-yl)methyl] sulfinyl]-1-methyl-1H- benzimidazole,

[3]6-(Difluoromethoxy)-2-[(RS)-[(3, 4-dimethoxypyridin-2-yl)methyl] sulfinyl]-1-methyl-1H- benzimidazole,

[4]5-(Difluoromethoxy)-2-[[(3,4-dimethoxy-2-pyridyl) methyl] thio]-1H benzimidazole.

NOTE — Related compound D and F peak observed in doublet peak.

Inject the reference solution. The test not valid unless the column efficiency is not less than 4000 theoretical plates, the tailing factor is not more than 2.0, and the relative standard deviation for replicate injection is not more than 5.0 per cent.

Inject the reference solution and the test solution, In the chromatogram obtained with the test solution, the area of any other secondary peak is not more than 5 times the area of the principle peak in the chromatogram obtained with the reference solution (0.5 per cent), the area of impurity A is not more than 5 times the area of principal peak in the chromatogram obtained with the reference solution (0.5 per cent), the area of impurity B should not more than 3 times the area of principal peak in the chromatogram obtained with the reference solution (0.3 per cent), the area of impurity D and F should not more than 7.5 times the area of principal peak in the chromatogram obtained with the reference solution (0.75 per cent), and the sum of the areas of all the secondary peaks is not more than 15 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent).

For Domperidone — Determine by liquid chromatography (2.4.14).

Solvent mixture. 0.1 M Methanolic sodium hydroxide prepared by dissolving 4.0 g of *sodium hydroxide* in 1000-ml volumetric flask, add 25.0 ml *water* and sonicate to dissolve the pellets then make up the volume with *methanol* and mix.

Test solution. Weigh a quantity of the mixed contents of 20 capsules containing about 150 mg of Domperidone into volumetric flask, add 75.0 ml of solvent mixture and sonicate for 15 minutes with intermittent shaking and make up to 100.0 ml with solvent mixture and mix.

NOTE—Use freshly prepared solution, maintain the sonicator temperature about 20 ± 5°.

Reference solution. 0.00045 per cent w/v solution of *domperidone IPRS* in solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as Inertsil, ODS-3V),
- column temperature: 25°,
- mobile phase: A. a mixture of 75 volumes of a buffer solution prepared by dissolving 6.8g *potassium dihydrogen orthophosphate* in 1000 ml *water*, adjusted

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to pH 7.2 with *0.2M sodium hydroxide* and 25 volumes of *acetonitrile*,

B. a mixture 45 volumes of a buffer solution prepared by dissolving 6.8 g *potassium dihydrogen orthophosphate* in 1000 ml *water*, adjusted to pH 7.2 with *0.2 M sodium hydroxide* and 55 volumes of *acetonitrile*,

- a gradient programme using the conditions given below,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 285 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
20	100	0
30	25	75
40	25	75
50	100	0
55	100	0

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2500 theoretical plates, the tailing factor is not more than 2.0, and the relative standard deviation for replicate injection is not more than 2.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 1.7 times the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than 6.66 times the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent). Ignore any peak with an area less than 0.17 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

NOTE -- Prepare the solutions immediately before use.

Solvent mixture. A mixture of 55 volumes of *water* and 45 volumes of *acetonitrile*.

Test solution. Weigh accurately a quantity of the mixed content of 20 capsules containing about 200 mg of Pantoprazole dissolved in 200.0 ml of *methanol* and sonicate for 30 minutes with intermittent shaking and make up the volume to 250.0 ml with *methanol*, mix and filter. Further dilute 5.0 ml of the solution to 100.0 ml with solvent mixture and mix.

Reference solution. Weigh and transfer accurately *pantoprazole sodium sesquihydrate IPRS* equivalent to 40 mg of *pantoprazole IPRS* and 30.0 mg of *domperidone IPRS* into a volumetric flask, add 70 ml of *methanol* and sonicate to dissolve and make up the volume to 100.0 ml with *methanol*. Further dilute 5.0 ml of the solution to 50.0 ml with solvent mixture and mix.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with base deactivated, end capped octadecylsilane silica gel bonded to porous silica (5 µm), (Such as Hypersil BDS),
- column temperature: 35°,
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 2.75 g of *potassium dihydrogen orthophosphate* and 0.53 g of *dipotassium hydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 7.4 with *potassium hydroxide solution* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 284 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0, and the relative standard deviation for replicate injection is not more than 2.0 per cent.

Inject the reference solution and the test solution.

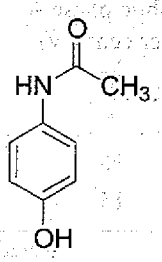
Calculate the content of $C_{16}H_{15}F_2N_3O_4S$ and $C_{22}H_{24}ClN_5O_2$ in the capsules.

Storage. Store protected from moisture, at a temperature not exceeding 25°.

Labelling. The label states the strength in terms of equivalent amount of pantoprazole and of domperidone.

Paracetamol

Acetaminophen



$C_8H_9NO_2$ Mol. Wt. 151.2

Paracetamol is 4-hydroxyacetanilide.

Paracetamol contains not less than 99.0 per cent and not more than 101.0 per cent of $C_8H_9NO_2$, calculated on the dried basis.

Category. Analgesic; antipyretic.

Description. White crystals or a white, crystalline powder.

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Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *paracetamol IPRS* or with the reference spectrum of paracetamol.

B. Dissolve 50 mg in sufficient *methanol* to produce 100 ml. To 1 ml of the solution add 0.5 ml of *0.1 M hydrochloric acid* and dilute to 100 ml with *methanol*. Protect the resulting solution from bright light and immediately measure the absorbance at the maximum at about 249 nm; absorbance at 249 nm, about 0.44 (2.4.7).

C. Boil 0.1 g in 1 ml of *hydrochloric acid* for 3 minutes, add 10 ml of *water* and cool; no precipitate is produced. Add 0.05 ml of *0.0167 M potassium dichromate*; a violet colour develops which does not turn red.

D. It gives the reaction of acetyl groups (2.3.1).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Note—Prepare the solutions immediately before use.

Test solution. Dissolve 0.2 g of the substance under examination in 2.5 ml of *methanol* containing 0.46 per cent w/v of *tetrabutylammonium hydroxide solution* (40 per cent w/v) and dilute to 10.0 ml with the solution containing equal volumes of 1.79 per cent w/v of *disodium hydrogen phosphate* and 0.78 per cent w/v of *sodium dihydrogen phosphate*.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (c). A solution containing 0.025 per cent w/v each of *4-aminophenol IPRS*, *paracetamol IPRS* and *chloroacetanilide IPRS* in *methanol*. Dilute 1.0 ml of the solution to 250.0 ml with the mobile phase.

Reference solution (d). Dissolve 20 mg of *4-nitrophenol IPRS* in 50.0 ml of *methanol*. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5µm) (Such as Zorbax C8),
- column temperature: 35°,
- mobile phase: a mixture of 37.5 volumes of a 1.79 per cent w/v solution of *disodium hydrogen phosphate*, 37.5 volumes of a 0.78 per cent w/v solution of *sodium dihydrogen phosphate* and 25 volumes of *methanol* containing 0.46 per cent v/v of *tetrabutylammonium hydroxide solution (40 per cent w/v)*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 20 µl.

The relative retention time with reference to paracetamol for 4-aminophenol is about 0.8, for 4-nitrophenol is about 3.0 and for chloroacetanilide is about 7.0.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to 4-aminophenol and paracetamol is not less than 4.0 and the signal-to-noise ratio of the peak due to chloroacetanilide is not less than 50.

Inject reference solution (a), (b), (c), (d) and the test solution. Run the chromatogram 12 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of the peak due to chloroacetanilide is not more than 0.2 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (10 ppm) and the area of the peak due to 4-aminophenol is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (50 ppm), the area of peak due to 4-nitrophenol is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent), the area of any other secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent) and the sum of areas of other secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.01 per cent).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

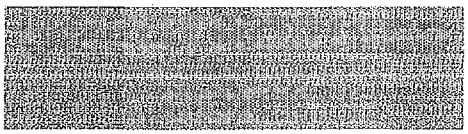
Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.5 g in a mixture of 10 ml of *water* and 50 ml of *1 M sulphuric acid*. Boil under a reflux condenser for 1 hour, cool and dilute to 100.0 ml with *water*. To 20.0 ml of the solution add 40 ml of *water*, 40 g of *water* in the form of ice, 15 ml of *2 M hydrochloric acid* and 0.1 ml of *ferroin solution* and titrate with *0.1 M ceric ammonium sulphate* until a yellow colour is produced. Carry out a blank titration.

1 ml of *0.1 M ceric ammonium sulphate* is equivalent to 0.00756 g of $C_8H_9No_2$.

Storage. Store protected from light and moisture.



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Paracetamol Infusion

Acetaminophen Infusion

Paracetamol Infusion is a sterile isotonic buffered solution for intravenous infusion consisting of paracetamol with the aid of a suitable auxiliary substances and alkali or acid.

Paracetamol Infusion contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of paracetamol $C_8H_9NO_2$.

Usual strength. 10 mg per ml.

Description. A clear, colourless solution.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution.

Tests

pH (2.4.24). 4.5 to 6.5.

Light absorption. The absorbance of the infusion at 500 nm (2.4.7) is not more than 0.04.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Use the infusion.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.002 per cent w/v each of *4-aminophenol IPRS* and *paracetamol IPRS* in the mobile phase.

Reference solution (c). A 0.00002 per cent w/v solution of *4- chloroacetanilide IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 25 volumes of *methanol* containing 0.115 g of *tetrabutylammonium hydroxide solution* (40 per cent w/v), with 37.5 volumes of *0.05 M disodium hydrogen orthophosphate* and 37.5 volumes of *0.05 M sodium dihydrogen orthophosphate*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 4.0.

Inject reference solution (a), (c) and the test solution. Run the chromatogram 12 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any peak corresponding to 4-aminophenol is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the area of any peak corresponding to 4- chloroacetanilide is not more than 0.001 times the area of the principal peak in the chromatogram obtained with reference solution (a) (10 ppm) and the area of any other secondary peak is not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent).

Bacterial endotoxins (2.2.3). Not more than 2.0 Endotoxin Units per ml.

Other tests. Comply with the tests stated under Parenteral Preparation (Infusions).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of injection with the mobile phase to obtain a solution containing 0.01 per cent w/v of Paracetamol.

Reference solution. A 0.01 per cent w/v solution of *paracetamol IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 20 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: *0.01 M sodium butanesulphonate* in a mixture of 85 volumes of *water*, 15 volumes of *methanol* and 0.4 volume of *formic acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 1500 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_9NO_2$ in the infusion.

Storage. Store protected from light.

Paracetamol Oral Suspension

Acetaminophen Oral Suspension

Paracetamol Oral Suspension is a suspension of Paracetamol in a suitable flavoured vehicle.

Paracetamol Oral Suspension contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of paracetamol, $C_8H_9NO_2$.

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Usual strength. 500 mg per 5 ml.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 50 volumes of *acetone*, 50 volumes of *toluene* and 1 volume of *glacial acetic acid.*

Test solution. Shake a quantity of the oral suspension containing about 125 mg of Paracetamol in 25 ml of *methanol* and dilute to 50 ml with *methanol*.

Reference solution. A 0.25 per cent w/v solution of *paracetamol IPRS* in *methanol.*

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use and protect from light.

Test solution. Shake a quantity of the suspension containing 0.5 g of Paracetamol in the mobile phase and dilute with the mobile phase to obtained a concentration of 0.5 per cent w/v of Paracetamol and filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.0005 per cent w/v, each of, *4-aminophenol IPRS* and *paracetamol IPRS* in the mobile phase.

Reference solution (c). A 0.000005 per cent w/v solution of *4-chloroacetanilide IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 250 volumes of *methanol* containing 1.15 g of a 40 per cent v/v solution of *tetrabutylammonium hydroxide* , 375 volumes of *0.05M disodium hydrogen orthophosphate* and 375 volumes of *0.05 M sodium dihydrogen orthophosphate,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 100 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 4.0.

Inject reference solution (a), (b), (c) and the test solution. Run the chromatogram 15 times the retention time of the principal peak. The area of any peak corresponding to 4-aminophenol is not more than 5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any peak corresponding to 4-chloroacetanilide is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (10 ppm) and the area of any other secondary peak is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent).

Other tests. Comply with the tests stated under Oral Suspension.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 0.4 volume of *formic acid*, 15 volumes of *methanol* and 85 volumes of *water.*

Test solution. Shake a quantity of the oral suspension containing about 25 mg of Paracetamol in 60 ml of the solvent mixture and dilute to 100.0 ml with the solvent mixture. Dilute 10.0 ml of the solution to 50.0 ml with the solvent mixture

Reference solution. A 0.005 per cent w/v solution of *paracetamol IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: *0.01M sodium butanesuphonate* in the solvent mixture,
- flow rate: 1 ml per minute,
- spectrophotometer set at 243 nm,
- injection volume: 20 µl.

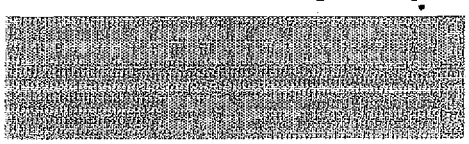
Inject the reference solution. The test is not valid unless the theoretical plates is not less than 2000, tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_9NO_2$.

Determine the weight per ml of the oral solution (2.4.29) and calculate the content of $C_8H_9NO_2$, weight in volume.

Storage. Store protected from light.



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Paracetamol Paediatric Oral Suspension

Acetaminophen Paediatric Oral Suspension; Paediatric Paracetamol Oral Suspension

Paracetamol paediatric oral suspension is a suspension of Paracetamol in a suitably flavoured vehicle. It should not be diluted.

Paracetamol Paediatric Oral Suspension contains not less than 95 per cent and not more than 105 per cent of the stated amount of paracetamol, $C_8H_9NO_2$.

Usual strengths. 125 mg per 5ml; 250 mg per 5 ml. In case of Paediatric drops 100 mg per ml and 150 mg per ml.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 0.5 volume of *glacial acetic acid*, 10 volumes of *toluene*, 25 volumes of *acetone* and 65 volumes of *dichloromethane*.

Test solution. Dilute the suspension under examination with *methanol* to produce a solution containing 0.24 per cent w/v of Paracetamol and filter.

Reference solution. A 0.24 per cent w/v solution of *paracetamol IPRS* in *methanol*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to the principal spot in the chromatogram obtained with the reference solution.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use and protect from light.

Test solution. Shake a quantity of the suspension containing 0.5 g of Paracetamol in the mobile phase and dilute with the mobile phase to obtained a concentration of 0.5 per cent w/v of Paracetamol and filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.0005 per cent w/v, each of, *4-aminophenol IPRS* and *paracetamol IPRS* in the mobile phase.

Reference solution (c). A 0.000005 per cent w/v solution of *4-chloroacetanilide IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 250 volumes of *methanol* containing 1.15 g of a 40 per cent v/v solution of *tetrabutylammonium hydroxide*, 375 volumes of *0.05M disodium hydrogen orthophosphate* and 375 volumes of *0.05 M sodium dihydrogen orthophosphate*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 100 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 4.0.

Inject reference solution (a), (b), (c) and the test solution. Run the chromatogram 15 times the retention time of the principal peak. The area of any peak corresponding to 4-aminophenol is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent), the area of any peak corresponding to 4-chloroacetanilide is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (10 ppm), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent), and the sum of the areas of other secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Mix a weighed quantity of the suspension under examination containing 24 mg of Paracetamol with 100 ml of the mobile phase, dilute to 200.0 ml with the mobile phase and filter.

Reference solution. A 0.012 per cent w/v solution of *paracetamol IPRS* in the mobile phase.

Use chromatographic system as described under Related substances.

Inject the reference solution and the test solution.

Determine the weight per ml of the suspension (2.4.29) and Calculate the content of $C_8H_9NO_2$ in the suspension.

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Paracetamol Paediatric Syrup

Paracetamol Paediatric Oral Solution; Acetaminophen Paediatric Syrup

Paracetamol Paediatric Syrup is a solution of Paracetamol in a suitable flavoured vehicle.

Paracetamol Paediatric Syrup contains not less than 95.0 per cent and not more than 105.0 per cent w/v solution of the stated amount of paracetamol, $C_8H_9NO_2$.

Usual strength. 125 mg per 5 ml.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 65 volumes of *chloroform*, 25 volumes of *acetone*, 10 volumes of *toluene* and 0.5 volumes of *glacial acetic acid*.

Test solution. Dilute a volume containing 25 mg of Paracetamol to 10 ml with *methanol* and filter if necessary.

Reference solution. A 0.25 per cent w/v solution of *paracetamol IPRS* in *methanol*

Apply to the plate 10 µl of each solution. After development, dry the plate in a current of warm air, examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution.

Tests

4-Aminophenol. Determine by liquid chromatography (2.4.14).

Test solution. Shake 5 ml of the preparation under examination with 15 ml of the mobile phase, dilute to 25 ml with the mobile phase and filter if necessary.

Reference solution. A 0.0025 per cent w/v solution of *4-aminophenol IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: *0.01 M sodium butanesulphonate* in a mixture of 85 volumes of *water*, 15 volumes of *methanol* and 0.4 volume of *formic acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 272 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution the area of any peak corresponding to 4-aminophenol is not more than the area of the peak in the chromatogram obtained with the reference solution (0.5 per cent). Peaks with a long retention time may occur due to preservatives in the preparations.

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Mix a weighed quantity of the preparation under examination containing 50 mg of Paracetamol in 70 ml of the mobile phase, dilute to 100.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 20.0 ml with the mobile phase.

Reference Solution. A 0.0125 per cent w/v solution of *paracetamol IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 20 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: *0.01 M sodium butanesulphonate* in a mixture of 85 volumes of *water*, 15 volumes of *methanol* and 0.4 volume of *formic acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 243 nm,
- injection volume: 20 µl.

Determine the weight per ml (2.4.29) of the syrup and calculate the content of $C_8H_9NO_2$, weight in volume.

Storage. Store protected from light and moisture.

Paracetamol Tablets

Acetaminophen Tablets

Paracetamol Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of paracetamol, $C_8H_9NO_2$.

Usual strengths. 500 mg; 650 mg.

Identification

Extract a quantity of the powdered tablets containing 0.5 g of Paracetamol with 20 ml of *acetone*, filter, evaporate the filtrate to dryness and dry at 105°. The residue complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *paracetamol IPRS* or with the reference spectrum of paracetamol.

B. Boil 0.1 g in 1 ml of *hydrochloric acid* for 3 minutes, add 10 ml of *water* and cool; no precipitate is produced. Add 0.05 ml of *0.0167 M potassium dichromate*; a violet colour develops which does not turn red.

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Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *phosphate buffer pH 5.8*
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter and dilute a suitable volume of the filtrate with the same solvent. Measure the absorbance of the resulting solution at the maximum at about 243 nm (2.4.7). Similarly measure the absorbance of a solution of known concentration of *paracetamol IPRS*. Calculate the content of $C_8H_9NO_2$.

Q. Not less than 80 per cent of the stated amount of $C_8H_9NO_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing about 0.2 g of Paracetamol in 10.0 ml of the mobile phase, filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.002 per cent w/v each of *4-aminophenol IPRS* and *paracetamol IPRS* in the mobile phase.

Reference solution (c).) A 0.00002 per cent w/v solution of *4- chloroacetanilide IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as Zorbax Rx C8),
- column temperature: 35 °,
- mobile phase: a mixture of 250 volumes of *methanol* containing 1.15 g of *tetrabutylammonium hydroxide solution* (40 per cent w/v), 375 volumes of *0.05M disodium hydrogen orthophosphate* and 375 volumes of *0.05M sodium dihydrogen orthophosphate*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 4.0.

Inject reference solution (a), (b), (c) and the test solution. Run the chromatogram 12 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of peak corresponding to 4-aminophenol is not more than the area of the corresponding peak in reference solution (b) (0.1 per cent), the area of peak corresponding to 4-chloroacetanilide is not more than the area of the principal peak in reference solution (c) (10 ppm) and the area of any other secondary peak is not more than the area of the principal peak obtained with reference solution (a) (0.25 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.15 g of Paracetamol, add 50 ml of *0.1 M sodium hydroxide*, dilute with 100 ml of *water*, shake for 15 minutes and add sufficient *water* to produce 200.0 ml. Mix, filter and dilute 10.0 ml of the solution to 100.0 ml with *water*. To 10.0 ml of the solution add 10 ml of *0.1 M sodium hydroxide*, dilute to 100.0 ml with *water* and mix. Measure the absorbance of the resulting solution at the maximum at about 257 nm (2.4.7). Calculate the content of $C_8H_9NO_2$ taking 715 as the specific absorbance at 257 nm.

Storage. Store protected from light and moisture.

Paracetamol and Caffeine Tablets

Paracetamol and CaffeineTablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of paracetamol, $C_8H_9NO_2$, and caffeine, $C_8H_{10}N_4O_2$.

Usual strengths. Paracetamol 650 mg and Caffeine 50 mg; Paracetamol 500 mg and Caffeine 25 mg .

Identification

A. Dissolve a quantity of the powdered tablets containing 500 mg of Paracetamol with 20 ml of *acetone*, filter and evaporate to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum obtained with *paracetamol IPRS* or with the reference spectrum of paracetamol.

B. Dissolve a quantity of the powder tablets containing 0.1 g of caffeine with 10 ml of *water*, shake for 5 minutes, filter and add 10 ml of *1M sodium hydroxide*. Extract with three 30 ml quantity of *dichloromethane*, washing each extract with the same 10 ml of *water*. Filter the combined extracts through absorbent cotton and evaporate the filtrate to dryness. Dissolve 10 mg of the residue in 1 ml of *hydrochloric acid*, add 0.1 g of *potassium chlorate*, evaporate to dryness in a porcelain dish and expose to *ammonia vapour*. A pink colour is observed.

C. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *phosphate buffer pH 5.8*,

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Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14), using the chromatographic system described under Assay of Paracetamol and Caffeine.

Test solution. Use the filtrate, dilute 10.0 ml of the solution to 100.0 ml with mobile phase.

Reference solution. A 0.0072 per cent w/v solution of *paracetamol IPRS* and 0.00055 per cent w/v solution of *caffeine IPRS* in the mobile phase .

Inject the reference solution and the test solution.

Calculate the content of $C_8H_9NO_2$ and $C_8H_{10}N_4O_2$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_8H_9NO_2$ and $C_8H_{10}N_4O_2$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use and protect from light.

Test solution. Weigh and powder 20 tablets. Dissolve a quantity of the powder containing 0.5 g of paracetamol with 50.0 ml of mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A 0.001 per cent w/v solution each of *4-aminophenol IPRS* and *paracetamol IPRS* in the mobile phase.

Reference solution (c). A 0.01 per cent w/v solution of *4-chloroacetanilide IPRS* in *methanol* and further dilute to obtain a solution of 0.00001 per cent w/v solution in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 250 volumes of *methanol* containing 1.15 g of a 40 per cent w/v solution of *tetrabutylammonium hydroxide,* 375 volumes of *0.05M disodium hydrogen orthophosphate* and 375 volumes of *0.05M sodium dihydrogen orthophosphate,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 50 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 4.0.

Inject reference solution (a), (b), (c) and the test solution. Run the chromatogram 12 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of peak corresponding to 4-aminophenol is not more than the area of the corresponding peak in reference solution (b) (0.1 per cent), the area of peak corresponding to 4- chloroacetanilide is not more than the area of the principal peak in reference solution (c) (0.001 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the total area of any other secondary peak is not more than 5 times the area of the peak due to paracetamol in the chromatogram obtained with reference solution (a) (0.5 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay for caffeine using following modifications.

Test solution. Disperse one tablet in 60 ml of the mobile phase and dilute to 100.0 ml with the same solvent and filter. Dilute a suitable volume of the solution with the mobile phase, to obtain a solution having concentration similar to the reference solution.

Reference solution. A 0.0025 per cent w/v solution of *caffeine IPRS* in the mobile phase.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{10}N_4O_2$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

For Paracetamol —

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 250 mg of Paracetamol with 75 ml of mobile phase, ultrasound for 10 minutes, dilute to 100.0 ml with same solvent and mix. Filter, rejecting the first 5.0 ml of the filtrate. Dilute 1.0 ml of the solution to 50-ml volumetric flask and dilute with same solvent.

Reference solution. A 0.005 per cent w/v solution of *paracetamol IPRS* in the mobile phase .

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a buffer solution prepared by dissolving 1.74 g of *sodium pentanesulfonate* in 220 volumes of *methanol* and 780 volumes of *water* and adjusted to pH 2.8 using *2 M hydrochloric acid,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 243 nm,
- injection volume: 20 µl.

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Inject the reference solution and the test solution.

Calculate the content of $C_8H_9NO_2$ in the tablets.

For Caffeine—

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 30 mg of Caffeine with 100.0 ml of mobile phase. Filter, dilute 5.0 ml of the solution to 50.0 ml with the same solvent.

Reference solution. A 0.003 per cent w/v solution of *caffeine IPRS* in the mobile phase .

Use the chromatographic system as described under Assay of paracetamol using a detection wavelength of 220 nm.

Calculate the content of $C_8H_{10}N_4O_2$ in the tablets.

Storage. Store protected from light and moisture.

Labelling. The label states the strength of paracetamol and caffeine.

Hard Paraffin

Hard Paraffin is a purified mixture of solid hydrocarbons obtained from petroleum or from shale oil.

Category. Pharmaceutical aid (stiffening agent).

Description. A white or colourless, translucent mass, frequently showing a crystalline structure; slightly greasy to the touch. Burns with a luminous flame. When melted, the liquid is free from fluorescence by daylight.

Tests

Acidity or alkalinity. To 10.0 g add 20 ml of boiling *water*, heat in a water-bath for 5 minutes, shake vigorously for 1 minute, cool, allow to separate and filter the aqueous layer. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution*. The solution is colourless and not more than 0.1 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution to pink.

Congealing range (2.4.10). 50° to 65°.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Liquid Paraffin

White Mineral Oil; Liquid Petrolatum

Liquid Paraffin is a purified mixture of liquid hydrocarbons obtained from petroleum to which not more than 10 ppm of tocopherol or of butylated hydroxytoluene may be added.

Category. Laxative; faecal softener.

Description. A transparent, colourless, oily liquid, free from fluorescence by daylight.

Tests

Weight per ml (2.4.29). 0.860 g to 0.904 g.

Viscosity (2.4.28). 110 mPas to 230 mPas.

Acidity or alkalinity. To 10.0 g add 20 ml of boiling *water*, heat in a water-bath for 5 minutes, shake vigorously for 1 minute, cool, allow to separate and filter the aqueous layer. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution*. The solution is colourless and not more than 0.1 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution to pink.

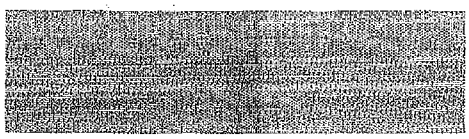
Light absorption. When examined in the range 240 nm to 280 nm (2.4.7), a 2.0 per cent w/v solution in *2,2,4-trimethylpentane* shows an absorption of not more than 0.1.

Readily carbonisable substances. Place 5 ml in a dry, heat-resistant glass-stoppered test-tube (125 mm x 18 mm) previously rinsed with chromic acid solution, then with *water* and dried. Add 5 ml of *nitrogen-free sulphuric acid* (containing 94.5 per cent to 95.5 per cent w/w of H_2SO_4), insert the stopper and shake as vigorously as possible in the longitudinal direction of the tube for 5 seconds. Loosen the stopper, immediately place the tube in a bath of boiling *water*, supporting it so as to prevent contact of the tube with the bottom or side of the bath and heat for 10 minutes. At the end of the second, fourth, sixth, and eighth minutes, remove the tube from the bath and shake as vigorously as possible in the longitudinal direction of the tube for 5 seconds. At the end of 10 minutes from the time the tube was placed in the bath remove the tube and allow to stand for 10 minutes. The lower acid layer is not more intensely coloured than a mixture of 3 ml of FCS, 1.5 ml of CCS and 0.5 ml of CSS (2.4.1), overlaid with 5 ml of liquid paraffin. If the sulphuric acid remains dispersed in the molten paraffin, the colour of the emulsion is not darker than that of the standard mixture when shaken vigorously.

Solid paraffins. Place a suitable quantity, previously dried by heating at 100° for 2 hours and cooled in a desiccator over sulphuric acid, in a glass cylindrical vessel having an internal diameter of approximately 25 mm. Close the vessel and immerse in a mixture of ice and *water*; after 4 hours the liquid is sufficiently clear that a black line, 0.5 mm in width, held vertically behind the vessel is easily seen.

Sulphur compounds. Mix 4 ml with 2 ml of *ethanol (95 per cent)*, and 2 drops of a clear, saturated solution of *lead monoxide* in *sodium hydroxide solution* and heat at 70° for 10 minutes with frequent shaking; the mixture remains colourless.

Storage. Store protected from light.



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Light Liquid Paraffin

Light Mineral Oil; Light Liquid Petrolatum

Light Liquid Paraffin is a purified mixture of liquid saturated hydrocarbons obtained from petroleum. It may contain a suitable stabiliser.

Category. Pharmaceutical aid (vehicle).

Description. A transparent, colourless, oily liquid, free from fluorescence by daylight.

Tests

Weight per ml (2.4.29). 0.820 g to 0.880 g.

Viscosity (2.4.28). 25 mPas to 80 mPas.

Acidity or alkalinity. To 10.0 g add 20 ml of boiling *water*, heat in a water-bath for 5 minutes, shake vigorously for 1 minute, cool, allow to separate and filter the aqueous layer. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution*. The solution is colourless and not more than 0.1 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution to pink.

Light absorption. When examined in the range 240 nm to 280 nm (2.4.7), a 2.0 per cent w/v solution in *2,2,4-trimethylpentane* shows an absorption of not more than 0.1.

Readily carbonisable substances. Place 5 ml in a dry, heat-resistant glass-stoppered test-tube (125 mm x 18 mm) previously rinsed with chromic acid solution, then with *water* and dried. Add 5 ml of *nitrogen-free sulphuric acid* (containing 94.5 per cent to 95.5 per cent w/w of H_2SO_4), insert the stopper and shake as vigorously as possible in the longitudinal direction of the tube for 5 seconds. Loosen the stopper, immediately place the tube in a bath of boiling *water*, supporting it so as to prevent contact of the tube with the bottom or side of the bath and heat for 10 minutes. At the end of the second, fourth, sixth, and eighth minutes, remove the tube from the bath and shake as vigorously as possible in the longitudinal direction of the tube for 5 seconds. At the end of 10 minutes from the time the tube was placed in the bath remove the tube and allow to stand for 10 minutes. The lower acid layer is not more intensely coloured than a mixture of 3 ml of FCS, 1.5 ml of CCS and 0.5 ml of CSS (2.4.1), overlaid with 5 ml of liquid paraffin. If the sulphuric acid remains dispersed in the molten paraffin, the colour of the emulsion is not darker than that of the standard mixture when shaken vigorously.

Solid paraffins. Place a suitable quantity, previously dried by heating at 100° for 2 hours and cooled in a desiccator over sulphuric acid, in a glass cylindrical vessel having an internal diameter of approximately 25 mm. Close the vessel and immerse in a mixture of ice and water; after 4 hours the liquid is sufficiently clear that a black line, 0.5 mm in width, held vertically behind the vessel is easily seen.

Sulphur compounds. Mix 4 ml with 2 ml of *ethanol (95 per cent)* and 2 drops of a clear, saturated solution of *lead monoxide* in *sodium hydroxide solution* and heat at 70° for 10 minutes with frequent shaking; the mixture remains colourless.

Storage. Store protected from light.

Liquid Paraffin Emulsion

Liquid Paraffin Oral Emulsion

Liquid Paraffin Emulsion is an oral emulsion of Liquid Paraffin in Purified Water.

Liquid Paraffin Emulsion contains not less than 44.0 per cent and not more than 49.0 per cent w/w of liquid paraffin.

Category. Laxative; faecal softener.

Tests

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Weigh 5.0 g, add 10 ml of *water*, extract with two quantities, each of 40 ml, of a mixture of 2 volumes of *ethanol (95 per cent)*, 3 volumes of *light petroleum (40° to 60°)* and 3 volumes of *ether* and then with 30 ml of a mixture of equal volumes of *light petroleum (40° to 60°)* and *ether*. Wash the combined extracts with 15 ml of *0.5 M sodium hydroxide* and then with 15 ml of *water*, evaporate the solvent, add 5 ml of *acetone* and evaporate again. Repeat the addition and evaporation of acetone until the residue is free from *water*, dry at 105° for 15 minutes and weigh.

Storage. Store protected from moisture.

White Soft Paraffin

White Petroleum Jelly

White Soft Paraffin is a purified, semi-solid mixture of hydrocarbons obtained from petroleum and bleached.

Category. Pharmaceutical aid (ointment base).

Description. A white, translucent, soft unctuous mass, retaining these characteristics on storage and when melted and allowed to cool without stirring; not more than slightly fluorescent by daylight, even melted.

Tests

Melting range (2.4.21). 38° to 56°, determined by Method IV.

Acidity or alkalinity. To 10.0 g add 20 ml of boiling *water*, heat in a water-bath for 5 minutes, shake vigorously for 1 minute,

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cool, allow to separate and filter the aqueous layer. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution*. The solution is colourless and not more than 0.1 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution to pink.

Light absorption (2.4.7). Absorbance of a 0.05 per cent w/v solution in *2,2,4-trimethylpentane* at about 290 nm, not more than 0.5.

Fixed oils, fats and resin. Digest 10 g with 50 ml of *sodium hydroxide solution* at 100° for 30 minutes and allow the aqueous layer to separate. On acidifying the aqueous layer with *dilute sulphuric acid*, no precipitate or oily matter is produced.

Foreign organic matter. Volatilises when heated, without emitting an acrid odour.

Consistency. 100 to 300, determined by the following method.

Apparatus. The apparatus is essentially in agreement with IS 4887:1980 and comprises a penetrometer fitted with a polished cone-shaped metal plunger weighing 150 g having a detachable steel tip of the following dimensions. The tip of the cone has an angle of 30°, the point being truncated to a diameter of 0.38 ± 0.08 mm, the base of the tip is 8.38 ± 0.13 mm in diameter and the length of the tip is 15 ± 0.25 mm. The remaining portion of the cone has an angle of 90°, is 28 to 29 mm in height, and has a maximum diameter of 65.1 mm at the base. The containers of the test are flat-bottomed metal or glass cylinders that are 102 ± 6 mm in diameter and not less than 60 mm in height.

Procedure. Melt a sufficient quantity at a temperature below 85° and pour into one or more of the containers filling to within 6 mm of the rim. Cool to 25°± 2.5° over a period of not less than 16 hours, protected from drafts. Two hours before the test, place the containers in a water-bath at 25° ± 0.5°. If the room temperature is below 23.5° or above 26.5°, adjust the temperature of the cone to 25° ± 0.5° by placing it in a water-bath.

Without disturbing the surface of the substance under examination, place the container on the penetrometer table, and lower the cone until the tip just touches the top surface of the test substance at a spot 25 mm to 38 mm from the edge of the container. Adjust the zero setting and quickly release the plunger, then hold it free for 5 seconds. Secure the plunger and read the total penetration from the scale. Make three or more trials, each so spaced that there is no overlapping of the areas of penetration. Where the penetration exceeds 20 mm, use a separate container of the test substance for each trial. Read the penetration to the nearest 0.1 mm. Calculate the average of the three or more readings and conduct further trials to a total of 10 if the individual results differ from the average by more than ± 3 per cent. The final average of the trials is not less than 10.0 mm and not more than 30.0 mm indicating a consistency value between 100 and 300.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Storage. Store protected from light and moisture.

Yellow Soft Paraffin

Yellow Petroleum Jelly

Yellow Soft Paraffin is a purified, semi-solid mixture of hydrocarbons obtained from petroleum.

Category. Pharmaceutical aid (ointment base).

Description. A pale yellow to yellow, translucent, soft unctuous mass, retaining these characteristics on storage and when melted and allowed to cool without stirring; not more than slightly fluorescent by daylight, even melted.

Tests

Melting range (2.4.21). 38° to 56°, determined by Method IV.

Light absorption (2.4.7). Absorbance of a 0.05 per cent w/v solution in *2,2,4-trimethylpentane* at about 290 nm, not more than 0.75.

Acidity or alkalinity. To 10.0 g add 20 ml of boiling *water*, heat in a water-bath for 5 minutes, shake vigorously for 1 minute, cool, allow to separate and filter the aqueous layer. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution*. The solution is colourless and not more than 0.1 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution to pink.

Fixed oils, fats and resin. Digest 10 g with 50 ml of *sodium hydroxide solution* at 100° for 30 minutes and allow the aqueous layer to separate. On acidifying the aqueous layer with *dilute sulphuric acid*, no precipitate or oily matter is produced.

Foreign organic matter. Volatilises when heated, without emitting an acrid odour.

Consistency. 100 to 300, determined by the following method.

Apparatus. The apparatus is essentially in agreement with IS 4887:1980 and comprises a penetrometer fitted with a polished cone-shaped metal plunger weighing 150 g having a detachable steel tip of the following dimensions. The tip of the cone has an angle of 30°, the point being truncated to a diameter of 0.38 ± 0.08 mm, the base of the tip is 8.38 ± 0.13 mm in diameter and the length of the tip is 15 ± 0.25 mm. The remaining portion of the cone has an angle of 90°, is 28 to 29 mm in height, and has a maximum diameter of 65.1 mm at the base. The containers of the test are flat-bottomed metal or glass cylinders that are 102 ± 6 mm in diameter and not less than 60 mm in height.

Procedure. Melt a sufficient quantity at a temperature below 85° and pour into one or more of the containers filling to within 6 mm of the rim. Cool to 25°± 2.5° over a period of not less than

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16 hours, protected from drafts. Two hours before the test, place the containers in a water-bath at 25° ± 0.5°. If the room temperature is below 23.5° or above 26.5°, adjust the temperature of the cone to 25° ± 0.5° by placing it in a water-bath.

Without disturbing the surface of the substance under examination, place the container on the penetrometer table, and lower the cone until the tip just touches the top surface of the test substance at a spot 25 mm to 38 mm from the edge of the container. Adjust the zero setting and quickly release the plunger, then hold it free for 5 seconds. Secure the plunger and read the total penetration from the scale. Make three or more trials, each so spaced that there is no overlapping of the areas of penetration. Where the penetration exceeds 20 mm, use a separate container of the test substance for each trial. Read the penetration to the nearest 0.1 mm. Calculate the average of the three or more readings and conduct further trials to a total of 10 if the individual results differ from the average by more than ± 3 per cent. The final average of the trials is not less than 10.0 mm and not more than 30.0 mm indicating a consistency value between 100 and 300.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Storage. Store protected from light and moisture.

Paraffin Ointment

Category. Pharmaceutical aid (ointment basis).

White Beeswax	20 g
Hard Paraffin	30 g
Cetostearyl Alcohol	50 g
White Soft Paraffin*	900 g

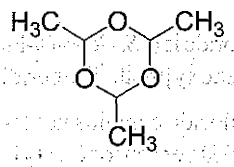
*May be replaced by Yellow Soft Paraffin if other medicaments to be incorporated are coloured.

Mix the ingredients, heat gently with stirring until homogeneous and stir until cold.

Tests

Paraffin Ointment complies with the tests stated under Ointments.

Paraldehyde



$C_6H_{12}O_3$ Mol. Wt. 132.7

Paraldehyde is 2,4,6-trimethyl-1,3,5-trioxane, the cyclic trimer of acetaldehyde. It may contain a suitable amount of antioxidant.

Category. Anticonvulsant in status epilepticus; hypnotic; sedative.

Description. A colourless or slightly yellow, transparent liquid. Solidifies at low temperature to form a crystalline mass.

Identification

A. Heat 5 ml with 0.1 ml of *1 M sulphuric acid*; acetaldehyde, recognisable by its odour, is evolved.

B. To 5 ml of a 10 per cent v/v solution add 5 ml of *ammoniacal silver nitrate solution* in a test-tube and heat on a water-bath; metallic silver is deposited as a mirror on the sides of the tube.

C. A 10 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), but becomes turbid on warming.

Tests

Congealing range (2.4.10). 10° to 13°.

Distillation range (2.4.8). Not more than 10 per cent distils below 123° and not less than 95 per cent distils below 126°.

Refractive index (2.4.27). 1.403 to 1.406.

Relative density (2.4.29). 0.991 to 0.996.

Acetaldehyde. Shake 5 ml with a mixture of 5 ml of *ethanol (60 per cent)*, 5 ml of *hydroxylamine hydrochloride reagent in ethanol (60 per cent)* and 2 drops of *methyl orange solution* and titrate with *0.5 M sodium hydroxide* to full yellow colour; not more than 0.8 ml of *0.5 M sodium hydroxide* is required.

Acidity. Mix 5 ml with 45 ml of *carbon dioxide-free water* and titrate with *0.1 M sodium hydroxide* using *phenolphthalein solution* as indicator; not more than 1.5 ml is required.

Chlorides. To 5 ml of a 1 per cent v/v solution add one drop of *nitric acid* and three drops of *silver nitrate solution*; no opalescence is produced immediately.

Sulphates. To 5 ml of a 1 per cent v/v solution add one drop of *hydrochloric acid* and three drops of *barium chloride solution*; no turbidity is produced.

Peroxides. In a stoppered vessel, dissolve 5 ml in sufficient of recently boiled and cooled *water* to produce 50 ml, add 5 ml of *dilute sulphuric acid* and 10 ml of *potassium iodide solution*. Close the flask and set aside in the dark for 15 minutes. Titrate with *0.1 M sodium thiosulphate* using *starch solution* as indicator; set aside for 5 minutes and, if necessary, complete the titration. Not more than 2.0 ml of *0.1 M sodium thiosulphate* is required.

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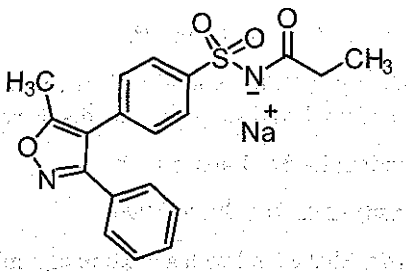
Non-volatile matter. Heat 5 ml in a small dish on a water-bath and dry at 105° for 1 hour; the residue weighs not more than 3 mg (0.06 per cent w/v).

Storage. Store protected from moisture, in complete darkness and at a temperature of 8° to 15°. If solidified, the whole of the contents of the container should be liquified by warming before use.

NOTE — Do not use Paraldehyde if it has a brownish colour or an odour of acetic acid. Avoid contact with rubber and plastics.

Labelling. The label states (1) the nature and the proportion of any antioxidant added; (2) that it may decompose on standing to form potentially harmful substances.

Parecoxib Sodium



$C_{19}H_{18}N_2O_4SNa$ Mol. Wt. 392.4

Parecoxib Sodium is *N*-[4-(5-Methyl-3-phenyl-1,2-oxazol-4-yl)phenylsulfonyl]propanamide sodium salt.

Parecoxib Sodium contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{19}H_{18}N_2O_4SNa$, calculated on the anhydrous basis.

Category. Non steroidal anti-inflammatory drug.

Description. A white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *parecoxib sodium IPRS* or with the reference spectrum of parecoxib sodium.

B. It gives the reaction (A) of sodium salts (2.3.1).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 45 volumes of *acetonitrile* and 55 volumes of *0.05M ammonium acetate*, adjusted to pH 5.0 with *acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 20 µl.

Inject the test solution. The test is not valid unless the column efficiency is not less than 2500 theoretical plates and the tailing factor is not more than 2.0.

Inject the test solution. The area of any secondary peak is not more than 0.5 per cent and the sum of the areas of all the secondary peaks is not more than 1.0 per cent, calculated by area normalisation.

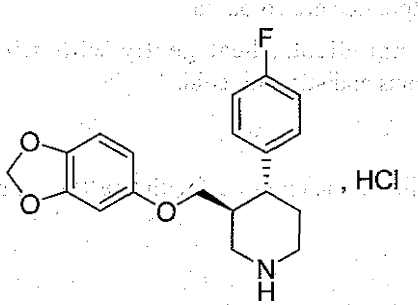
Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not more than 1.0 per cent, determined on 0.1 g, using Method 1.

Assay. Dissolve 0.3 g in 50 ml of *glacial acetic acid*. Titrate with *0.1 M perchloric acid* and determine the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.039241 of $C_{19}H_{18}N_2O_4SNa$.

Paroxetine Hydrochloride



$C_{19}H_{20}FNO_3,HCl$ Mol. Wt. 365.8

Paroxetine Hydrochloride is (3*S*,4*R*)-4-(4-Fluorophenyl)-3-[(3,4-methylenedioxy)phenoxy]methyl]piperidine hydrochloride.

Paroxetine Hydrochloride contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{19}H_{20}FNO_3,HCl$, calculated on the anhydrous basis.

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Category. Antidepressant.

Description. A white or almost white, hygroscopic, crystalline powder. It shows polymorphism (2.5.11).

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *paroxetine hydrochloride IPRS* or with the reference spectrum of paroxetine hydrochloride.

B. It gives reaction (A) of chlorides (2.3.1).

Tests

Impurity D. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 5 ml of *methanol* and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of *paroxetine impurity D IPRS ((3R,4S)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine ((+)-trans-paroxetine) IPRS)* in 2 ml of *methanol* and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 10 cm x 4.0 mm, packed with silica gel AGP for chiral chromatography (5 µm),
- mobile phase: a mixture of 93 volumes of buffer solution prepared by dissolving 8.7 g of *dipotassium hydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.5 with *orthophosphoric acid* and 7 volumes of *acetonitrile*,
- flow rate: 0.9 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram 2.5 times the retention time of principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak corresponding to paroxetine hydrochloride impurity D is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 10 volumes of *tetrahydrofuran* and 90 volumes of *water*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). Dilute 5.0 ml of the test solution to 50.0 ml with the solvent mixture.

Reference solution (b). Dissolve 5 mg of *paroxetine hydrochloride impurity H IPRS* in 25 ml of *tetrahydrofuran* and dilute to 50.0 ml with *water*.

Reference solution (c). Dissolve 5 mg of *paroxetine hydrochloride impurity C IPRS* in 25 ml of *tetrahydrofuran* and dilute to 50.0 ml with *water*.

Reference solution (d). To 5.0 ml of reference solution (a), add 1.0 ml of reference solution (b) and dilute to 100.0 ml with the solvent mixture.

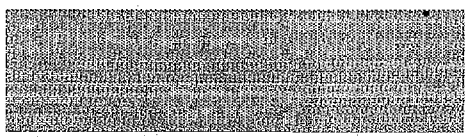
Reference solution (e). To 5.0 ml of reference solution (a), add 5.0 ml of reference solution (b) and 5.0 ml of reference solution (c), dilute to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution (f). Dissolve 2.5 mg of *paroxetine impurity E IPRS* in the solvent mixture, add 2.5 ml of the test solution and dilute to 100.0 ml with the solvent mixture.

Reference solution (g). Dissolve 5 mg of *paroxetine impurity A IPRS* in the solvent mixture and dilute to 50 ml with the solvent mixture. (Use this solution to identify the peak due to impurity A).

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with end capped octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 0.5 volume of *trifluoroacetic acid*, 10 volumes of *tetrahydrofuran* and 90 volumes of *water*,
  B. a mixture of 0.5 volume of *trifluoroacetic acid*, 10 volumes of *tetrahydrofuran* and 90 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
30	80	20
50	20	80
55	20	80
60	80	20
65	80	20



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Name	Relative retention time	Correction factor
Paroxetine impurity A[1]	0.8	—
Paroxetine impurity E[2]	0.9	—
Paroxetine impurity F[3] (with reference to impurity C)	0.97	1.7
Paroxetine (Retention time: about 28 minutes)	1.0	—
Paroxetine impurity J[4] (with reference to impurity C)	1.02	1.3
Paroxetine impurity C[5]	1.5	1.6

[1]desfluoroparoxetine,

[2]*cis*-paroxetine,

[3]*N*-benzyldesfluoroparoxetine,

[4](3*S*,4*R*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4'-fluorobiphenyl-3-yl)piperidine,

[5]*N*-benzylparoxetine.

Inject reference solutions (e) and (f). The test is not valid unless the resolution between the peaks corresponding to paroxetine impurity E and paroxetine in the chromatogram obtained with reference solution (f) is not less than 3.5. The signal-to-noise ratio for the principal peak in the chromatogram obtained with reference solution (e) is not less than 3.

Inject reference solution (d), (e) and the test solution. In the chromatogram obtained with the test solution, the area of the peak corresponding to paroxetine impurity A is not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.3 per cent), the area of any peaks corresponding to paroxetine impurities C, F, J, each of, is not more than 0.2 times the area of the peak due to paroxetine in the chromatogram obtained with reference solution (d) (0.1 per cent), the area of any other secondary peak is not more than 0.2 times the area of the peak due to paroxetine in the chromatogram obtained with reference solution (d) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent). Ignore any peak with the area less than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.05 per cent).

Impurities H and I. Determine by liquid chromatography (2.4.14) as described under Related substances with the following modifications.

- spectrophotometer set at 263 nm,

Name	Relative retention time
Paroxetine hydrochloride impurity I[1]	0.2
Paroxetine hydrochloride impurity H[2]	0.4
Paroxetine hydrochloride (Retention time: about 28 minutes)	1.0

[1][(3*S*,4*R*)-4-(4-fluorophenyl)piperidin-3-yl]methanol,

[2][(3*S*,4*R*)-1-benzyl-4-(4-fluorophenyl)piperidin-3-yl]methanol.

Inject reference solution (e). The test is not valid unless, the signal-to-noise ratio for the peak due to paroxetine impurity H is not less than 3.0.

Inject reference solution (d) and the test solution. In the chromatogram obtained with the test solution, the area of the peak corresponding to paroxetine impurities H and I is not more than the area of the peak corresponding to paroxetine impurity H in the chromatogram obtained with reference solution (d) (0.1 per cent).

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent, determined in platinum crucible.

Water (2.3.43). Not more than 1.5 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in *water* and dilute to 100.0 ml with *water*.

Reference solution (a). A 0.0512 per cent w/v solution of *paroxetine hydrochloride hemihydrate IPRS* in *water*.

Reference solution (b). A solution containing 0.05 per cent w/v of *paroxetine hydrochloride hemihydrate IPRS* and *paroxetine impurity A IPRS* in *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with trimethylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 3.85 g of *ammonium acetate* in 500 ml of *water*, adjusted to pH 5.5 with *anhydrous acetic acid* and dilute to 600 ml with *water*, add 400 ml of *acetonitrile* slowly add with stirring, 10 ml of *triethylamine* and adjusted to pH 5.5 with *anhydrous acetic acid* ,
- flow rate: 1 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks corresponding to paroxetine and paroxetine impurity A is not less than 2.0.

Inject reference solution (a) and the test solution. Run the chromatograms twice the retention time of the principal peak.

Calculate the content of $C_{19}H_{21}ClFNO_3$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

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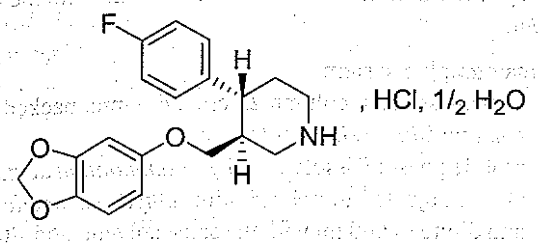
Paroxetine Hydrochloride Hemihydrate



$C_{19}H_{21}ClFNO_3,½H_2O$ Mol.Wt. 374.8

Paroxetine Hydrochloride Hemihydrate is (3*S*,4*R*)-3-[(1,3-Benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine hydrochloride hemihydrate.

Paroxetine Hydrochloride Hemihydrate contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{19}H_{21}ClFNO_3$, calculated on the anhydrous basis.

Category. Antidepressant.

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *paroxetine hydrochloride hemihydrate IPRS* or with the reference spectrum of paroxetine hydrochloride hemihydrate.

If the spectra obtained show differences, dissolve 1 part of the substance under examination and 1 part of the reference substance separately in 10 parts of a mixture of 1 volume of *water* and 9 volumes of *2-propanol* and heat to 70 ° to dissolve. Recrystallise and record new spectra using the residue.

B. It gives reaction (B) of chlorides (2.3.1).

C. In the test for impurity D, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with reference solution (c).

Tests

Impurity D. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in 20 ml of *methanol* and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *paroxetine impurity D IPRS ((3R,4S)-3-[(1,3-benzodioxol-5-yloxy) methyl]-4-(4-fluorophenyl)piperidine ((+)-trans-paroxetine))* and 5.0 mg of *paroxetine hydrochloride hemihydrate IPRS* in 2.0 ml of *methanol* and dilute to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 10 mg of *paroxetine hydrochloride hemihydrate IPRS* in 2 ml of *methanol* and dilute to 10.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 10 cm x 4.0 mm, packed with silica gel AGP for chiral chromatography (5 µm),
- mobile phase: a mixture of 20 volumes of *methanol,* 80 volumes of *sodium chloride* solution prepared by dissolving 5.8 g *sodium chloride* in 1000 ml of *water*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 10 µl.

The retention time of paroxetine is about 30 minutes. Run the chromatogram 2.5 times the retention time of paroxetine.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to paroxetine impurity D and paroxetine is not less than 2.2.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of paroxetine impurity D is not more than twice the area of the peak in the chromatogram obtained with reference solution (a) (0.2 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. A mixture of 10 volumes of *tetrahydrofuran and* 90 volumes of *water*.

Test solution. Dissolve 50.0 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the same solvent mixture.

Reference solution (a). Dilute 5.0 ml of the test solution to 50.0 ml with the solvent mixture. Dilute 2.0 ml of the solution to 200.0 ml with the solvent mixture.

Reference solution (b). Dissolve 2.0 mg of *paroxetine IPRS* containing *impurity C IPRS ((3S,4R)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-ethoxyphenyl)piperidine)* in the solvent mixture and dilute to 10.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10 ml with the solvent mixture.

Reference solution (c). Dissolve 2.0 mg of *paroxetine impurity A IPRS ((3S,4R)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-phenylpiperidine) or desfluoroparoxetine)* in the solvent mixture and dilute to 20 ml with the same solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.0 mm, packed with endcapped octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,

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– mobile phase: A. mix 5 volumes of *trifluoroacetic acid*, 10 volumes of *tetrahydrofuran* and 90 volumes of *water*, B. mix 5 volumes of *trifluoroacetic acid*, 10 volumes of *tetrahydrofuran* and 90 volumes of *acetonitrile*,
– a gradient programme using the conditions given below,
– flow rate: 1 ml per minute,
– spectrophotometer set at 295 nm,
– injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
30	80	20
50	20	80
60	20	80
65	80	20
70	80	20

The relative retention time with reference to paroxetine for paroxetine impurity A is about 0.8.

Inject reference solution (b).The test is not valid unless the resolution between the peaks due to paroxetine impurity C and paroxetine is not less than 3.5.

Inject reference solution (c) to identify peak of impurity A.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of paroxetine impurity A is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). The area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). 2.2 per cent to 2.7 per cent, determined on 0.3 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50.0 mg of the substance under examination in *water* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 50.0 mg of *paroxetine hydrochloride hemihydrate IPRS* in *water* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dissolve 5.0 mg of *paroxetine hydrochloride hemihydrate IPRS* and 5.0 mg of *paroxetine impurity A IPRS* in *water* and dilute to 10.0 ml with the same solvent.

Chromatographic system
– a stainless steel column 25 cm x 4.6 mm, packed with *trimethylsilane silica gel* (5 µm),
– mobile phase: Dissolve 3.85 g of *ammonium acetate* in *water*, adjusted to pH 5.5 with *anhydrous acetic acid* and dilute to 600 ml with the same solvent; add 400 ml of *acetonitrile*; slowly add, with stirring, 10 ml of *triethylamine* and read just to pH 5.5 with *anhydrous acetic acid*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 295 nm,
– injection volume: 10 µl.

Inject reference solution (b). Run the chromatogram twice the retention time of paroxetine peak. The test is not valid unless the resolution between the peaks due to impurity A and paroxetine is not less than 2.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{19}H_{21}ClFNO_3$.

Storage. Store protected from light.

Paroxetine Prolonged-release Tablets

Paroxetine Sustained-release Tablets; Paroxetine Extended-release Tablets

Paroxetine Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same..

Paroxetine Prolonged-release Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of paroxetine, $C_{19}H_{20}FNO_3$.

Usual strengths. 12.5 mg; 25mg; 37.5 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2). Complies with the test stated under Tablets.

Related substances. Determine by liquid chromatography (2.4.14).

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Solvent mixture. 80 volumes of *acetonitrile* and 20 volumes of *water.*

Test solution. Disperse a quantity of the powdered tablets containing 75 mg of Paroxetine in 75 ml of solvent mixture and sonicate for 45 minutes, cool and dilute to 100.0 ml with the solvent mixture.

Reference solution. A 0.000375 per cent w/v solution of *paroxetine hydrochloride hemihydrates IPRS* equivalent to Paroxetine in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as Inertsil C8-3),
- column temperature: 40°,
- mobile phase: A. a mixture of 900 volumes of *water*, 100 volumes of *tetrahydrofuran* and 5 volumes of *trifluroacetic acid,*
  B. a mixture of 900 volumes of *acetonitrile*, 100 volumes of *tetrahydrofuran* and 5 volumes of *trifluroacetic acid,*
- a gradient programme using the conditions given below,
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
30	80	20
40	20	80
50	80	20
60	80	20

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with the reference solution.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (1.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of *acetonitrile* and 20 volumes of *water.*

*Test solution .*Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 37.5 mg of Paroxetine, add 75 ml of solvent mixture and sonicate for 45 minutes,cool and dilute to 100.0 ml with the solvent mixture Centrifuge to obtain a clear solution. Dilute 10.0 ml of the solution to 100.0 ml with solvent mixture.

Reference solution. A 0.00375 per cent w/v solution of *paroxetine hydrochloride hemihydrates IPRS* equivalent to Paroxetine in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Zorbax TMS),
- mobile phase: a mixture of 45 volumes of buffer solution prepared by dissolving 3.854 g of *ammonium acetate* in 1000 ml of *water*, add 1 ml of *triethylamine*, adjusted to pH 4.5 with *glacial acetic acid*, and 55 volumes of *acetonitrile,* adjusted to pH 5.5 with *glacial acetic acid,*
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{20}FNO_3$ in the tablets.

Storage. Store at a temperature not exceeding 30°, protected from light and moisture.

Labelling. The label states the strength in terms of the equivalent of amount of Paroxetine.

Paroxetine Tablets

Paroxetine Hydrochloride Tablets

Paroxetine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of paroxetine, $C_{19}H_{20}FNO_3$.

Usual strengths. 10 mg; 20 mg; 30 mg.

Identification

Disperse a quantity of the powdered tablets containing 90 mg of Paroxetine with 100 ml of *0.1 M hydrochloric acid*, and stir for 1 hour. Transfer the mixture to a separating funnel, and add 1.5 ml of *ammonium hydroxide* to make the solution alkaline. Add 100 ml of *ethyl ether* to the funnel, and shake for 2 minutes. Transfer the organic layer into the necessary number

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of centrifuge tubes, and centrifuge for 10 minutes. Recombine the clarified extracts, add 1 drop of *water* and 0.5 ml of *hydrochloric acid*, stir and evaporate to dryness under a stream of nitrogen. Dry the residue in an oven at 90° for 1 hour. The residue complies with the following test.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *paroxetine hydrochloride IPRS*, treated in same manner or with the reference spectrum of paroxetine.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of simulated gastric juice, artificial (without enzyme),
Speed and time. 60 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.063 per cent w/v solution of *paroxetine hydrochloride IPRS* prepared by dissolving in minimum quantity of *methanol* and diluted with the dissolution medium to obtain a solution having a known concentration similar to the expected concentration of test solution.

Use chromatographic system as described under Assay, using 20 µl injection volume:

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{20}FNO_3$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{19}H_{20}FNO_3$.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay using the following solution.

Test solution. Disperse one tablet in 0.7 per cent v/v solution of *hydrochloric acid* and dilute with *methanol* to obtain a solution containing 0.01 per cent w/v of paroxetine and centrifuge.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 100 mg of paroxetine in *methanol* and dilute to 200 ml with *methanol*. Centrifuge a portion of the solution for 6 minutes. Dilute 20.0 ml of the supernatant liquid to 100.0 ml with *methanol*.

Reference solution. A 0.011 per cent w/v solution of *paroxetine hydrochloride IPRS* in *methanol*.

Chromatographic system
- a stainless steel column 3.3 cm x 4.6 mm, packed with octylsilane bonded to porous silica (3 µm),
- mobile phase: a mixture of 70 volumes of buffer solution prepared by mixing 100 ml of *water*, 0.6 ml of *orthophosphoric acid*, and 0.3 ml of *triethylamine* and 30 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 295 nm,
- Injection volume: 5 µl.

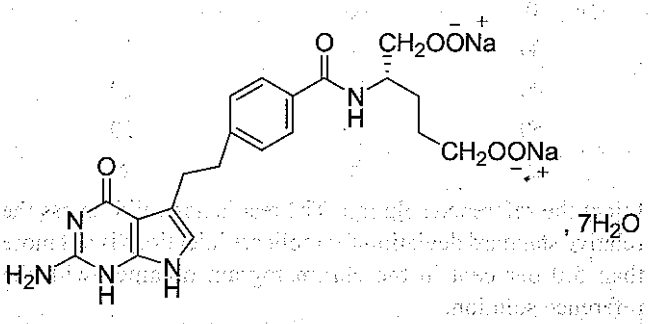
Inject the reference solution. The test is not valid unless the column efficiency is not less than 750 theoretical plates, the tailing factor is not more than 4.0 and and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{20}FNO_3$ in the tablets.

Storage. Store protected from moisture.

Pemetrexed Disodium Heptahydrate



$C_{20}H_{19}N_5Na_2O_6,7H_2O$ Mol. Wt. 597.5

Pemetrexed Disodium Heptahydrate is a disodium (2*S*)-2-[[4-[2-(2-amino-4-oxo-4,7-dihydro-1*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]amino]pentanedioate heptahydrate.

Pemetrexed Disodium Heptahydrate contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{20}H_{19}N_5Na_2O_6$, calculated on the anhydrous basis.

Category. Thymidylate synthetase inhibitor; cytostatic.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pemetrexed*

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disodium heptahydrate IPRS or with the reference spectrum of pemetrexed disodium heptahydrate.

B. It gives reaction (A) of sodium salts (2.3.1).

C. Enantiomeric purity (see Tests).

D. Water (see Tests).

Tests

Appearance of solution. A 5.6 per cent w/v solution in *carbon dioxide-free water* (Solution A) is not more opalescent than opalescence standard OS2 (2.4.1), and not more intensely coloured than reference solution GYS_4 or YS_4 (2.4.1).

pH (2.4.24). 7.5 to 8.4 for solution A.

Enantiomeric purity. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use or store them at 2-8° for not more than 24 hours.

Test solution. Dissolve 12 mg of the substance under examination in *water* and dilute to 50.0 ml with *water*.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with *water*. Dilute 3.0 ml of the solution to 10.0 ml with *water*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) with a pore size of 12 nm,
- column temperature: 40°,
- mobile phase: a mixture of 5 volumes of *acetonitrile* and 95 volumes of a solution prepared by dissolving 8 g of *β-cyclodextrin* in 900 ml of *water*, add 15 ml of *triethylamine* and 6 ml of *phosphoric acid*, adjusted to pH 6.0 with *orthophosphoric acid*, dilute to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 50 µl.

The relative retention time with reference to pemetrexed (retention time : about 30 minutes) for pemetrexed impurity E is about 0.94.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak due to (2*R*)-2-[[4-[2-(2-amino-4-oxo-4,7-dihydro-1*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]amino] pentanedioic acid (pemetrexed disodium impurity E) is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.3 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use or store them at 2 to 8° for not more than 24 hours.

Test solution. Dissolve 20 mg of the substance under examination in *water* and dilute to 100.0 ml with *water*.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *water*. Dilute 1.0 ml of the solution to 10.0 ml with *water*.

Reference solution (b). In order to prepare impurities B and C in situ, dissolve 30 mg of the substance under examination in 10.0 ml of a 0.4 per cent w/v solution of *sodium hydroxide*, heat at 70° for 40 minutes and allow to cool. Dilute 1.0 ml of the solution to 10.0 ml with *water*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm packed with octylsilane bonded to porous silica (3.5 µm),
- mobile phase: A. a mixture of 5 volumes of *acetonitrile* and 95 volumes of buffer solution containing 0.145 per cent w/v solution of *ammonium formate* in *water*, adjusted to pH 3.5 with *anhydrous formic acid*,
  B. a mixture of 30 volumes of *acetonitrile* and 70 volumes of buffer solution containing 0.145 per cent w/v solution of *ammonium formate* in *water*, adjusted to pH 3.5 with *anhydrous formic acid*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 250 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
3	100	0
45	0	100
48	100	0

Name	Relative retention time
Pemetrexed impurity A[1]	0.82
Pemetrexed impurity B[2]	0.87
Pemetrexed impurity C[3]	0.88
Pemetrexed impurity D[4]	0.90
Pemetrexed (Retention time: about 26 minute)	1.0

[1](2*S*)-2-[[4-[2-(2-amino-1-methyl-4-oxo-4,7-dihydro-1*H*-pyrrolo[2,3-*d*]pyrmidin-5 yl)ethyl]benzoyl]amino]- pentanedioic acid,

[2](2*S*, 2'*S*)-2,2'-[[(5*R*)- 2,2'- diamino- 4,4'6-trioxo-1,4,4',6,7,7'-hexahydro-1'*H*, 5*H*-5,6'-bipyrollol[2,3-*d*]pyrimidine-5,5'-diyl] bis (ethylenebenzene-4,1diylcarbonylimin)]dipentanedioic acid,

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[3](2*S*, 2'*S*)-2,2'-[[(5*S*)- 2,2'-diamino-4,4'6-trioxo-1,4,4',6,7,7'-hexahydro-1'*H*, 5*H*-5,6'-bipyrrollol[2,3-*d*]pyrimidine-5,5'-diyl] bis (ethylenebenzene-4,1diylcarbonylimin)]dipentanedioic acid,

[4](2S)-2-[[(4S)-4-[[4-[2-(2-amino-4-oxo-4,7-dihydro-1*H*-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl]amino]-4-carboxybutanoyl]amino] pentanedioic acid.

Inject the reference solution (b). The test is not valid unless the peak-to-valley ratio is not less than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any peak corresponding to pemetrexed impurity A and pemetrexed impurity D is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent). Ignore any peak with an area less than 0.5 times of the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). 19.5 per cent to 22.1 per cent, determined on 0.05 g.

Bacterial endotoxins (2.2.3). Not more than 0.17 Endotoxin Units per mg of pemetrexed.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use or store them at 2-8° for not more than 24 hours.

Test solution. Dissolve 30 mg of the substance under examination in *water* and dilute to 200.0 ml with *water*.

Reference solution. Dissolve 30 mg of *pemetrexed disodium heptahydrate IPRS* in *water* and dilute to 200.0 ml with *water*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with base deactivated octylsilane bonded to porous silica (3.5 µm),
- column temperature: 30°,
- mobile phase: a mixture of 11 volumes of *acetonitrile* and 89 volumes of buffer solution prepared by diluting 1.7 ml of *glacial acetic acid* to 900 ml of *water*, adjusted to pH 5.3 with a 76.0 per cent w/v solution of *sodium hydroxide* in *water* and dilute to 1000 ml with *water*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 285 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, tailing factor is not more than 2.0 and the relative standard deviation of replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{19}N_5Na_2O_6$.

Pemetrexed Injection

Pemetrexed Injection is a sterile material consisting of pemetrexed Disodium heptahydrate with or without buffering agents and other excipients. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections or with suitable diluents immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Pemetrexed Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pemetrexed, $C_{20}H_{21}N_5O_6$.

Usual strengths. 100 mg; 500 mg.

The contents of the sealed container comply with the requirements stated under Parental Preparations (Powder for Injections) and with the following requirements.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponding to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 6.6 to 7.8.

Related substances. Determine by liquid chromatography (2.4.14).

Buffer solution. A 0.17 per cent v/v of *glacial acetic acid* in *water* adjusted to pH 5.5 with *50 per cent sodium hydroxide solution*.

Test solution. Dilute the injection with *water* to produce a solution containing 0.02 per cent w/v of pemetrexed.

Reference solution (a). A solution of *pemetrexed disodium IPRS* in *water* containing 0.00001 per cent w/v solution of pemetrexed.

Reference solution (b). Dissolve 28 mg of *pemetrexed disodium IPRS* in 3 per cent *hydrogen peroxide* equivalent to 10 per cent of the final volume and diluted with *water* to 10.0 ml, heat this solution for 2-5 hours at 75°, allow it to cool at room temperature, dilute 5.0 ml of the solution to 50.0 ml with

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water.(NOTE — The solution preparation forms ketopemetrexed(4-{2-[(RS)-2-Amino-4,6-dioxo-4,5,6,7-tetrahydro-3H-pyrrolo[2,3-d]pyrimidin-5yl]ethyl}benzoyl)-L-glutamic acid, pemetrexed R-dimer and pemetrexed S-dimer.)

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (3.5 µm),
- column temperature: 35°,
- sample temperature: 2° to 8°,
- mobile phase: A. a mixture of 97 volumes of buffer solution and 3 volumes of *acetonitrile*,
  B. a mixture of 87.5 volumes of buffer solution and 12.5 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 250 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
40	0	100
45	0	100
47	100	0

The relative retention time with reference to pemetrexed for ketopemetrexed is about 0.31, for pemetrexed R- dimer about 0.67 and for pemetrexed S- dimer peaks is 0.71.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the pemetrexed R-dimer and pemetrexed S-dimer obtained with reference solution (b) is not less than 2.0. The signal to noise ratio for the principal peak in the chromatogram obtained with reference solution (a) is not less than 10.

Inject the test solution. The area of any peak due to ketopemetrexed multiplied with correction factor of 1.64 is not more than 0.6 per cent, the area of any secondary peak is not more than 0.24 per cent and the sum of the areas of all the secondary of such peaks is not more than 1.50 per cent, calculated by area normalisation. Ignore any peak with an area less than 0.05 per cent of that of the principal peak.

Other tests. Comply with the tests stated under Parenteral Preparation (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Buffer solution. A 0.17 per cent v/v of *glacial acetic acid* in *water* adjusted to pH 5.3 with *50 per cent sodium hydroxide solution*.

Test solution. Dilute the injection with *water* to produce a solution containing 0.01 per cent w/v of pemetrexed.

Reference solution. A 0.014 per cent w/v solution of *pemetrexed disodium IPRS* in *water*.

Chromatographic system
- a stainless steel column 7.5 cm x 4.6 mm, packed with octylsilane bonded to porous silica (3.5 µm),
- column temperature: 30°,
- mobile phase: a mixture of 11 volumes of *acetonitrile* and 89 volumes of buffer solution,
- flow rate: 1 ml per minute,
- spectrophotometer set at 285 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation of replicate injections is not more than 1.5 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{21}N_5O_6$ in the injection.

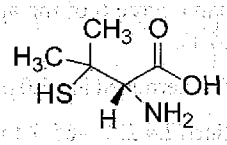
Bacterial endotoxins (2.2.3). Not more than 0.17 Endotoxin Units per mg of pemetrexed.

Sterlity (2.2.11). Complies with the test for sterility.

Storage. Store protected from moisture and at a temperature not exceeding 30°.

Penicillamine

D-Penicillamine



$C_5H_{11}NO_2S$ Mol. Wt. 149.2

Penicillamine is 3-mercapto-D-valine.

Penicillamine contains not less than 98.0 per cent and not more than 101.0 per cent of $C_5H_{11}NO_2S$, calculated on the dried basis.

Category. Chelating agent in copper and lead poisoning; antirheumatoid arthritic.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if test B, C and D carried out. Test D may be omitted if test A, B and C are carried out.

A. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 40 volumes of *1-butanol*, 10 volumes of *glacial acetic acid* and 10 volumes of *water*.

Test solution. Dissolve 0.25 g of the substance under examination in 100 ml of *water*.

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Reference solution. A 0.25 per cent w/v solution of *penicillamine IPRS* in *water.*

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 10 cm. Dry the plate at 105° for 5 to 10 minutes and expose to iodine vapour for 5 to 10 minutes. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. Dissolve 0.5 g in a mixture of 0.5 ml of *hydrochloric acid* and 4 ml of warm *acetone*, cool in ice and scratch the inside of the tube with a glass rod to initiate crystallisation; a white precipitate is produced. Filter under vacuum, wash the precipitate with *acetone* and dry with suction. A 1 per cent w/v solution of the dried material is dextrorotatory.

C. To 4 ml of a 1 per cent w/v solution add 2 ml of *phosphotungstic acid solution* and heat nearly to boiling; a blue colour is produced.

D. In the test for Penicillamine disulphide, the principal peak in the chromatogram obtained with test solution (b) corresponds to the peak due to penicillamine in the chromatogram obtained with reference solution (a).

Tests

Appearance of solution. A 10.0 per cent w/v solution in *carbon dioxide-free water* (solution A) is clear (2.4.1), and not more intensely coloured than degree 6 of the appropriate range of reference solution (2.4.1).

pH (2.4.24). 4.5 to 5.5, determined in a 1.0 per cent w/v solution.

Specific optical rotation (2.4.22). −65.0° to −61.0°, determined in a 5.0 per cent w/v solution in *1 M sodium hydroxide.*

Heavy metals (2.3.13). 12.0 ml of solution A, complies with the limit test for heavy metals, Method D (20 ppm). Use *lead standard solution (2 ppm Pb)* to prepare the standard.

Mercuric salts. Determine by atomic absorption spectrophotometry (2.4.2), using a solution prepared in the following manner. To 1.0 g of the substance under examination add 10 ml of *water* and 0.15 ml of *perchloric acid* and swirl until dissolution is complete. Add 1 ml of *ammonium pyrrolidinedithiocarbamate solution* that has been washed three times immediately before use, each time with an equal volume of *4-methyl-2-pentanone*. Mix, add 2 ml of *4-methyl-2-pentanone*, shake for 1 minute, dilute to 25 ml with *water*, allow the layers to separate and use the 4-methyl-2-pentanone layer. Measure the absorbance at 254 nm using a mercury hollow-cathode lamp and an air-acetylene flame and setting the zero using a 4-methyl-2-pentanone layer obtained by repeating the procedure described above but omitting the substance under examination. For the standard solution dissolve 0.108 g of *yellow mercuric oxide* in the minimum volume of *2 M hydrochloric acid*, add sufficient *water* to produce 1000.0 ml and treat suitable volumes in the same manner as the solution of the substance under examination (10 ppm).

Penicillamine disulphide. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 40 mg of the substance under examination in mobile phase and dilute to 10 ml with the mobile phase and mix.

Reference solution (a). A 0.4 per cent w/v solution of *penicillamine IPRS* in the mobile phase.

Reference solution (b). A 0.004 per cent w/v solution of *penicillamine disulphide IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 5 mm, packed with octylsilane bonded to porous silica (5 to 10 µm),
- mobile phase: an equal volume of 0.2 per cent w/v solution of *methanesulphonic acid* and 0.01 per cent w/v solution of *disodium edetate,*
- flow rate: 2 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

In the chromatogram obtained with test solution , the area of any peak corresponding to penicillamine disulphide is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent).

Penicillin. Carry out the following procedure in a penicillin-free atmosphere and with equipment reserved for the test. Sterilise the equipment at 180° for 3 hours and the buffer solutions at 121° for 20 minutes before use.

Liquefy a suitable nutrient medium such as that described below and inoculate at a suitable temperature with a culture of *Micrococcus flavus* (ATCC 9341) to give 5 x 10^4 micro-organisms per ml or a quantity necessary to obtain the required sensitivity and formation of clearly defined inhibition zones of suitable diameter. Immediately pour the inoculated medium into five Petri dishes (10 cm in diameter) to give uniform layers 2 to 5 mm in depth. Alternatively, the medium may consist of two layers, only the upper layer being inoculated. Store the dishes so that no appreciable growth or death of micro-organisms occurs before use and so that the surface of the medium is dry at the time of use. In each dish, place five stainless steel hollow cylinders (6 mm in diameter) on the surface of the medium evenly spaced on a circle with a radius of about 25 mm and concentric with the dish. For each dish, place in separate cylinders 0.15 ml of each of the following five solutions.

For solution (1) dissolve 1.0 g of the substance under examination in 8 ml of *phosphate buffer pH 2.5*, add 8 ml of *ether* and shake vigorously for 1 minute. Repeat the extraction

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and combine the ether layers. Add 8 ml of *phosphate buffer pH 2.5*, shake for 1 minute, allow to settle and separate the ether layer quantitatively, taking care to eliminate the aqueous phase completely. *(Penicillin is unstable at pH 2.5; carry out the operations at this pH within 6 to 7 minutes)*. Add 8 ml of *phosphate buffer pH 6.0*, shake for 5 minutes, allow to settle, separate the aqueous layer and check that the pH is 6.0. For solution (2) add 20 µl of *penicillinase solution* to 2 ml of solution (1) and incubate at 37° for 1 hour. For solution (3) dissolve 5 mg of *benzylpenicillin sodium* in 500 ml of phosphate buffer pH 6.0 and dilute 0.25 ml of the solution to 200 ml with *phosphate buffer pH 2.5*. Carry out the extraction procedure described under solution (1) using 8 ml of the solution and beginning at the words "add 8 ml of *ether*...". For solution (4) add 20 ml of *penicillinase solution* to 2 ml of solution (3) and incubate at 37° for 1 hour. Prepare solution (5) in the same manner as solution (1) but omitting the substance under examination.

Maintain the dishes at 30° for at least 24 hours. Measure the diameters of the zones of inhibition to within 0.1 mm. The test is not valid unless solution (3) gives a clear zone of inhibition and solution (4) and (5) give no zones of inhibition. If solution (1) gives a zone of inhibition it is caused by penicillin provided solution (2) gives no zone of inhibition. If this is the case, the average diameter of the zones of inhibition given by solution (1) for the five Petri dishes is less than that given by solution (3) (0.1 ppm).

Nutrient medium

Peptone	5 g
Yeast extract	1.5 g
Meat extract	1.5 g
Sodium chloride	3.5 g
Agar	15 g
Distilled water	1000 ml
Adjusted to pH	6.0

Penilloic acid. Absorbance of a 0.2 per cent w/v solution at about 268 nm, not more than 0.07 (2.4.7) (about 0.5 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 60° over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa.

Assay. Dissolve 0.1 g in 30 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01492 g of $C_5H_{11}NO_2S$.

Storage. Store protected from moisture.

Penicillamine Tablets

D-Penicillamine Tablets

Penicillamine Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of penicillamine, $C_5H_{11}NO_2S$. The tablets are coated.

Usual strengths. 50 mg; 125 mg; 250 mg.

Identification

A. Shake a quantity of the powdered tablets containing 20 mg of Penicillamine with 4 ml of *water* and filter. Add to the filtrate 2 ml of *phosphotungstic acid solution* and allow to stand for 5 minutes; a blue colour is produced.

B. Dissolve a quantity of the powdered tablets containing 10 mg of Penicillamine in 5 ml of *water* and add 0.3 ml of *5 M sodium hydroxide* and 20 mg of *ninhydrin*; an intense blue or violet-blue colour is produced immediately.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of a solution containing 0.5 per cent w/v solution of *disodium edetate* and 0.05 per cent w/v solution of *sodium lauryl sulphate*,
Speed and time. 100 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a suitable quantity of *penicillamine IPRS* in dissolution medium to obtain a solution of a known concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 30 cm × 3.9 mm, packed with octadecylsilane bonded to porous silica (5µm),
- mobile phase: a mixture of 60 volumes of *0.01 M dibasic sodium phosphate* and 40 volumes of *0.01 M monobasic potassium phosphate*, adjusted to pH 7.0 with *0.01 M dibasic sodium phosphate* or *0.01 M monobasic potassium phosphate*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 80 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injection is not more than 2.0 per cent.

Inject the reference solution and the test solution.



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Calculate the content of $C_5H_{11}NO_2S$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_5H_{11}NO_2S$.

Mercuric salts. Disperse a quantity of the powdered tablets containing 1 g of Penicillamine in 10 ml of *water* in a stoppered flask, add 0.2 ml of *9 M perchloric acid* and swirl to dissolve. Add 1 ml of *ammonium pyrrolidinedithiocarbamate solution*, mix, add 2 ml of *4-methyl-2-pentanone*, shake for 1 minute and add sufficient *water* to produce 25 ml. Determine by atomic absorption spectrophotometry (2.4.2), using a mercury hollow-cathode lamp and an air-acetylene flame and setting the zero using a *4-methyl-2-pentanone* layer obtained by repeating the procedure described above but omitting the substance under examination, measuring at 254 nm. Use *mercury solution AAS*, suitably diluted with *water*, for the standard solutions, adjusted to contain the same concentrations of *9 M perchloric acid, ammonium pyrrolidinedithiocarbamate solution* and *4-methyl-2-pentanone* as the solution under examination (10 ppm).

Penicillamine disulphide. Determine by liquid chromatography (2.4.14).

Test solution. Shake quantity of the powdered tablets containing 40 mg of Penicillamine with 10 ml of the mobile phase, filter and use the filtrate.

Reference solution. A 0.004 per cent w/v solution of *penicillamine disulphide IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 5 mm, packed with octylsilane bonded to porous silica (5 to 10 µm),
- mobile phase: an equal volume of 0.2 per cent w/v solution of *methanesulphonic acid* and 0.01 per cent w/v solution of *disodium edetate*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

In the chromatogram obtained with the test solution the area of any peak corresponding to penicillamine disulphide is not greater than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. A 0.5 per cent w/v solution of *disodium edetate* in *water*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 0.25 g of Penicillamine in 150 ml of the solvent mixture, shake for 5 minutes, allow to stand for 90 minutes and dilute to 200.0 ml with the solvent mixture.

Reference solution (a). A 0.125 per cent w/v solution of *penicillamine IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.1 per cent w/v of *penicillamine IPRS* and 0.01 per cent w/v of *penicillamine disulfide IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 6.9 g of *monobasic sodium phosphate* and 0.2 g of *sodium 1-hexanesulphonate*, in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 1.6 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

The relative retention time with reference to penicillamine disulfide for penicillamine is about 0.7.

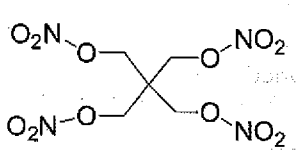
Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to penicillamine and penicillamine disulfide is not less than 3.0 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injection is not more than 1.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_5H_{11}NO_2S$ in the tablets.

Storage. Store protected from moisture.

Diluted Pentaerythritol Tetranitrate



$C_5H_8N_4O_{12}$ Mol. Wt. 316.1

Diluted Pentaerythritol Tetranitrate is a dry mixture of 2,2-bis(hydroxymethyl)propane-1,3-diol tetranitrate with Lactose or Mannitol or a mixture of Lactose and Starch or any other suitable inert excipients which permit safe handling.

Diluted Pentaerythritol Tetranitrate contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of pentaerythritol tetranitrate, $C_5H_8N_4O_{12}$.

Category. Antianginal.

Description. A white or almost white, powder.

Identification

A. Transfer a quantity of powder containing 10 mg of pentaerythritol tetranitrate to a medium porosity sintered-glass filter, add 5 ml of dry *acetone* and collect the filtrate. Repeat

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with two further quantities, each of 5 ml, of dry *acetone* and evaporate the combined filtrate at a temperature not exceeding 60°, with the aid of a gentle current of air, and dry the residue at 60° for 4 hours; the residue melts at 138° to 142° (2.4.21).

B. Suspend 10 mg of the residue obtained in test A in a mixture of 2 ml of *sulphuric acid* and 1 ml of *water;* cool and carefully overlay with 3 ml of *ferrous sulphate solution*; a reddish brown colour is produced at the interface of the two liquids.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 20 volumes of *ethyl acetate* and 80 volumes of *toluene*.

Test solution. Dissolve a quantity of the substance under examination containing about 10 mg of Pentaerythritol Tetranitrate in 10 ml of *ethanol (95 per cent)*, filter.

Reference solution. Shake a quantity of *diluted pentaerythritol tetranitrate IPRS* containing about 10 mg of Pentaerythritol Tetranitrate with 10 ml of *ethanol (95 per cent)*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine at 254 nm, The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Impurity A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 15 volumes of *glacial acetic acid*, 30 volumes of *acetone* and 60 volumes of *toluene*.

Test solution. Shake a quantity of powder containing 0.1 g of pentaerythritol tetranitrate in 5.0 ml of *ethanol (95 per cent)*, filter.

Reference solution. Dissolve about 10 mg of *potassium nitrate* in 1 ml of *water* and dilute to 100 ml with *ethanol (95 per cent)*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 8 cm. Dry the plate in air and spray with freshly prepared *potassium iodide* and *starch solution* and examine at 254 nm. The spot due to nitrate in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent, calculated as potassium nitrate).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution (a). Shake a quantity of powder containing 25 mg of pentaerythritol tetranitrate in 20 ml of the mobile phase for 15 minutes and dilute to 25.0 ml with the mobile phase, filter.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve a quantity of *diluted pentaerythritol tetranitrate IPRS* containing about 25 mg of Pentaerythritol Tetranitrate in 20 ml of the mobile phase, sonicate for 15 minutes and dilute to 25.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (c). Dilute 0.3 ml of reference solution (b) to 10.0 ml with the mobile phase.

Reference solution (d). Dilute 200 µl of *glyceryl trinitrate solution IPRS* to 25.0 ml with the mobile phase.

Reference solution (e). To 1 ml of reference solution (b), add 1 ml of reference solution (d) and dilute to 10.0 ml with the mobile phase.

Reference solution (f). Dilute 1.0 ml of reference solution (a) to 20.0 ml with the mobile phase. Dilute 0.5 ml of the solution to 50.0 ml with the mobile phase.

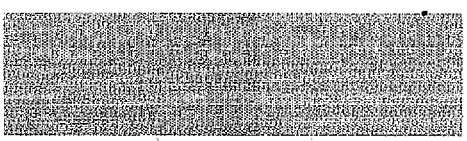
Chromatographic system

- a stainless steel column 15 cm x 3.9 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of *water* and 65 volumes of *acetonitrile*,
- flow rate: 1.4 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject reference solution (e). The test is not valid unless the resolution between the peaks due to glyceryl trinitrate and pentaerythritol tetranitrate is not less than 3.0. The relative retention time with reference to pentaerythritol tetranitrate for pentaerythritol tetranitrate impurity B is about 0.7 and for pentaerythritol tetranitrate impurity C is about 3.0.

Inject reference solution (c), (f) and test solution (a). Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with test solution (a) the area of the peak due to pentaerythritol tetranitrate impurity C is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent), the area of any other secondary peak is not more than not more than twice the area of the principal peak in the chromatogram obtained with reference solution (f) (0.1 per cent) and the sum of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.6 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (f) (0.05 per cent).

Heavy metals (2.3.13). 2.0 g complies with limit test for heavy metals, Method B (10 ppm).



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Assay. Determine by liquid chromatography (2.4.14), as described under test for Related substances.

Inject reference solution (b) and test solution (b).

Calculate the content of $C_5H_8N_4O_{12}$.

Storage. Store protected from light and moisture.

NOTE — Undiluted pentaerythritol tetranitrate is a powerful explosive. It can be exploded with percussion or excessive heat. Great care and appropriate precautions should be taken in handling and only exceedingly small amounts should be isolated.

Labelling. The label states the content of pentaerythritol tetranitrate.

Pentaerythritol Tetranitrate Tablets

Pentaerythritol Tetranitrate Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pentaerythritol tetranitrate, $C_5H_8N_4O_{12}$.

Usual strengths. 10 mg; 30 mg.

Identification

A. Transfer a quantity of powder containing 10 mg of pentaerythritol tetranitrate to a medium porosity sintered-glass filter, add 5 ml of dry *acetone* and collect the filtrate. Repeat with two further quantities, each of 5 ml, of dry *acetone* and evaporate the combined filtrate at a temperature not exceeding 60°, with the aid of a gentle current of air, and dry the residue at 60° for 4 hours; the residue melts at 138° to 142° (2.4.21).

Tests

Uniformity of content. Complies with the test stated under Tablets.

Crush one tablet and transfer to a 50-ml volumetric flask with the aid of 15 ml of *acetone*. Add sufficient *acetone* to produce 25 ml, heat the mixture on a water-bath at a temperature not exceeding 60° and boil gently, with occasional swirling, for 5 minutes. Cool, dilute to volume with *acetone* and mix. Transfer a portion of the mixture to a glass-stoppered centrifuge tube and centrifuge at 1500 rpm for 5 minutes. Transfer 2.5 ml of the supernatant solution to a 100-ml volumetric flask and evaporate at 35° with the aid of a current of air to dryness. To the residue add 1.0 ml of *glacial acetic acid* and swirl to dissolve. Add 2 ml of *phenoldisulphonic acid solution*, mix and allow to stand for 5 minutes. Add 25 ml of *water* and 10 ml of *strong ammonia solution*, cool, dilute to volume with *water* and mix. Measure the absorbance of the resulting solution at the maximum at about 409 nm (2.4.7), using *water* as the blank.

Weigh 0.130 g of *potassium nitrate*, previously dried at 105° for 4 hours, dissolve in 3 ml of *water*, dilute with sufficient *glacial acetic acid* to produce 200.0 ml and mix well. Using 1.0 ml of the solution repeat the procedure beginning at the words "Add 2 ml of *phenoldisulphonic acid solution*,.....". Calculate the content of $C_5H_8N_4O_{12}$ in the tablet.

1 ml of the potassium nitrate solution is equivalent to 0.000503 g of $C_5H_8N_4O_{12}$.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 50 mg of pentaerythritol tetranitrate and transfer to a 100-ml volumetric flask with the aid of about 30 ml of *acetone*. Add sufficient *acetone* to produce 50 ml and warm on a water-bath at a temperature not exceeding 60° and boil gently, with occasional swirling, for 5 minutes. Cool, dilute to volume with *acetone* and mix. Transfer a portion of the mixture to a glass-stoppered centrifuge tube and centrifuge at 1500 rpm for 5 minutes. Transfer 1.0 ml of the supernatant solution to a 100-ml volumetric flask and evaporate at 35° with the aid of a current of air to dryness. To the residue add 1.0 ml of *glacial acetic acid* and swirl to dissolve. Add 2 ml of *phenoldisulphonic acid solution*, mix and allow to stand for 5 minutes. Add 25 ml of *water* and 10 ml of *strong ammonia solution*, cool, dilute to volume with *water* and mix. Measure the absorbance of the resulting solution at the maximum at about 409 nm (2.4.7), using *water* as the blank.

Weigh 0.13 g of *potassium nitrate*, previously dried at 105° for 4 hours, dissolve in 3 ml of *water*, dilute with sufficient *glacial acetic acid* to produce 200.0 ml and mix well. Using 1.0 ml of the solution repeat the procedure beginning at the words "Add 2 ml of *phenoldisulphonic acid solution*,.....".

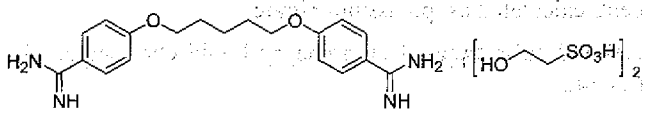
Calculate the content of $C_5H_8N_4O_{12}$ from the values of the absorbances so obtained.

1 ml of the potassium nitrate solution is equivalent to 0.000503 g of $C_5H_8N_4O_{12}$.

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of the equivalent amount of pentaerythritol tetranitrate.

Pentamidine Isethionate



$C_{19}H_{24}N_4O_2,2C_2H_6O_4S$ Mol. Wt. 592.7

Pentamidine Isethionate is 4,4′-[pentane-1,5-diylbis(oxy)] bisbenzenecarboximidamide di(2-hydroxyethanesulphonate).

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Pentamidine Isethionate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{19}H_{24}N_4O_2$, $2C_2H_6O_4S$, calculated on the dried basis.

Category. Antiprotozoal.

Description. A white or almost white powder or crystals; hygroscopic.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pentamidine isethionate IPRS* or with the reference spectrum of pentamidine isethionate.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *0.01 M hydrochloric acid* shows an absorption maximum only at about 262 nm; absorbance at about 262 nm, about 0.46).

C. To 10 ml of a 0.05 per cent w/v solution add 1 ml of a 0.1 per cent w/v solution of *glyoxal sodium bisulphite* and 1 ml of a solution prepared by dissolving 4 g of *boric acid* in a mixture of 27 ml of *1 M sodium hydroxide* and sufficient *water* to produce 100 ml. Heat on a water-bath for 10 minutes; a magenta colour is produced.

Tests

pH (2.4.24). 4.5 to 6.5, determined in a 5.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 0.1 g of the substance under examination in 100.0 ml of the mobile phase.

Reference solution (a). Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). To 0.1 g in a conical flask, add 40 ml of *water* and glass beads, adjusted to pH 10.5 with dilute *sodium hydroxide solution* and boil under reflux for 20 minutes. Cool and dilute to 50 ml with *water.* Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of *methanol* and 35 volumes of 3 per cent w/v solution of *ammonium acetate*, adjusted to pH 7.5 with *triethylamine*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the resolution between the 2 principal peaks is not less than 2.0. Inject reference solution (a) and the test solution. Run the chromatogram 3.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the sum of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Ammonium isethionate. To 1.0 g in a test-tube (about 4 cm in diameter) add 10 ml of *water* and 20 ml of *1 M sodium hydroxide.* Immediately attach a bung carrying a splash head and an aspirator tube (about 5 mm in diameter). Connect the splash head to two test-tubes in series, each containing 20 ml of *0.01 M sulphuric acid.* Heat the tube containing the substance under examination in a water-bath at 45° to 50° and, maintaining this temperature, draw a current of air, previously passed through *1 M sulphuric acid*, through the liquids in a series of tubes for 3 hours at such a rate that the bubbles are just too rapid to count. Titrate the combined solutions from the two absorption tubes with *0.02 M sodium hydroxide* using *methyl red-methylene blue solution* as indicator; not less than 36.5 ml of *0.02 M sodium hydroxide* is required.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 4.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.250 g in 50 ml of *dimethylformamide.* Add 0.25 ml of *thymol blue solution* and titrate with *0.1 M tetrabutylammonium hydroxide*, under a current of *nitrogen*, until the colour of the indicator changes to blue. Carry out a blank titration.

1 ml of *0.1 M tetrabutylammonium hydroxide* is equivalent to 0.02963 g of $C_{23}H_{36}N_4O_{10}S_2$.

Storage. Store protected from moisture.

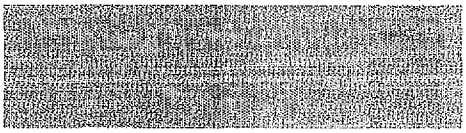
Pentamidine Injection

Pentamidine Isethionate Injection

Pentamidine Injection is a sterile material consisting of Pentamidine Isethionate with or without buffering agents and other excipients. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).



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Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Pentamidine Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of pentamidine isethionate, $C_{19}H_{24}N_4O_2, 2C_2H_6O_4S$.

Usual strength. 200 mg.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pentamidine isethionate IPRS* or with the reference spectrum of pentamidine isethionate.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *0.01 M hydrochloric acid* shows an absorption maximum only at about 262 nm; absorbance at about 262 nm, about 0.46.

C. To 10 ml of a 0.05 per cent w/v solution add 1 ml of a 0.1 per cent w/v solution of *glyoxal sodium bisulphite* and 1 ml of a solution prepared by dissolving 4 g of *boric acid* in a mixture of 27 ml of *1 M sodium hydroxide* and sufficient *water* to produce 100 ml. Heat on a water-bath for 10 minutes; a magenta colour is produced.

Tests

pH (2.4.24). 4.5 to 6.5, determined in a 5.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of substance under examination in 100.0 ml of the mobile phase.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with the mobile phase.

Reference solution (b). Dissolve 0.1 g of substance under examination in 40 ml of *water*, adjusted to pH 10.5 with *2 M sodium hydroxide*, heat under a reflux condenser for 20 minutes, cool and dilute to 50 ml with *water*. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Spherisorb ODS 1),
- mobile phase: a mixture of 130 volumes of *methanol* and 70 volumes of 3 per cent w/v solution of *ammonium acetate*, adjusted to pH 7.5 with *triethylamine*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 2.0.

Inject reference solution (a) and the test solution. Run the chromatogram 3.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any secondary peak is not more 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent).

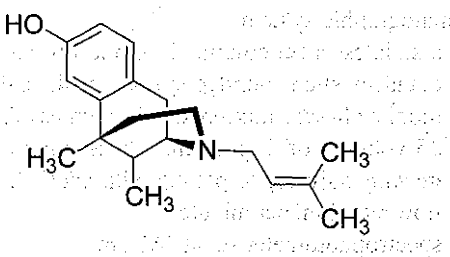
Ammonium isethionate. To 1.0 g in a test-tube (about 4 cm in diameter) add 10 ml of *water* and 20 ml of *1 M sodium hydroxide*. Immediately attach a bung carrying a splash head and an aspirator tube (about 5 mm in diameter). Connect the splash head to two test-tubes in series, each containing 20 ml of *0.01 M sulphuric acid*. Heat the tube containing the substance under examination in a water-bath at 45° to 50° and, maintaining this temperature, draw a current of air, previously passed through *1 M sulphuric acid*, through the liquids in a series of tubes for 3 hours at such a rate that the bubbles are just too rapid to count. Titrate the combined solutions from the two absorption tubes with *0.02 M sodium hydroxide* using *methyl red-methylene blue solution* as indicator; not less than 36.5 ml of *0.02 M sodium hydroxide* is required.

Assay. Dissolve 0.25 g of the mixed contents of 10 containers in 50 ml of *dimethylformamide*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M tetrabutylammonium hydroxide* is equivalent to 0.02963 g of $C_{19}H_{24}N_4O_2, 2C_2H_6O_4S$.

Storage. Store in single dose containers.

Pentazocine



$C_{19}H_{27}NO$ Mol. Wt. 285.4

Pentazocine is (2*RS*,6*RS*,11*RS*)-6,11-dimethyl-3-(3-methylbut-2-enyl)-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol.

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Pentazocine contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{19}H_{27}NO$, calculated on the dried basis.

Category. Narcotic analgesic.

Description. A white or pale cream powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pentazocine IPRS* or with the reference spectrum of pentazocine.

B. To 1 mg in a porcelain crucible add 0.5 ml of a solution of *sulphuric acid* containing 1 per cent w/v solution of *ammonium molybdate*; an intense blue colour is produced which changes to bluish green, green and finally, on standing, yellow.

C. Dissolve 5 mg in 5 ml of *sulphuric acid*, add 0.05 ml of *ferric chloride solution* and mix; a yellow colour is produced which deepens slightly in intensity on warming. On the addition of *0.05 ml of nitric acid* the yellow colour is unchanged.

Tests

Light absorption (2.4.7). To 0.1 g add 20 ml of *water* and 10 ml of *1 M hydrochloric acid*, shake to dissolve and add sufficient *water* to produce 100 ml. Dilute 10 ml to 100 ml with *water*. The absorbance of the resulting solution at the maximum at about 278 nm, 0.67 to 0.71.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Mobile phase. A mixture of 94 volumes of *chloroform*, 3 volumes of *2-propylamine* and 3 volumes of *methanol*.

Test solution. Dissolve 0.2 g of the substance under examination in 10 ml of *chloroform*.

Reference solution (a). A 0.02 per cent w/v solution of the substance under examination in *chloroform*.

Reference solution (b). A 0.01 per cent w/v solution of the substance under examination in *chloroform*.

Reference solution (c). A 0.005 per cent w/v solution of the substance under examination in *chloroform*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Heat the plate at 105° for 15 minutes, allow to cool, expose to iodine vapour and re-examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a); not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) and not more than four such spots are more intense than the spot in the chromatogram obtained with reference solution (c).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

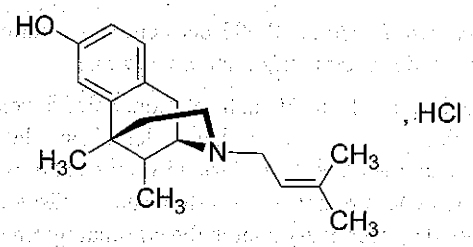
Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 4 hours.

Assay. Dissolve 0.6 g in 50 ml of *anhydrous glacial acetic acid* and titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02854 g of $C_{19}H_{27}NO$.

Storage. Store protected from light and moisture.

Pentazocine Hydrochloride



$C_{19}H_{27}NO,HCl$ Mol. Wt. 321.9

Pentazocine Hydrochloride is (2*RS*,6*RS*,11*RS*)-6,11-dimethyl-3-(3-methylbut-2-enyl)-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol hydrochloride.

Pentazocine Hydrochloride contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{19}H_{27}NO,HCl$, calculated on the dried basis.

Category. Narcotic analgesic.

Description. A white or pale cream-coloured, crystalline powder. It shows polymorphism (2.5.11).

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pentazocine hydrochloride IPRS* or with the reference spectrum of pentazocine hydrochloride.

B. To 1 mg in a porcelain crucible add 0.5 ml of a solution of *sulphuric acid* containing 1 per cent w/v solution of *ammonium molybdate*; an intense blue colour is produced which changes to bluish green, green and finally, on standing, yellow.

C. It gives reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 4.0 to 6.0, determined in a 1.0 per cent w/v solution.

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Light absorption (2.4.7). Dissolve 0.1 g in 10 ml of *1 M hydrochloric acid* and add sufficient *water* to produce 100 ml; dilute 10.0 ml of the solution to 100.0 ml with *water*. Absorbance of the resulting solution at the maximum at about 278 nm, 0.59 to 0.63.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Mobile phase. A mixture of 94 volumes of *chloroform*, 3 volumes of *2-propylamine* and 3 volumes of *methanol*.

Test solution. Dissolve 0.2 g of the substance under examination in 10 ml of *chloroform*.

Reference solution (a). A 0.02 per cent w/v solution of the substance under examination in *chloroform*.

Reference solution (b). A 0.01 per cent w/v solution of the substance under examination in *chloroform*.

Reference solution (c). A 0.005 per cent w/v solution of the substance under examination in *chloroform*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Heat the plate at 105° for 15 minutes, allow to cool, expose to iodine vapour and re-examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a); not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) and not more than four such spots are more intense than the spot in the chromatogram obtained with reference solution (c).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 100° at a pressure not exceeding 0.7 kPa.

Assay. Dissolve 0.5 g in 50 ml of *anhydrous glacial acetic acid*, add 10.0 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03219 g of $C_{19}H_{27}NO,HCl$.

Storage. Store protected from light and moisture.

Pentazocine Tablets

Pentazocine Hydrochloride Tablets

Pentazocine Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of pentazocine hydrochloride, $C_{19}H_{27}NO,HCl$.

Usual strength. 25 mg.

Identification

A. Shake a quantity of the powdered tablets containing 0.1 g of Pentazocine Hydrochloride with 10 ml of *water*, filter, add 1 ml of *1 M sodium hydroxide* and shake the resulting solution with 20 ml of *chloroform*. Wash the chloroform extract with 5 ml of *water*, dry over *anhydrous sodium sulphate* and filter. Evaporate the chloroform using a rotary evaporator and dry the oily residue at a temperature not exceeding 25° at a pressure of 2 kPa for 1 hour.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pentazocine IPRS* or with the reference spectrum of pentazocine.

B. To a quantity of the powdered tablets containing 50 mg of Pentazocine Hydrochloride add 70 ml of *water*, shake for 15 minutes, add sufficient *water* to produce 100 ml and filter. To 10 ml of the filtrate add 10 ml of *1 M sodium hydroxide* and sufficient *water* to produce 100 ml. When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows absorption maxima at about 238 nm and 298 nm.

C. Shake a quantity of the powdered tablets containing 25 mg of Pentazocine Hydrochloride with 5 ml of *water* and 0.5 ml of *2 M nitric acid* for 1 minute and filter. The filtrate gives reaction (A) of chlorides (2.3.1).

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Mobile phase. A mixture of 94 volumes of *chloroform*, 3 volumes of *2-propylamine* and 3 volumes of *methanol*.

Test solution. Shake a quantity of the powdered tablets containing 0.2 g of Pentazocine Hydrochloride with 10 ml of *0.1 M methanolic ammonia* for 10 minutes, centrifuge and use the supernatant liquid.

Reference solution (a). Dilute 1 volume of test solution to 100 volumes with *0.1 M methanolic ammonia*.

Reference solution (b). Dilute 1 volume of test solution to 200 volumes with *0.1 M methanolic ammonia*.

Reference solution (c). Dilute 1 volume of test solution to 400 volumes with *0.1 M methanolic ammonia*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Heat the plate at 105° for 15 minutes, allow to cool, expose to iodine vapour and re-examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a), not more than one such spot is more intense than the spot in the

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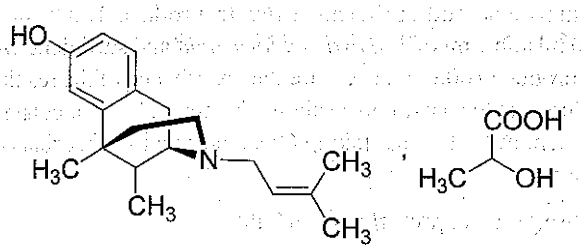
chromatogram obtained with reference solution (b) and not more than four such spots are more intense than the spot in the chromatogram obtained with reference solution (c).

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 25 mg of Pentazocine Hydrochloride, shake with 100 ml of *water* for 15 minutes, add 2.5 ml of *1 M hydrochloric acid* and sufficient *water* to produce 250.0 ml and filter. Measure the absorbance of the filtrate at the maximum at about 278 nm (2.4.7). Calculate the content of $C_{19}H_{27}NO,HCl$ taking 61.2 as the specific absorbance at 278 nm.

Storage. Store protected from light and moisture.

Pentazocine Lactate



$C_{19}H_{27}NO,C_3H_6O_3$ Mol. Wt. 375.4

Pentazocine Lactate is (2*RS*,6*RS*,11*RS*)-6,11-dimethyl-3-(3-methylbut-2-enyl)-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol lactate.

Pentazocine Lactate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{19}H_{27}NO,C_3H_6O_3$, calculated on the dried basis.

Category. Narcotic analgesic.

Description. A white or pale cream-coloured, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pentazocine lactate IPRS* or with the reference spectrum of pentazocine lactate.

B. To 1 mg in a porcelain crucible add 0.5 ml of a solution of *sulphuric acid* containing 1 per cent w/v solution of *ammonium molybdate*; an intense blue colour is produced which changes to bluish green, green and finally, on standing, yellow.

C. It gives reaction (A) of lactates (2.3.1).

Tests

pH (2.4.24). 5.5 to 6.5, determined in a 1.0 per cent w/v solution.

Light absorption (2.4.7). Dissolve 0.1 g in 10 ml of *1 M hydrochloric acid* and add sufficient *water* to produce 100 ml; dilute 10 ml to 100 ml with *water*. Absorbance of the resulting solution at the maximum at about 278 nm, 0.50 to 0.54.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Mobile phase. A mixture of 94 volumes of *chloroform*, 3 volumes of *2-propylamine* and 3 volumes of *methanol*.

Test solution. Dissolve 0.2 g of the substance under examination in 10 ml of *chloroform*.

Reference solution (a). A 0.02 per cent w/v solution of the substance under examination in *chloroform*.

Reference solution (b). A 0.01 per cent w/v solution of the substance under examination in *chloroform*.

Reference solution (c). A 0.005 per cent w/v solution of the substance under examination in *chloroform*.

Apply to the plate 25 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Heat the plate at 105° for 15 minutes, allow to cool, expose to iodine vapour and re-examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a); not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) and not more than four such spots are more intense than the spot in the chromatogram obtained with reference solution (c).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 16 hours.

Assay. Weigh 0.75 g of the substance under examination, dissolve in 50 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03754 g of $C_{19}H_{27}NO,C_3H_6O_3$.

Storage. Store protected from light and moisture.

Pentazocine Injection

Pentazocine Lactate Injection

Pentazocine Injection is a sterile solution in Water for Injections of either Pentazocine Lactate or pentazocine lactate prepared by the interaction of Pentazocine and Lactic Acid.

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Pentazocine Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of pentazocine, $C_{19}H_{27}NO$.

Usual strength. The equivalent of 30 mg of pentazocine per ml; the equivalent of 60 mg of pentazocine per ml.

Description. A clear, colourless or almost colourless liquid.

Identification

A. To a volume containing 90 mg of pentazocine add 5 ml of *0.1 M sodium hydroxide* and shake the resulting solution with 5 ml of *chloroform*. Wash the chloroform extract with 2 ml of *water*, dry over *anhydrous sodium sulphate* and filter. Evaporate the chloroform without applying heat and dry the oily residue at a temperature not exceeding 25° and at a pressure of 2 kPa for 1 hour.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pentazocine IPRS* or with the reference spectrum of pentazocine (form B).

B. Dilute a volume containing 30 mg of pentazocine to 100 ml with *water*. To 10 ml add 10 ml of *1 M sodium hydroxide* and sufficient *water* to produce 100 ml. When examined in the range 230 nm to 360 nm (2.4.7), the solution shows absorption maxima, at about 238 nm and 298 nm.

C. To a volume containing 30 mg of pentazocine add 2 ml of *0.1 M sodium hydroxide*, extract with 2 ml of *chloroform* and evaporate 0.1 ml of the *chloroform* extract to dryness in a porcelain crucible. Add to the residue 0.5 ml of a 1 per cent w/v solution of *ammonium molybdate* in *sulphuric acid*; an intense blue colour is produced which changes to bluish green, green and finally, on standing, yellow.

Tests

pH (2.4.24). 4.0 to 5.0.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Mobile phase. A mixture of 94 volumes of *chloroform*, 3 volumes of *2-propylamine* and 3 volumes of *methanol*.

Test solution. Dilute a volume of the injection with sufficient *ethanol (95 per cent)* to produce a solution containing 2.0 per cent w/v solution of pentazocine.

Reference solution (a). Dilute 1 volume of the test solution to 100 volumes with *ethanol (95 per cent)*.

Reference solution (b). Dilute 1 volume of the test solution to 200 volumes with *ethanol (95 per cent)*.

Reference solution (c). Dilute 1 volume of the test solution to 400 volumes with *ethanol (95 per cent)*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Heat the plate at 105° for 15 minutes, allow to cool, expose to iodine vapour and re-examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a), not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) and not more than four such spots are more intense than the spot in the chromatogram obtained with reference solution (c).

Bacterial endotoxins (2.2.3). Not more than 5.8 Endotoxin Units per mg of pentazocine.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

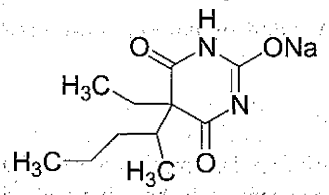
Assay. To a measured volume containing about 0.15 g of pentazocine add sufficient *water* to produce 100.0 ml. To 5.0 ml add 1 ml of *1 M hydrochloric acid* and sufficient *water* to produce 100.0 ml. Measure the absorbance of the resulting solution at the maximum at about 278 nm (2.4.7). Calculate the content of $C_{19}H_{27}NO$, taking 69 as the specific absorbance at 278 nm.

Storage. Store protected from light.

Labelling. The label states the strength in terms of the equivalent amount of pentazocine in a suitable dose-volume.

Pentobarbitone Sodium

Soluble Pentobarbitone; Pentobarbital Sodium



$C_{11}H_{17}N_2NaO_3$ Mol. Wt. 248.3

Pentobarbitone Sodium is sodium 5-ethyl-5-[(1*RS*)-1-methylbutyl]barbiturate.

Pentobarbitone Sodium contains not less than 99.0 per cent and not more than 101.5 per cent of $C_{11}H_{17}N_2NaO_3$, calculated on the dried basis.

Category. Hypnotic.

Description. A white, crystalline powder or granules; hygroscopic.

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Identification

A. To 10 ml of a 10 per cent w/v solution add 5 ml of *2 M acetic acid*; a white, crystalline precipitate is produced. Filter, wash the precipitate with *water* and dry at 105°. Determine the melting point of the dried precipitate (2.4.21). Mix equal parts of the dried precipitate and *pentobarbitone IPRS* and determine the melting point (2.4.21).. The difference between the melting points (which are about 131°) is not greater than 2°.

B. Complies with the test for identification of barbiturates (2.3.1), using the dried precipitate obtained in test A for preparing the test solution.

C. To 10 mg add 10 mg of *vanillin* and 2 ml of *sulphuric acid*, mix and heat on a water-bath for 2 minutes; a reddish-brown colour is produced. Cool and add 5 ml of *ethanol*; the colour changes to violet and then blue.

D. Ignite 1 g; the residue gives reaction (A) of sodium salts (2.3.1).

Tests

Appearance of solution. Prepare freshly a 10.0 per cent w/v solution in *carbon dioxide-free water* (solution A). Solution A is clear (2.4.1).

pH (2.4.24). 9.6 to 11.0, determined in solution A.

Related substances. Complies with the test for related substances in barbiturates (2.3.4), but applying 10 µl of each of the following solutions.

Test solution. A 2.0 per cent w/v solution of the substance under examination.

Reference solution. A 0.01 per cent w/v solution of the substance under examination.

Do not ignore any spot remaining on the line of application.

Free pentobarbitone. Not more than 3.5 per cent, determined by the following method. Weigh 2.0 g and dissolve in 75 ml of *dimethylformamide*, heating gently if necessary. Add 0.25 ml of a 1 per cent w/v solution of *thymol blue* in *dimethylformamide* and titrate with *0.1 M sodium methoxide* to a blue end-point. Carry out a blank titration.

1 ml of *0.1 M sodium methoxide* is equivalent to 0.02263 g of pentobarbitone.

Isomer. Dissolve 0.3 g in 5 ml of a 5 per cent w/v solution of *anhydrous sodium carbonate* and add 0.3 g of *4-nitrobenzyl bromide* dissolved in 10 ml of *ethanol (95 per cent)*. Heat under a reflux condenser for 30 minutes, cool to 25° scratch the side of the vessel with a glass rod if necessary to induce crystallisation and filter. Wash the residue with five quantities, each of 5 ml, of *water*. Transfer the residue as completely as possible to a small flask, add 25 ml of *ethanol (95 per cent)* and heat under a reflux condenser for 10 minutes; the solid dissolves completely. Cool to 25° and scratch the side of the flask with a glass rod to induce crystallisation. Filter, wash the residue with two quantities, each of 5 ml, of *water* and dry at 105° for 30 minutes. The dried residue melts at 136° to 148° (2.4.21).

Heavy metals (2.3.13). 0.67 g complies with the limit test for heavy metals, Method B (30 ppm).

Loss on drying (2.4.19). Not more than 3.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.4 g in 25 ml of a 12.75 per cent w/v solution of *silver nitrate* in *pyridine* and titrate with *0.1 M ethanolic sodium hydroxide* using 0.5 ml of *thymolphthalein solution* as indicator, until a pure blue colour is obtained. Carry out a blank titration.

1 ml of *0.1 M ethanolic sodium hydroxide* is equivalent to 0.02483 g of $C_{11}H_{17}N_2NaO_3$.

Storage. Store protected from moisture.

Pentobarbitone Tablets

Pentobarbitone Sodium Tablets; Pentobarbital Sodium Tablets

Pentobarbitone Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of pentobarbitone sodium, $C_{11}H_{17}N_2NaO_3$.

Usual strength. 100 mg.

Identification

A. Shake a quantity of the powdered tablets containing 0.1 g of Pentobarbitone Sodium with 10 ml of a 10 per cent w/v solution of *pyridine* and filter. Add to the filtrate 1 ml of *cupric sulphate* with *pyridine solution* and set aside for 10 minutes; a reddish violet precipitate is produced.

B. Shake a quantity of the powdered tablets containing 0.1 g of Pentobarbitone Sodium with 10 ml of *water* and filter. To the filtrate add 2 ml of *hydrochloric acid*; a white precipitate is produced (distinction from pentobarbitone).

C. The residue obtained in the Assay melts at 127° to 130°(2.4.21).

D. The powdered tablets, when moistened with *hydrochloric acid* and introduced on a platinum wire into a flame, impart a yellow colour to the flame.

Tests

Isomer. Dissolve a quantity of the powdered tablets containing 0.3 g of Pentobarbitone Sodium in 5 ml of a 5 per cent w/v

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solution of *anhydrous sodium carbonate* and add 0.3 g of *4-nitrobenzyl bromide* dissolved in 10 ml of *ethanol (95 per cent)*. Heat under a reflux condenser for 30 minutes, cool to 25° scratch the side of the vessel with a glass rod if necessary to induce crystallisation and filter. Wash the residue with five quantities, each of 5 ml, of *water*. Transfer the residue as completely as possible to a small flask, add 25 ml of *ethanol (95 per cent)* and heat under a reflux condenser for 10 minutes; filter the hot solution. Cool to 25° and scratch the side of the flask with a glass rod to induce crystallisation. Filter, wash the residue with two quantities, each of 5 ml, of *water* and dry at 105° for 30 minutes. The dried residue melts at 136° to 148° (2.4.21).

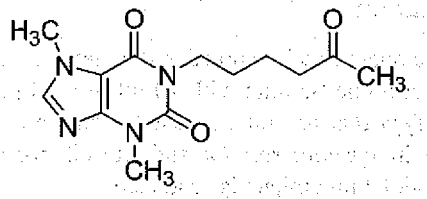
Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.3 g of Pentobarbitone Sodium, dissolve as completely as possible in 10 ml of a 2 per cent w/v solution of *sodium hydroxide*, saturate with *sodium chloride*, acidify with *hydrochloric acid* and extract with successive quantities, each of 15 ml, of *ether* until complete extraction is effected. Wash the combined extracts with two quantities, each of 2 ml, of *water* and extract the combined washings with 10 ml of *ether*. Add the ether to the main ether layer, filter and wash the filter with *ether*. Evaporate the solvent and dry the residue to constant weight at 105°.

1 g of residue is equivalent to 1.097 g of $C_{11}H_{17}N_2NaO_3$.

Storage. Store protected from moisture.

Pentoxifylline



$C_{13}H_{18}N_4O_3$ Mol. Wt. 278.3

Pentoxifylline is 1 *H*-purine-2,6-dione, 3,7-dihydro-3,7-dimethyl-1-(5-oxohexyl).

Pentoxifylline contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{13}H_{18}N_4O_3$, calculated on the dried basis.

Category. Vasodilator.

Description. A white to almost white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pentoxifylline IPRS* or with the reference spectrum of pentoxifylline.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (b).

Tests

Appearance of solution. A 2.0 per cent w/v solution in *carbon dioxide-free water*, is clear (2.4.1) and colourless (2.4.1).

Acidity. A 2.0 per cent w/v solution in *carbon dioxide free water*, add 1 drop of *bromothymol blue solution*.

Not more than 0.2 ml of *0.01 M sodium hydroxide* is required to produce a colour change to blue.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Dissolve 35 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). A solution containing 0.00007 per cent w/v of *caffeine* and 0.035 per cent w/v of *pentoxifylline IPRS* in the mobile phase.

Reference solution (b). A 0.00007 per cent w/v solution of *pentoxifylline IPRS* in the mobile phase.

Chromatographic system

– injection volume: 20 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to caffeine and pentoxyfylline is not less than 10 with reference solution (a) and the relative standard deviation for replicate injections is not more than 5.0 with reference solution (b).

Inject reference solution (b) and the test solution. Run the chromatogram 5 times the retention time of the principal peak for the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy Metals (2.3.13). 2.0 g complies with the limit test for heavy metals, method B (10 ppm).

Chlorides (2.3.12). 2.5 g complies with the limit test for chlorides (100 ppm).

Sulphates (2.3.17). 0.75 g complies with the limit test for sulphates (200 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, by drying under vaccum at 60° for 3 hours.

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Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 100.0 ml of the mobile phase and dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (a). A solution containing 0.0024 per cent w/v of *caffeine* and 0.0048 per cent w/v of *pentoxifylline IPRS* in the mobile phase.

Reference solution (b). A 0.005 per cent w/v solution of *pentoxifylline IPRS in* the mobile phase.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 2 volumes of *methanol*, 2.5 volumes of *tetrahydrofuran*, 15 volumes of *acetonitrile* and 80 volumes of 0.1 per cent w/v solution of *perchloric acid*,
- flow rate: 0.7 ml per minute,
- spectrophotometer set at 273 nm,
- injection volume: 10 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to caffeine and pentoxyfylline is not less than 10 with reference solution (a) and the relative standard deviation for replicate injections is not more than 2.0 with reference solution (b).

Inject reference solution (b) and the test solution.

Calculate the content of $C_{13}H_{18}N_4O_3$.

Storage: Store protected from moisture.

Pentoxifylline Prolonged-release Tablets

Pentoxifylline Extended-release Tablets; Pentoxifylline Sustained-release Tablets.

Pentoxifylline Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Pentoxifylline Prolonged-release Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of pentoxifylline, $C_{13}H_{18}N_4O_3$.

Usual strength. 400 mg.

Identification

A. Weigh and powder five tablets (a coarse screen may be used to separate the powder from the tablet film-coating if necessary). Weigh a quantity of powder containing 200 mg of pentoxifylline to a 15 ml centrifuge tube, add about 10 ml of *methanol*, cap the tube, and shake vigorously for about 5 minutes. Centrifuge for about 5 minutes to allow undissolved material to settle. Decant the supernatant into a suitable beaker, and evaporate the solution with the aid of a current of air to dryness at about 35°. Dissolve the residue in about 15 ml of *methylene chloride*, transfer to a separatory funnel, add about 10 ml of *water* and shake. Allow the layers to separate, transfer the methylene chloride layer, and pass through *anhydrous sodium sulphate*, collecting the filtrate in a small beaker. Evaporate the solution with the aid of a current of air to dryness at about 35°. Dissolve the residue in 8 to 10 ml of *ether*, and then chill in an ice bath, if necessary, to induce crystallization. Collect the crystals on filter paper, wash with about 2 ml of cold *ether*, and allow to dry in air. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pentoxifylline IPRS* or with the reference spectrum of pentoxifylline.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (b).

Tests

Dissolution (2.5.2). Complies with the test stated under Tablets.

Chromatographic purity. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Transfer a quantity of powder containing 40 mg of pentoxifylline to a 50-ml volumetric flask. Add 0.4 ml of *methanol* and swirl for at least 1 minute. Add about 30 ml of solvent mixture and sonicate for 60 minutes with occasional swirling of the flask. Add an additional 15 ml of solvent mixture, allow cooling to room temperature, diluting with solvent mixture to volume, mix and centrifuging. Dilute 10.0 ml of the solution to a 25-ml volumetric flask, with solvent mixture to volume and mix to obtain a solution containing 0.032 per cent w/v of pentoxifylline.

Reference solution. Dissolve an accurately weighed quantity of *pentoxifylline IPRS* in solvent mixture containing an amount of *methanol* equal to 0.8 per cent of the total volume, and dilute with solvent mixture to obtain a solution containing 0.000096 per cent w/v of pentoxfylline.

Inject the reference solution. The test is not valid unless the relative standard deviation is not more than 5.0 per cent.

Inject the reference solution and the test solution. Run the chromatogram five times the retention time of the principal peak. In the chromatogram obtained with the test solution the

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area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (0.3 per cent) and the sum of the areas of all the secondary peaks is not more than 3.3 times the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Perchloric acid solution. Dissolve 1g of *perchloric acid* in 1000 ml of *water* and mix.

Solvent mixture. 70 volumes of *water* and 30 volumes of *ethanol.*

Test solution. Weigh and powder 20 tablets. Transfer a quantity of powder containing 40 mg of Pentoxifylline to a 50-ml volumetric flask. Add 0.4 ml of *methanol*, and swirl for at least 1 minute. Add about 30 ml of the solvent mixture, and sonicate for 60 minutes with occasional swirling of the flask. Add an additional 15 ml of the solvent mixture, allow cooling to room temperature, diluting with the solvent mixture to volume and mix and centrifuging Dilute 3.0 ml of the solution to 50.0 ml with solvent mixture.

Reference solution (a). Dissolve 20 mg of *pentoxifylline IPRS* and 10 mg of *caffeine IPRS* in 0.2 ml of *methanol,* and dilute to 25.0 ml with the solvent mixture, and mix. Dilute 3.0 ml of the solution to 50 ml with the solvent mixture and mix.

Reference solution (b). Dissolve a quantity of *pentoxifylline IPRS* in solvent mixture containing an amount of *methanol* equal to 0.8 per cent of the total volume and dilute with solvent mixture to obtain a solution containing 0.0048 per cent w/v of pentoxfylline.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 80 volumes of *perchloric acid solution,*15 volumes of *acetonitrile,* 2.5 volumes of *tetrahydrofuran* and 2 volumes of *methanol,*
- flow rate: 0.7 ml per minute,
- spectrophotometer set at 273 nm,
- injection volume: 10 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between pentoxifylline and caffeine is not less than 10 with reference solution (a) and the relative standard deviation for replicate injections is not more than 2.0 per cent with reference solution (b).

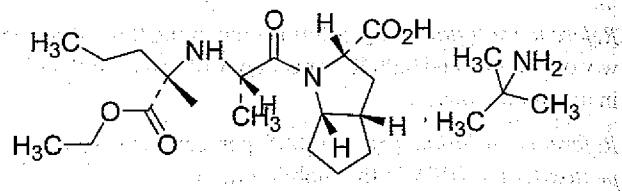
Inject reference solution (b) and the test solution.

Calculate the content of $C_{13}H_{18}N_4O_3$ in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Perindopril Erbumine

Perindopril tert-Butylamine



$C_{23}H_{43}N_3O_5$ Mol. Wt. 441.6

Perindopril Erbumine is 2-methylpropan-2-amine(2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[[(1*S*)-1(ethoxycarbonyl)butyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylate.

Perindopril Erbumine contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{23}H_{43}N_3O_5$, calculated on the anhydrous basis.

Category. Antihypertensive.

Description. A white or almost white, slightly hygroscopic, crystalline powder. It shows polymorphism (2.5.11).

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *Perindopril erbumine IPRS* or with the reference spectrum of perindopril erbumine.

If the spectra obtained show differences, dissolve the substance under examination and the reference substance separately in *methylene chloride*, evaporate to dryness and record new spectra using the residues.

B. Specific optical rotation. See Test

Tests

Specific optical rotation. (2.4.22). – 69° to – 66°, determined on 1.0 per cent w/v solution in *ethanol (95 per cent)*, calculated on anhydrous basis at 20°.

Impurity A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G.*

Mobile phase. A mixture of 1 volume of *glacial acetic acid,* 40 volumes of *toluene* and 60 volumes of *methanol.*

Test solution. Dissolve 200 mg of the substance under examination in *methanol* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *perindopril impurity A IPRS (2S,3aS,7aS)-octahydro-1H-indole-2-carboxylic acid)* in *methanol* and dilute to 25.0 ml with the same solvent.

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Reference solution (b). Dilute 5.0 ml of reference solution (a) to 20.0 ml with *methanol*.

Reference solution (c). To 5.0 ml of reference solution (a), add 5 ml of a 2 per cent w/v solution of *1,1-dimethylethylamine* in *methanol*.

Apply to the plate 10 µl of the test solution, reference solution (b) and reference solution (c). Allow the mobile phase to rise 8 cm, dry the plate in a current of warm air and expose to iodine vapour for at least 20 hours. Any spot due to perindopril impurity A is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Stereochemical purity. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in *ethanol (95 per cent)* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *ethanol (95 per cent)*. Dilute 1.0 ml of the solution to 10.0 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *perindopril for stereochemical purity IPRS* (containing perindopril impurity I (2*RS*,3a*RS*,7a*RS*)-1-[(2*RS*)-2-[[(1*SR*)-1-(ethoxycarbonyl) butyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid ((±)-13 -*epi*-perindopril)) in *ethanol (95 per cent) R* and dilute to 5.0 ml with the same solvent.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with spherical octadecylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: a mixture of 21.7 volumes of *acetonitrile*, 0.3 volumes of *1-pentanol* and 78 volumes of a 0.15 per cent w/v solution of *sodium heptanesulphonate* previously adjusted to pH 2.0 with a mixture of equal volumes of *perchloric acid* and *water*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 215 nm,
- equilibration time 240 minutes,
- injection volume: 10 µl.

Run the chromatogram 1.5 times the retention time of principal peak.

The relative retention time with reference to perindopril (retention time is about 100 minutes): perindopril impurity I is about 0.9.

Inject reference solution (a). The signal to noise ratio is not less than 3 for the principal peak.

Inject reference solution (b) the peak to valley ratio is not less than 3, where H_p = height above the baseline of the peak due to perindopril impurity I and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to perindopril.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of perindopril impurity I is not more than the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent). The area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent). Ignore any peak with a relative retention with reference to the principal peak of less than 0.6 or more than 1.4.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE —Prepare the solutions immediately before use or maintain at a temperature below 10 .

Test solution. Dissolve 60 mg of the substance under examination in mobile phase A and dilute to 20.0 ml with mobile phase A.

Reference solution (a). Dissolve 3 mg of *perindopril for peak identification IPRS* (containing impurities B, E, F, H and K) in 1 ml of mobile phase A.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with mobile phase A.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10.0 ml with mobile phase A.

Chromatographic system
- a stainless steel column 15 cm × 4.0 mm, spherical end-capped octylsilane bonded to porous silica (5 µm),
- column temperature: 60°,
- mobile phase: A. *water* adjusted to pH 2.5 with a mixture of equal volumes of *perchloric acid* and *water*,
  B. 0.03 per cent v/v solution of *perchloric acid* in *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
5	95	5
60	40	60
65	95	5
70	95	5

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Name	Relative retention time
Perindopril impurity B[1]	0.68
Perindopril impurity K[2]	0.72
Perindopril (Retention time is about 25 minutes)	1.0
Perindopril impurity E[3]	1.2
Perindopril impurity F[4]	1.6
Perindopril impurity H[5] (may be eluted as 1 or 2 peaks)	1.8

[1](2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[[(1*S*)-1-carboxybutyl] amino]propanoyl] octahydro-1*H*-indole-2-carboxylic acid (perindoprilat),

[2](3*S*,5a*S*,9a*S*,10a*S*)-3-methyldecahydropyrazino[1,2-*a*]indole-1,4-dione,

[3](2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[[(1*S*)-1-[(1-methylethoxy)carbonyl] butyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid,

[4]ethyl (2*S*)-2-[(3*S*,5a*S*,9a*S*,10a*S*)-3-methyl-1,4-dioxodecahydropyrazino[1,2-*a*]indol-2(1*H*)-yl]pentanoate,

[5](2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[(5*RS*)-3-cyclohexyl-2-(cyclohexylimino)-4-oxo-5-propylimidazolidin-1-yl]propanoyl]octahydro-1*H*-indole-2-carboxylic acid.

Inject reference solution (a). The peak to valley ratio between the peaks due to perindopril impurity B and perindopril impurity K is not less than 3.

Inject reference solution (b), (c) and the test solution. In the chromatogram obtained with the test solution, the area of any peak due to perindopril impurity E is not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent), the area of any peak due to perindopril impurity B is not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent), the area of any peak due to perindopril impurity F and H for each impurity is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent). The area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (b) (1.0 per cent). Ignore any peak with an area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.0 per cent determined on 0.5 g.

Assay. Dissolve 0.16 g in 50 ml of *anhydrous acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02208 g of $C_{23}H_{43}N_3O_5$.

Storage. Store protected from moisture.

Perindopril Erbumine Tablets

Perindopril Erbumine Tablets contain not less than 92.5 per cent and not more than 105.0 per cent of the stated amount of perindopril erbumine, $C_{19}H_{32}N_2O_5,C_4H_{11}N$.

Usual strength. 8 mg.

Identification

A. Dissolve a quantity of the powdered tablets containing 20 mg of perindopril erbumine in 10 ml of *dichloromethane* and centrifuge for 5 minutes, filter. Extract the filtrate with 10 ml of *water* and wash the upper aqueous layer with two 10 ml quantities of *hexane*. Evaporate the aqueous layer to dryness on a water bath and dry the residue at 60° at a pressure not exceeding 0.7 kPa.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *perindopril erbumine IPRS* or with the reference spectrum of perindopril erbumine.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 500 ml of *0.05 M hydrochloric acid*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with *0.05 M hydrochloric acid*, to produce a solution containing 0.0004 per cent w/v of Perindopril Erbumine.

Reference solution. A 0.0004 per cent w/v solution of *perindopril erbumine IPRS* in *0.05 M hydrochloric acid*.

Chromatographic system

- a stainless steel column 7.5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm) (Such as Nucleosil 3 C18),
- column temperature: 40°,
- mobile phase: a mixture of 34 volumes of *acetonitrile* and 66 volumes of *water*, adjusted to pH 2.0 with a mixture equal volume of *perchloric acid* and *water*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 40 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{32}N_2O_5,C_4H_{11}N$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{19}H_{32}N_2O_5,C_4H_{11}N$.

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Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use or maintain at a temperature below 10°.

Test solution. Disperse a quantity of powdered tablets containing 16 mg of Perindopril Erbumine in 7 ml of mobile phase A with the aid of ultrasound, dilute to 10.0 ml with mobile phase A and filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with mobile phase A.

Reference solution (b). A 0.16 per cent w/v solution of *perindopril IPRS* for peak identification in the mobile phase A.

Reference solution (c). A 0.01 per cent w/v solution of *perindopril impurity IPRS* in mobile phase A.

Reference solution (d). Dilute 2.0 ml of reference solution (a) to 10.0 ml with mobile phase A.

Chromatographic system

- a stainless steel column 15 cm x 4.0 mm, spherical octylsilane bonded to porous silica (5 µm) (Such as Inersil C 8),
- column temperature: 60°,
- mobile phase: A. *water* adjusted to pH 2.5 with a mixture of equal volumes of *perchloric acid* and *water*; B. A 0.03 per cent v/v solution of *perchloric acid* in *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
5	95	5
60	40	60
65	95	5
70	95	5

Name	Relative retention time
Perindopril impurity B[1]	0.68
Perindopril impurity K[2]	0.72
Perindopril (Retention time is about 25 minutes)	1.0
Perindopril impurity E[3]	1.2
Perindopril impurity C[4]	1.25
Perindopril impurity D[5]	1.3
Perindopril impurity F[6]	1.6
Perindopril impurity H[7] (may be eluted as 1 or 2 peaks)	1.8

[1](2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[[(1*S*)-1-carboxybutyl]amino]propanoyl] octahydro-1*H*-indole-2-carboxylic acid (perindoprilat),

[2](3*S*,5a*S*,9a*S*,10a*S*)-3-methyldecahydropyrazino[1,2-*a*]indole-1,4-dione,

[3](2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[[(1*S*)-1-[(1-methylethoxy)carbonyl] butyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid,

[4](2*S*)-2-[(3*S*,5a*S*,9a*S*,10a*S*)-3-methyl-1,4-dioxodecahydropyrazino [1,2-*a*]indol-2(1*H*)-yl]pentanoic acid,

[5](2*S*)-2-[(3*S*,5a*S*,9a*S*,10a*R*)-3-methyl-1,4-dioxodecahydropyrazino [1,2-*a*]indol-2(1*H*)-yl]pentanoic acid,

[6]ethyl (2*S*)-2-[(3*S*,5a*S*,9a*S*,10a*S*)-3-methyl-1,4-dioxodecahydropyrazino [1,2-*a*]indol-2(1*H*)-yl]pentanoate,

[7](2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[(5*RS*)-3-cyclohexyl-2-(cyclohexylimino)-4-oxo-5-propylimidazolidin-1-yl]propanoyl]octahydro-1*H*-indole-2-carboxylic acid.

Inject reference solution (b). In the chromatogram obtained with reference solution (b) the peak to valley ratio between the peaks due to perindopril impurity B and perindopril impurity K is not less than 3.

Inject reference solution (c) for identification of peak due to perindopril impurity C and D.

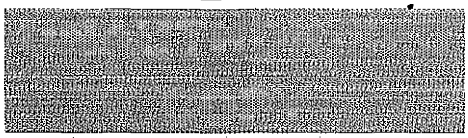
Inject reference solution (a), (d) and the test solution. In the chromatogram obtained with the test solution, the area of the any peak due to perindopril impurity E is not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent), the area of any peak due to perindopril impurity B or F is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent). The area of any peak due to perindopril impurity C or D is not more than 1.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent). The area of any other secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent) and the sum of the areas of all the secondary peaks, other than the peak due to perindopril impurities B, C, D, E and F is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay, using the following solution as the test solution.

Test solution. Disperse 1 tablet in the mobile phase and dilute with the mobile phase to obtain a solution containing 0.004 per cent w/v of perindopril erbumine.

Calculate the content of $C_{19}H_{32}N_2O_5$,$C_4H_{11}N$ in the tablet.



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Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Dissolve a quantity of the powder containing 20 mg of Perindopril Erbumine in 100.0 ml of mobile phase with the aid of ultrasound and filter. Dilute with the same solvent to get 0.004 per cent w/v of perindopril erbumine.

Reference solution. A 0.004 per cent w/v solution of *Perindopril erbumine IPRS* in the mobile phase.

Use chromatographic system as described under Dissolution, using 20 µl injection volume.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{32}N_2O_5,C_4H_{11}N$ in the Tablets.

Peritoneal Dialysis Solutions

Intraperitoneal Dialysis Fluids

Peritoneal Dialysis Solutions are sterile preparations for intraperitoneal use containing electrolytes with a concentration close to the electrolytic composition of plasma. They contain dextrose in varying concentrations and/or other suitable osmotic agents. They do not contain antioxidants such as metabisulphite salts.

Peritoneal Dialysis Solutions contain not less than 97.5 per cent and not more than 102.5 per cent of the stated amount of sodium, Na, not less than 95.0 per cent and not more than 105.0 per cent of the stated amounts of potassium, K, calcium, Ca, magnesium, Mg, chloride, Cl, acetate, $C_2H_3O_2$, lactate, $C_3H_5O_3$, sodium bicarbonate, $NaHCO_3$, bicarbonate, HCO_3 and dextrose, $C_6H_{12}O_6$.

Usual strengths: Several formulations are used. The concentrations of the components per litre of solution are usually in the following range.

	Concentration in mmol/litre
Sodium	125 – 150
Potassium	0 – 4.5
Calcium	0 – 2.5
Magnesium	0.25 – 1.5
Acetate and/or Lactate and/or Bicarbonate	30 – 60
Chloride	90 – 120
Dextrose	25 – 250

When bicarbonate is present, the solution of sodium bicarbonate is supplied in a separate container or a separate compartment and is added to the electrolyte solution immediately before use.

Unless otherwise justified and authorised, antioxidants such as metabisulphite salts are not added to the solutions.

Description. Clear, colourless or faintly straw-coloured solutions.

Identification

A. To 5 ml of the solution under examination, add 2 ml of *dilute sodium hydroxide solution* and 0.05 ml of *potassium cupri-tartrate solution*; the solution remains blue and clear. Heat to boiling; a copious red precipitate is formed.

B. 20 ml gives reactions of chlorides, sodium salts, potassium salts and calcium salts (2.3.1).

C. To 5 ml add 1 ml of *hydrochloric acid* in a test-tube fitted with a stopper and a bent tube, heat and collect a few ml of the distillate. The distillate gives reaction (C) of acetates (2.3.1).

D. To 0.1 ml of *titan yellow solution* add 10 ml of *water*, 2 ml of the solution under examination and 1 ml of *1 M sodium hydroxide*; a pink colour is produced if magnesium salts are present.

E. Lactates and bicarbonates are identified together with the Assay for lactate and bicarbonate.

Tests

Appearance of solution. The solution under examination is clear (2.4.1), and not more intensely coloured than reference solution YS4 (2.4.1).

pH (2.4.24). 4.5 to 6.5. If the solution contains bicarbonate, 6.5 to 8.0.

5-Hydroxymethylfurfural and Related substances. Dilute a volume containing 1.0 g of Dextrose to 250.0 ml with *water* and measure the absorbance of the resulting solution (2.4.7) at the maximum at about 284 nm; absorbance at about 284 nm, not more than 0.25.

Aluminium. Adjust the pH of 400 ml of the solution under examination to pH 6.0 and add 10 ml of *acetate buffer pH 6.0*. Extract the resulting solution with successive quantities of 20, 20 and 10 ml of a 0.5 per cent w/v solution of *8-hydroxyquinoline* in *chloroform* and dilute the combined extracts to 50.0 ml with *chloroform*. Use as the blank a mixture of 10 ml of *acetate buffer pH 6.0* and 100 ml of *water* treated in the same manner and as the standard solution a mixture of 2.0 ml of *aluminium standard solution (2 ppm Al)*, 10 ml of *acetate buffer pH 6.0* and 90 ml of *water* treated in the same manner. Measure the fluorescence of the test solution (I_1), of the standard solution (I_2) and of the blank (I_3), (2.4.5), using an excitation wavelength of 392 nm and a secondary filter with a

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transmission band centred at 518 nm, or a monochromator set to transmit at this wavelength. The fluorescence of the test solution ($I_1 - I_3$) is not greater than that of the standard solution ($I_2 - I_3$).

Particulate contamination (2.5.9). Carry out the test using 50 ml of the solution under examination.

The preparation meets the requirements of the test if it contains particles within the maximum limits shown below.

Particle size in µm (Equal to or larger than)	Maximum number of particles per ml
10	25
25	3

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Bacterial endotoxins (2.2.3). Not more than 0.25 Endotoxin Unit per ml.

Pyrogens (2.2.8). Solutions for which a validated test for bacterial endotoxins cannot be carried out, comply with the test for pyrogens, injecting 10 ml of the solution per kg of the rabbit's body weight.

Sterility (2.2.11). Complies with the test for sterility.

Assay. *For sodium* — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 589 nm and using sodium solution FP, or sodium solution AAS respectively, suitably diluted with *water* for the standard solutions.

For potassium — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 767 nm and using potassium solution FP or potassium solution AAS respectively, suitably diluted with *water* for the standard solutions.

For calcium — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 422.7 nm and using calcium solution FP or calcium solution AAS respectively, suitably diluted with *water* for the standard solutions.

For magnesium — To 50.0 ml add 50 ml of *water* and 5 ml of *strong ammonia-ammonium chloride solution* and titrate with *0.005 M disodium edetate* using 50 mg of *eriochrome black T mixture* as indicator.

1 ml of *0.005 M disodium edetate* is equivalent to 0.1215 mg of Mg.

For total chloride — Dilute a measured volume containing about 60 mg of chloride to 50.0 ml with *water*. Add 25.0 ml of *0.1 M silver nitrate* and 2 ml of *nitric acid*. Filter, wash the precipitate with *water* slightly acidified with *nitric acid* and titrate the excess of silver nitrate with *0.1 M ammonium thiocyanate* using *ferric ammonium sulphate solution* as indicator until a reddish yellow colour is produced. Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of total chloride, calculated as Cl.

For acetate (if present) — Determine by liquid chromatography (2.4.14).

Test solution. Dilute a measured volume of the preparation under examination quantitatively with *water* to obtain a solution containing about 1.0 mg of acetate per ml.

Reference solution. Dissolve a weighed quantity of *sodium acetate* in *water* to obtain a solution having a known concentration of about 0.12 per cent w/v of *sodium acetate*.

Chromatographic system
- a stainless steel column 30 cm x 7.8 mm, packed with a strong cation-exchange resin consisting of sulphonated cross-linked styrene-divinylbenzene copolymer in the hydrogen form (7 µm),
- mobile phase: a filtered and degassed *0.1 M sulphuric acid*,
- flow rate: 0.8 ml per minute,
- column temperature: 60°,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution and record the chromatograms. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject separately the test and standard solutions and record the chromatograms. Measure the responses for the major peak and calculate the content of acetate in the preparation under examination.

For lactate (if present) — Determine by liquid chromatography (2.4.14).

Test solution. Use the preparation under examination.

Reference solution(a). A 0.2 per cent w/v solution of *sodium lactate IPRS* in *water*.

Reference solution (b). Prepare a solution in *water* containing about 3 mg of *anhydrous sodium acetate* and 3 mg of *sodium lactate IPRS* per ml.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm),
- mobile phase: a filtered and degassed solution in *water* containing about 1 ml of *formic acid* and 1 ml of *dicyclohexylamine* in 1000 ml,

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- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject separately reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to acetate and lactate is not less than 2.0, the tailing factor for the analyte peak is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject separately the test solution and reference solution (a), and record the chromatograms. Measure the responses for the major peak and calculate the content of lactate in the preparation under examination.

For sodium bicarbonate — Titrate with *0.1 M hydrochloric acid* a volume of the preparation under examination containing about 0.1 g of *sodium bicarbonate*, determining the end-point potentiometrically (2.4.25).

1 ml of *0.1 M hydrochloric acid* is equivalent to 8.40 mg of $NaHCO_3$.

For lactate and bicarbonate — Determine by liquid chromatography (2.4.14).

Test solution. Use the preparation under examination.

Reference solutions. Dissolve weighed quantities of lactates and bicarbonates in order to obtain solutions having concentrations of about 90 per cent, 100 per cent and 110 per cent of the claim in 100 ml of *water*.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm, packed with a cation-exchange resin (9 µm),
- column temperature: 85°,
- mobile phase: a filtered and degassed *0.005 M sulphuric acid*,
- flow rate: 0.6 ml per minute,
- differential refractometer detector,
- injection volume: 20 µl.

Inject separately the test solution and the reference solutions in duplicate, and record the chromatograms in the prescribed conditions. The peaks elute in the following order, lactates, then bicarbonates. Determine the concentration of lactates and bicarbonates in the test solution by interpolating the peak area for lactate and the peak height for bicarbonate from the linear regression curve obtained with the solutions prepared as reference solutions.

For dextrose — Transfer a volume of the preparation under examination containing about 25 mg of Dextrose to a 250-ml conical flask with a ground-glass neck and add 25.0 ml of *cupri-citric solution*. Add a few grains of pumice, fit a reflux condenser, heat so that boiling occurs within 2 minutes and boil for exactly 10 minutes. Cool and add 3 g of *potassium iodide* dissolved in 3 ml of *water*. Carefully add, in small amounts, 25 ml of a 25 per cent w/w solution of *sulphuric acid*. Titrate with *0.1 M sodium thiosulphate* using *starch solution* as indicator. Carry out a blank titration using 25 ml of *water*.

Calculate the content of anhydrous dextrose, $C_6H_{12}O_6$, from the following Table.

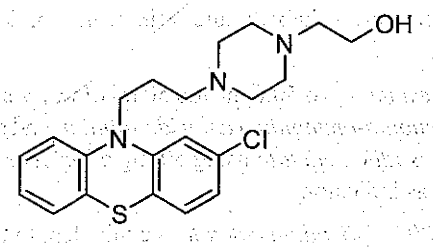
Volume of *0.1 M sodium thiosulphate* consumed (ml)	Anhydrous dextrose (mg)
8	19.8
9	22.4
10	25.0
11	27.6
12	30.3
13	33.0
14	35.7
15	38.5
16	41.3

Storage. Peritoneal Dialysis Solutions are supplied in rigid or semi-rigid plastic containers, in flexible plastic containers fitted with a special connecting device (these are generally filled to a volume below their nominal capacity and presented in closed protective envelopes) or in glass containers. Store at a temperature not exceeding 30°.

CAUTION — Exposure to temperatures below 4° may cause crystallisation and separation of solid particles rendering the preparation unsuitable for use.

Labelling. The label states (1) the formula of the solution for peritoneal dialysis, expressed in grams per litre and in millimoles per litre; (2) the total osmolar concentration in mOsmol per litre; (3) the nominal volume of the solution in the container; (4) that the solution is free from bacterial endotoxins, or where applicable, that it is apyrogenic; (5) that the solution is not to be used for intravenous infusion; (6) that any unused portion of the solution is to be discarded; (7) that the solution containing visible particles should not be used; (8) the storage conditions.

Perphenazine



$C_{21}H_{26}ClN_3OS$ Mol. Wt. 404.0

Perphenazine is 2-[4-[3-(2-chloro-10*H*-phenothiazin-10-yl)propyl]piperazin-1-yl]ethanol.

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Perphenazine contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{21}H_{26}ClN_3OS$, calculated on the dried basis.

Category. Dopamine receptor antagonist; neuroleptic.

Description. A white or yellowish-white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *perphenazine IPRS* or with the reference spectrum of perphenazine.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *kieselguhr G*.

Mobile phase. A mixture of 2 volumes of *diethylamine* and 100 volumes of *light petroleum*, saturated with *phenoxyethanol* (add 6 volumes *phenoxyethanol* to 8 volumes of the above mixture until there is a persistent cloudiness after shaking, decant, and use the supernatant liquid, even if it is cloudy).

Test solution. Dissolve 20 mg of the substance under examination in 10 ml of *chloroform*.

Reference solution. A 0.2 per cent w/v solution of *perphenazine IPRS* in *chloroform*.

Impregnate the plate by placing it in a closed tank containing the necessary quantity of the impregnation mixture containing 2.5 per cent v/v of *phenoxyethanol* and 7.5 per cent v/v of *formamide* in *acetone* so that the plate dips about 5 mm beneath the surface of the liquid. When the impregnation mixture has risen at least 17 cm from the lower edge of the plate, remove the plate and use immediately. Carry out the chromatography in the same direction as the impregnation.

Apply to the plate 2 µl of each solution. Develop in the dark and allow the mobile phase to rise 15 cm. Expose the plate to ultraviolet light at 365 nm and examine after a few minutes. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution. Dry the plate at 120° for 20 minutes, allow to cool and spray with a 10 per cent v/v solution of *sulphuric acid* in *ethanol (95 per cent)*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

C. When examined in the range 230 nm to 350 nm (2.4.7), a 0.001 per cent w/v solution in *methanol* shows absorption maxima at 257 nm and 313 nm. The ratio of the absorbance measured at the maximum at 313 nm to that measured at the maximum at 257 nm is 0.120 to 0.128.

D. Melting range (2.4.21). 96° to 100°.

Tests

Appearance of solution. A 2.0 per cent w/v solution in *methanol* is clear (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Prepare the solutions immediately before use. Carry out the test protected from light.

Test solution. Dissolve 20 mg of the substance under examination in mobile phase A and dilute to 10.0 ml with mobile phase A.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with mobile phase A.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with mobile phase A.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with base deactivated octylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 35 volumes of *acetonitrile* and 65 volumes of a 0.7 per cent w/v solution of *sodium dihydrogen phosphate*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 10 µl.

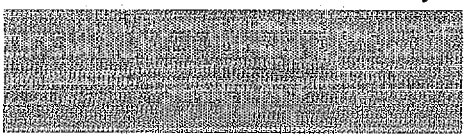
Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	100	0
10	80	20
33	30	70
48	100	0

Name	Relative retention time	Correction factor
Perphenazine impurity A[1]	0.3	0.6
Perphenazine impurity B[2]	0.8	—
Perphenazine (Retention time is about 12 minutes)	1.0	—

[1]2-[4-[3-(2-chloro-5-oxido-10*H*-phenothiazin-10-yl)propyl]piperazin-1-yl]ethanol,

[2]2-[4-[3-(10*H*-phenothiazin-10-yl)propyl]piperazin-1-yl]ethanol.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.



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Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to perphenazine impurity A is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any peak corresponding to perphenazine impurity B is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in vacuum at 65° for 4 hours.

Assay. Weigh 0.15 g and dissolve in 25 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.0202 g of $C_{21}H_{26}ClN_3OS$.

Calculate the content of $C_{21}H_{26}ClN_3OS$.

Storage. Store protected from light.

Perphenazine Tablets

Perphenazine Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of perphenazine, $C_{21}H_{26}ClN_3OS$.

Usual strengths. 2 mg; 4 mg.

Identification

A. To a quantity of the powdered tablets containing 40 mg of Perphenazine, add 10 ml of *water* and 2 ml of *1 M sodium hydroxide*, shake and extract with 15 ml of *ether*. Wash the ether layer with 5 ml of *water*, dry with *anhydrous sodium sulphate* and evaporate the *ether* to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *perphenazine IPRS* or with the reference spectrum of perphenazine.

B. Extract a quantity of the powdered tablets containing 20 mg of Perphenazine with 10 ml of *chloroform*, filter and evaporate the filtrate to dryness. Dissolve the residue in 2 ml of *methanol*, pour into a 4.0 per cent w/v solution of *picric acid* in *methanol* at 50°, cool and allow to stand for 4 hours. After recrystallisation from *methanol*, the precipitate melts at about 248° (2.4.21) with decomposition.

C. To a quantity of the powdered tablets containing 5 mg of Perphenazine, add 5 ml of *sulphuric acid* and allow to stand for 5 minutes. A red colour is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1M hydrochloric acid*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the medium, if necessary, at the maxima at about 257 nm (2.4.7). Calculate the content of $C_{21}H_{26}ClN_3OS$ in the medium from the absorbance obtained from a solution of known concentration of *perphenazine IPRS* in the dissolution medium.

Q. Not less than 75 per cent of the stated amount of $C_{21}H_{26}ClN_3OS$.

Related substances. Comply with the test for Related substances in Phenothiazines (2.3.5) with the following modifications.

Use mobile phase (c) and applying to the plate 50 µl of each of the following freshly prepared solutions.

Test solution. Extract a quantity of the powdered tablets containing 20 mg of Perphenazine with 10 ml of *ethanol* (*95 per cent*) and filter.

Reference solution. Dilute 1 ml of the test solution to 200 ml with the *ethanol* (*95 per cent*).

Uniformity of content. Complies with the test stated under Tablets.

NOTE — Carry out the test protected from light.

Transfer one tablet to a 100-ml volumetric flask and add 5 ml of *water*, mix with the aid of ultrasound for 15 minutes or until the tablet has completely disintegrated, heat on a water-bath for 3 minutes, swirling continuously and cool. Add 50 ml of *ethanol* (*95 per cent*), mix with the aid of ultrasound for 2 minutes, shake for 5 minutes, dilute to 100 ml with *ethanol* (*95 per cent*) and filter through a glass microfibre filter paper. Dilute a volume of the solution with sufficient *ethanol* (*95 per cent*) to produce a solution containing 0.001 per cent w/v of Perphenazine. Record the second-derivative ultraviolet absorption spectra of the resulting solution in the range 210 to 290 nm (2.4.7). Measure the amplitude from the peak at 265 nm to the trough at 255 nm.

Calculate the content of $C_{21}H_{26}N_3OS$ in the tablet from the absorbance obtained by repeating the operation using

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perphenazine IPRS in place of the substance under examination.

Other tests. Comply with the tests stated under Tablets.

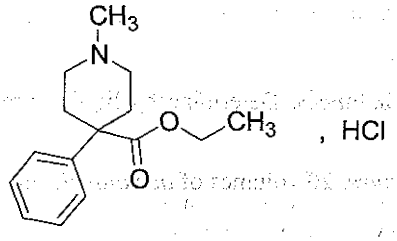
Assay. *NOTE—Carry out the test protected from light.*

Shake 10 whole tablets with 50 ml of *water* and mix with the aid of ultrasound for 15 minutes or until the tablets have completely disintegrated, heat on a water-bath for 3 minutes, swirling continuously, cool, add 400 ml of *ethanol (95 per cent)*, mix with the aid of ultrasound for 2 minutes, shake for 5 minutes, dilute to 500 ml with *ethanol (95 per cent)* and filter through a glass microfibre filter paper. Dilute the filtrate with *ethanol (95 per cent)* to produce a solution containing 0.001 per cent w/v of Perphenazine. Record second-derivative ultraviolet absorption spectra of the solutions in the range 210 to 290 nm (2.4.7). Measure the amplitude from the peak at 265 nm to the trough at 255 nm.

Calculate the content of $C_{21}H_{26}N_3OS$ in the tablet from the absorbance obtained by repeating the operation using *perphenazine IPRS* in place of the substance under examination.

Pethidine Hydrochloride

Meperidine Hydrochloride



$C_{15}H_{21}NO_2,HCl$ Mol. Wt. 283.8

Pethidine Hydrochloride is ethyl 1-methyl-4-phenylpiperidine-4-carboxylate hydrochloride.

Pethidine Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{15}H_{21}NO_2,HCl$, calculated on the dried basis.

Category. Narcotic analgesic.

Description. Colourless crystals or a white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pethidine hydrochloride IPRS* or with the reference spectrum of pethidine hydrochloride.

B. To 5 ml of a 1 per cent w/v solution add a few drops of *potassium mercuri-iodide solution*; a cream-coloured precipitate is produced.

C. Dissolve 5 mg in 0.5 ml of *water* and add 0.1 ml of *formaldehyde solution* and 2 ml of *sulphuric acid*; an orange-red colour is produced.

D. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 2.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. To 10 ml of a 2.0 per cent w/v solution in *carbon dioxide-free water* add 0.2 ml of *methyl red solution* and 0.2 ml of *0.01 M sodium hydroxide*; the solution is yellow. Add 0.3 ml of *0.01 M hydrochloric acid*; the solution is red.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 20 volumes of *acetonitrile* and 80 volumes of *water*.

Test solution (a). Dissolve about 0.1 g of the substance under examination in 25.0 ml of the solvent mixture.

Test solution (b). Dissolve about 0.125 g of the substance under examination in 10.0 ml of the solvent mixture.

Reference solution (a). Dilute 0.5 ml of test solution (a) to 100.0 ml with the solvent mixture.

Reference solution (b). Dissolve 10 mg of *1-methyl-4-phenylpiperidine IPRS (pethidine impurity A IPRS)* to 100.0 ml with the solvent mixture.

Reference solution (c). Dissolve 12.5 mg of *1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine IPRS (pethidine impurity B IPRS)* in 10.0 ml of the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution (d). Dilute 5.0 ml of reference solution (b) and 1.0 ml of reference solution (c) to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm packed with endcapped octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS2),
- mobile phase: A. a mixture of equal volumes of 4.2 per cent w/v solution of *sodium perchlorate* and 1.2 per cent v/v solution of *orthophosphoric acid*, adjusted to pH 2.0 with *triethylamine*,

B. *acetonitrile*,

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– a gradient programme using the conditions given below,
– flow rate: 1 ml per minute,
– spectrophotometer set at 210 nm,
– injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
15	75	25
31	55	45
40	55	45
41	80	20
50	80	20

The relative retention time with reference to pethidine for pethidine impurity B is about 0.66 and for pethidine impurity A is about 0.68.

Inject reference solution (c). The test is not valid unless the signal-to-noise ratio for the first peak is not less than 10 and peak-to-valley ratio where *Hp* is height above the baseline, and *Hv* is height above the baseline of the lowest point of the curve separating this peak from the peak due to pethidine impurity A is not less than 4.0.

Inject reference solution (a), (d) and test solution (a) and (b). In the chromatogram obtained with test solution (b), the area of the peak corresponding to pethidine impurity B is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (10 ppm). In the chromatogram obtained with test solution (a), the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.5 g in 30 ml of *anhydrous glacial acetic acid*, add 12 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02838 g of $C_{15}H_{21}NO_2,HCl$.

Storage. Store protected from light and moisture.

Pethidine Injection

Pethidine Hydrochloride Injection; Meperidine Hydrochloride Injection

Pethidine Injection is a sterile solution of Pethidine Hydrochloride in Water for Injections.

Pethidine Injection contains not less than 95.0 per cent and not more than 105.0 per cent of pethidine hydrochloride, $C_{15}H_{21}NO_2,HCl$.

Usual strength. 50 mg per ml; 100 mg per ml.

Identification

A. To a volume containing 50 mg of Pethidine Hydrochloride add sufficient *1 M sodium hydroxide* to make strongly alkaline to litmus paper and extract with two quantities, each of 10 ml, of *chloroform*. Wash the combined extracts with 5 ml of *water*, dry over *anhydrous sodium sulphate*, filter and evaporate the filtrate to dryness. Remove the last traces of chloroform by drying the residual oil at 60° at a pressure not exceeding 0.7 kPa. On the oily residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pethidine hydrochloride IPRS* treated in the same manner or with the reference spectrum of pethidine.

B. To 0.5 ml add 0.1 ml of *formaldehyde solution* and 2 ml of *sulphuric acid*; an orange-red colour is produced.

C. It gives the reactions of chlorides (2.3.1).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 20 volumes of *acetonitrile* and 80 volumes of *water*.

Test solution. Dilute a volume of the injection containing 0.1 g of Pethidine Hydrochloride with 25.0 ml of the solvent mixture.

Reference solution. Dilute 0.5 ml of the test solution to 100.0 ml with the solvent mixture.

Chromatographic system
– a stainless steel column 25 cm x 4.0 mm packed with endcapped octadecylsilane bonded to porous silica (5 µm) (Such as Kromasil C18),
– mobile phase: A. a mixture of equal volumes of 4.2 per cent w/v solution of *sodium perchlorate* and 1.2 per cent v/v solution of *orthophosphoric acid*, adjusted to pH 2.0 with *triethylamine*,
B. *acetonitrile*,
– a gradient programme using the conditions given below,
– flow rate: 1 ml per minute,

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- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
15	75	25
31	55	45
40	55	45
41	80	20
50	80	20

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *Determine by liquid chromatography* (2.4.14).

Test solution. Dilute a volume of the injection containing about 0.1 g of Pethidine Hydrochloride with 100.0 ml of the *water*. Dilute 3.0 ml of the solution to 25.0 ml with the mobile phase.

Reference solution. A 0.1 per cent w/v solution of *pethidine hydrochloride IPRS*. Dilute 3.0 ml of the solution to 25.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with endcapped octadecylsilane bonded to porous silica (5 µm) (Such as Spherisorb ODS1),
- column temperature: 40°,
- mobile phase: a mixture of 45 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, add 10 ml *triethylamine*, adjusted to pH 7.0 with *orthophosphoric acid* and 55 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{21}NO_2$,HCl in the injection.

Storage. Store protected from light.

Pethidine Tablets

Pethidine Hydrochloride Tablets; Meperidine Hydrochloride Tablets

Pethidine Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of pethidine hydrochloride, $C_{15}H_{21}NO_2$,HCl.

Usual strengths. 25 mg; 50 mg.

Identification

A. Shake a quantity of the powdered tablets containing 50 mg of Pethidine Hydrochloride with 20 ml of *chloroform*, filter, evaporate the filtrate to dryness and dry the residue at a pressure of 2 kPa.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pethidine hydrochloride IPRS* or with the reference spectrum of pethidine hydrochloride.

B. Shake a quantity of the powdered tablets containing 0.2 g of Pethidine Hydrochloride with 20 ml of *water* and filter. To 5 ml of the filtrate add 10 ml of picric acid solution. The crystals so obtained, after washing with *water* and drying, melt at about 190° (2.4.21).

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *kieselguhr G*.

Mobile phase. The upper layer obtained by shaking together 100 volumes of *light petroleum (50° to 70°)*, 8 volumes of *2-phenoxyethanol* and 1 volume of *diethylamine*.

Test solution. The upper layer obtained by shaking a quantity of the powdered tablets containing 0.1 g of Pethidine Hydrochloride with 5 ml of *water*, filtering, shaking the filtrate with 0.5 ml of *5 M sodium hydroxide* and 2 ml of *ether* and allowing the layers to separate.

Reference solution. Dilute 0.5 ml of the test solution to 50 ml with *ether*.

Impregnate the dry plate by placing it in a closed tank containing a mixture of 90 volumes of *acetone* and 10 volumes of *2-phenoxyethanol* so that the plate dips about 5 mm beneath the surface of the liquid, allowing the impregnating solvent to ascend at least 15 cm, removing the plate from the tank and drying in a current of air. Use immediately, with the flow of the mobile phase in the same direction as the impregnation.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in air for 10 minutes, return the plate to the tank and repeat the development. Remove the plate, allow it to dry in air for 10 minutes and spray with a

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0.2 per cent w/v solution of *2,7-dichlorofluorescein* in *methanol*. Allow to stand for 5 minutes and spray with *water* until the background is white to pale yellow. Examine in daylight. The chromatograms show red or orange spots. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution. Examine without delay under ultraviolet light at 365 nm. The chromatograms show spots with intense yellow fluorescence. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.3 g of Pethidine Hydrochloride in 40 ml of *water*, add 2 ml of *5 M sodium hydroxide* and extract immediately with successive quantities of 25, 10 and 10 ml of *chloroform*. Wash each extract with the same 15 ml of *water* and filter into a dry flask. Combine the extracts (which should be clear and free from droplets of *water*). Titrate with *0.05 M perchloric acid*, using 0.15 ml of *1-naphtholbenzein solution* as indicator. Carry out a blank titration.

1 ml of *0.05 M perchloric acid* is equivalent to 0.01419 g of $C_{15}H_{21}NO_2,HCl$.

Storage. Store protected from light and moisture.

Petrolatum

Petrolatum is a purified mixture of semisolid hydrocarbons obtained from petroleum. It may contain a suitable stabilizer.

Category. Pharmaceutical aid.

Tests

Melting range (2.4.21). 38° to 60°.

Alkalinity. To 35 g, add 100 ml of boiling *water* in a suitable beaker, cover, and place on a stirring hot-plate maintained at the boiling point of water. After 5 minutes, allow the phases to separate. Draw off the separated water into a casserole, wash the petrolatum further with two 50-ml portions of boiling water, and add the washings to the casserole. To the pooled washings, add 1 drop of *phenolphthalein TS*, and boil; the solution does not acquire a pink colour.

Acidity. If the addition of *phenolphthalein TS* in the test for alkalinity produces no pink colour, add 0.1 ml of *methyl orange TS*; no red or pink colour is produced.

Consistency. A penetrometer fitted with a polished cone-shaped metal plunger weighing 150 g, having a detachable steel tip of the following dimensions: the tip of the cone has an angle of 30°, the point of the tip is truncated to a diameter of 0.381 mm, the base of the tip is 8.38 mm in diameter, and the length of the tip is 14.94 mm.

The remaining portion of the cone has an angle of 90°, is 28 mm in height, and has a maximum diameter at the base of 65 mm. The containers for the test are flat-bottom metal cylinders that are 100 mm in diameter and not less than 65 mm in height. They are constructed of at least 1.6 mm (16-gauge) metal, and are provided with well-fitting, water-tight covers.

Place the required number of containers in an oven, bring them and a quantity of the sample to a temperature of 82°, and pour the sample into one or more of the containers, filling to within 6 mm of the rim. Cool to 25° over a period of not less than 16 hours, protected from drafts. Two hours before the test, place the containers in a water-bath at 25°. If the room temperature is below 23.5° or above 26.5°, adjust the temperature of the cone to 25° by placing it in the water-bath without disturbing the surface of the substance under test, place the container on the penetrometer table, and lower the cone until the tip just touches the top surface of the test substance at a spot 25 to 38 mm from the edge of the container. Adjust the zero setting and quickly release the plunger, then hold it free for 5 seconds. Secure the plunger, and read the total penetration from the scale. Make three or more trials, each so spaced that there is no overlapping of the areas of penetration. Where the penetration exceeds 20 mm, use a separate container of the test substance for each trial. Read the penetration to the nearest 0.1 mm. Calculate the average of three or more readings, and conduct further trials to a total of 10 if the individual results differ from the average by more than ±3 per cent.

Final average of the trials is not less than 10.0 mm and not more than 30.0 mm, indicating a consistency value of 100 to 300.

Organic acids. Dissolve 20.0 g in 100 ml of a mixture of 10 volumes of *ethanol (95 per cent)* and 20 volumes of *water*. Agitate thoroughly, and heat to boiling. Add 1 ml of *phenolphthalein TS* and titrate rapidly with *0.1 M sodium hydroxide*, with vigorous agitation to the production of a sharp pink endpoint, noting the colour change in the alcohol–water layer; not more than 0.4 ml of *0.1 M sodium hydroxide* is required.

Colour

Test solution. Melt 10 g on a steam-bath, and pour 5 ml of the liquid into a clear-glass 15-mm x 150-mm test tube, keeping the petrolatum melted.

Reference solution. A mixture of 3.2 volumes of *ferric chloride CS* and 1.2 volumes of *cobaltous chloride CS*.

The warm, melted liquid is not darker than 5 ml of the reference solution in a similar tube; the comparison of the two being

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made in reflected light against a white background and the petrolatum tube being held directly against the background at such an angle that there is no fluorescence.

Relative density (2.4.29). 0.815 to 0.880 at 60°.

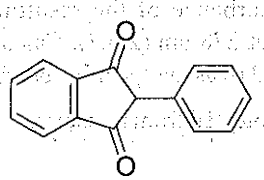
Fixed oils, fats and resin. Digest 10 g with 50 ml of *5 M sodium hydroxide* at 100° for 30 minutes. Separate the water layer, and acidify it with *2.5 M sulphuric acid*; no oily or solid matter separates.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Storage. Store protected from moisture.

Labelling. Label it to indicate the name and proportion of any added stabilizer.

Phenindione



$C_{15}H_{10}O_2$ Mol. Wt. 222.2

Phenindione is 2-phenylindane-1,3-dione.

Phenindione contains not less than 98.0 per cent and not more than 100.5 per cent of $C_{15}H_{10}O_2$, calculated on the dried basis.

Category. Anticoagulant.

Description. Soft, white or creamy-white crystals.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenindione IPRS* or with the reference spectrum of phenindione.

B. Dissolve 0.1 g in 30 ml of *ethanol (95 per cent)* with the aid of heat, cool and add sufficient *ethanol (95 per cent)* to produce 50 ml. Dilute 10 ml of the solution to 250 ml with *0.1 M sodium hydroxide* and further dilute 5 ml to 100 ml with *0.1 M sodium hydroxide*. When examined in the range 230 nm and 360 nm (2.4.7), the solution shows absorption maxima at about 278 nm and at about 330 nm; absorbance at about 278 nm, about 0.55 and at about 330 nm, about 0.16.

C. To 1 g add 50 ml of *ethanol (95 per cent)* and 0.5 ml of *aniline*, heat gently under a reflux condenser for 3 hours, cool in ice and filter. The residue, after washing with 2 ml of *ethanol (95 per cent)* and recrystallisation from *chloroform*, melts at about 225° (2.4.21).

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A 0.02 per cent w/v solution of *butylated hydroxytoluene* in a mixture of 80 volumes of *toluene*, 20 volumes of *ethyl acetate* and 4 volumes of *glacial acetic acid*.

Test solution. Dissolve 0.1 g of the substance under examination in 10 ml of *dichloromethane*.

Reference solution (a). A 0.02 per cent w/v solution of the substance under examination in *dichloromethane*.

Reference solution (b). A 0.005 per cent w/v solution of the substance under examination in *dichloromethane*.

Allow the mobile phase to ascend 4 cm, remove the plate and dry it in a current of cold air for 1 minute. Immediately apply 10 µl of each solution. Dry the plate in warm air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in oven at 105° for 2 hours.

Assay. Weigh 0.3 g, add 50 ml of *ethanol (95 per cent)* and warm until solution is effected. Cool to room temperature, add 10 ml of a 10 per cent v/v solution of *bromine* in *ethanol (95 per cent)* and allow to stand for 10 minutes, shaking occasionally. Add 1 g of *2-naphthol* and shake until the colour of the bromine is discharged. Remove any vapour of bromine in the flask with a current of air, add 50 ml of *water* and 10 ml of *dilute potassium iodide solution* and titrate the liberated iodine with *0.1 M sodium thiosulphate* using *starch solution* as indicator.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.01111 g of $C_{15}H_{10}O_2$.

Storage. Store protected from moisture.

Phenindione Tablets

Phenindione Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of phenindione, $C_{15}H_{10}O_2$.

Usual strength. 50 mg.

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Identification

Shake a quantity of the powdered tablets containing 0.2 g of Phenindione with 50 ml of *chloroform*, filter and evaporate the filtrate to dryness. Recrystallise the residue from *ethanol (95 per cent)*. The crystals complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenindione IPRS* or with the reference spectrum of phenindione..

B. Dissolve 0.1 g in 30 ml of *ethanol (95 per cent)* with the aid of heat, cool and add sufficient *ethanol (95 per cent)* to produce 50 ml. Dilute 10 ml of the solution to 250 ml with *0.1 M sodium hydroxide* and dilute 5 ml of the solution to 100 ml with *0.1 M sodium hydroxide*. When examined in the range 230 nm to 360 nm (2.4.7), the solution shows absorption maxima at about 278 nm and 330 nm; absorbance at about 278. about 0.55 and at about 330 nm, about 0.16.

C. To 50 mg add 1 ml of *sulphuric acid*; a deep blue to violet solution is produced. On dilution with *water* the solution becomes colourless and a white precipitate is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* and 1.8 g of *sodium hydroxide* in 900 ml of *water,* adjusted to pH 8.0 and dilute to 1000 ml with *water*,
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium, filter. Measure the absorbance of the filtrate suitably diluted with the medium, if necessary at the maximum at about 328 nm (2.4.7). Calculate the content of $C_{15}H_{10}O_2$ in the medium from the absorbance obtained from a solution of known concentration of *phenindione IPRS* in the dissolution medium.

Q. Not less than 70 per cent of the stated amount of $C_{15}H_{10}O_2$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A 0.02 per cent w/v solution of *butylated hydroxytoluene* in a mixture of 80 volumes of *toluene*, 20 volumes of *ethyl acetate* and 4 volumes of *glacial acetic acid*.

Test solution. Shake a quantity of the powdered tablets containing 50 mg of Phenindione with 15 ml of *dichloromethane*, filter, evaporate the filtrate to dryness and dissolve the residue in 5 ml of *dichloromethane*.

Reference solution (a). Dilute 1 volume of the test solution to 50 volumes with *dichloromethane*.

Reference solution (b). Dilute 1 volume of the test solution to 200 volumes with *dichloromethane*.

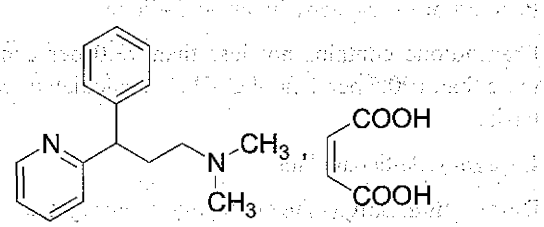
Allow the mobile phase to ascend 4 cm, remove the plate and dry it in a current of cold air for 1 minute. Immediately apply 10 µl of each solution. Dry the plate in warm air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b)..

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 Tablets. Weigh a quantity of the powder containing about 50 mg of Phenindione and shake with 150 ml of *0.1 M sodium hydroxide* for 1 hour, add sufficient *0.1 M sodium hydroxide* to produce 250.0 ml, filter and dilute 5.0 ml of the filtrate to 250.0 ml with *0.1 M sodium hydroxide*. Measure the absorbance of the resulting solution at the maximum at about 278 nm (2.4.7). Calculate the content of $C_{15}H_{10}O_2$ taking 1310 as the specific absorbance at 278 nm.

Storage. Store protected from moisture.

Pheniramine Maleate



$C_{16}H_{20}N_2,C_4H_4O_4$ Mol. Wt. 356.4

Pheniramine Maleate is (3*RS*)-*N*,*N*-dimethyl-3-phenyl-3-(pyridin-2-yl)propan-1-amine hydrogen maleate..

Pheniramine Maleate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{16}H_{20}N_2,C_4H_4O_4$, calculated on the dried basis.

Category. Antihistaminic.

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pheniramine maleate IPRS* or with the reference spectrum of pheniramine maleate.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution in *0.1 M hydrochloric acid* shows

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an inflection at about 262 nm; absorbance at about 265 nm, about 0.42.

Tests

pH (2.4.24). 4.5 to 5.5, determined in a 1.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. A mixture of 10 volumes of *acetonitrile* and 90 volumes of mobile phase A.

Test solution. Dissolve 10 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). Dissolve 10 mg of *2-benzylpyridine (pheniramine impurity A)* in 10.0 ml of the test solution and dilute to 100.0 ml with the solvent mixture.

Reference solution (b). Dilute 2.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm, packed with dimethyloctadecylsilane bonded to porous silica (10 µm),
- mobile phase: A. a 0.51 per cent w/v solution of *sodium heptanesulphonate*, adjusted to pH 2.5 with *orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1ml per minute,
- spectrophotometer set at 264 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
35	62	38
37	90	10

The order of elution of three principal peaks is maleic acid, pheniramine impurity A and pheniramine.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to pheniramine impurity A and pheniramine is not less than 8.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore the peak due to maleic acid and peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Heavy metals (2.3.13). Dissolve 1.0 g in 10 ml of *water* and add 2 ml of *acetic acid* and sufficient *water* to produce 25.0 ml. The resulting solution complies with the limit test for heavy metals, Method A (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 60° at a pressure not exceeding 0.7 kPa.

Assay. Dissolve 0.4 g in 20 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, using *1- naphthol-benzein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01782 g of $C_{16}H_{20}N_2,C_4H_4O_4$.

Storage. Store protected from light and moisture.

Pheniramine Injection

Pheniramine Maleate Injection

Pheniramine Injection is a sterile solution of Pheniramine Maleate in Water for Injections.

Pheniramine Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pheniramine maleate, $C_{16}H_{20}N_2$, $C_4H_4O_4$.

Usual strength. 22.75 mg per ml.

Identification

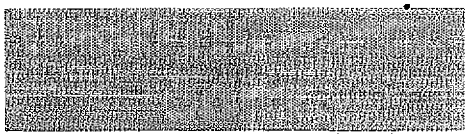
Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 50 volumes of *cyclohexane*, 40 volumes of *chloroform* and 10 volumes of *diethylamine*.

Test solution. Evaporate an appropriate volume of the injection to dryness in a current of nitrogen using the minimum amount of heat, dissolve the residue in sufficient *chloroform* to produce a solution containing 2.0 per cent w/v solution of Pheniramine Maleate and centrifuge.

Reference solution. A 2.0 per cent w/v solution of *pheniramine maleate IPRS* in *chloroform*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The two principal spots in the chromatogram obtained with the test solution correspond to those in the chromatogram obtained with the reference solution. Spray the plate with *dilute potassium iodobismuthate solution*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.



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Tests

pH (2.4.24). 4.5 to 5.5.

Related substances. Determine by the method described under the Identification test using as the reference solution a solution prepared by diluting 1 volume of the test solution to 500 volumes with *chloroform*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. To a measured volume containing about 0.11 g of Pheniramine Maleate add sufficient *water* to produce 50.0 ml and mix well. To 20.0 ml add sufficient *1 M sodium hydroxide* to make the solution just alkaline to *litmus paper*, add 2 ml in excess and extract with two quantities, each of 50 ml, of *ether*. Wash each ether extract in succession with 20, 20 and 5 ml of *0.1 M hydrochloric acid*, dilute the combined extracts to 100.0 ml with *0.1 M hydrochloric acid* and mix. Dilute 5.0 ml of the solution to 100.0 ml with *0.1 M hydrochloric acid* and measure the absorbance of the resulting solution at the maximum at about 265 nm (2.4.7), using *0.1 M hydrochloric acid* as the blank. Calculate the content of $C_{16}H_{20}N_2$, $C_4H_4O_4$ taking 210 as the specific absorbance at 265 nm.

Storage. Store protected from light.

Pheniramine Tablets

Pheniramine Maleate Tablets

Pheniramine Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of pheniramine maleate, $C_{16}H_{20}N_2$, $C_4H_4O_4$.

Usual strengths. 12.5 mg; 25 mg; 50 mg.

Identification

Boil a quantity of the powdered tablets containing about 0.5 g of Pheniramine Maleate with 150 ml of *acetone* under a reflux condenser for about 45 minutes. Filter and evaporate the filtrate to dryness on a water-bath. The residue complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pheniramine maleate IPRS* or with the reference spectrum of pheniramine maleate.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution in *0.1 M hydrochloric acid* shows an inflection at about 262 nm; absorbance at about 265 nm, about 0.42.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 50 volumes of *cyclohexane*, 40 volumes of *chloroform* and 10 volumes of *diethylamine*.

Test solution. Shake a quantity of the powdered tablets containing 20 mg of Pheniramine Maleate with 10 ml of *methanol*, centrifuge and use the supernatant liquid.

Reference solution (a). Dilute 1 volume of the test solution to 100 volumes with *methanol*.

Reference solution (b). Dilute 1 volume of reference solution (a) to 20 volumes with *methanol*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air, spray with *dilute potassium iodobismuthate solution*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b).

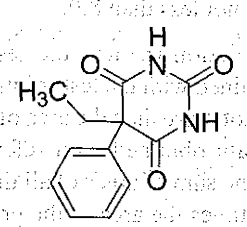
Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 45 mg of Pheniramine Maleate, shake with 20 ml of *0.1 M hydrochloric acid*, centrifuge and transfer the supernatant liquid to a 100-ml volumetric flask. Repeat the extraction with three further quantities, each of 20 ml, of *0.1 M hydrochloric acid*. Combine the extracts and add sufficient *0.1 M hydrochloric acid* to produce 100.0 ml. Mix and dilute 5.0 ml of the solution to 100.0 ml with *0.1 M hydrochloric acid*; measure the absorbance of the resulting solution at the maximum at about 265 nm (2.4.7), using *0.1 M hydrochloric acid* as the blank. Calculate the content of $C_{16}H_{20}N_2$,$C_4H_4O_4$ taking 210 as the specific absorbance at 265 nm.

Storage. Store protected from light and moisture.

Phenobarbitone

Phenobarbital



$C_{12}H_{12}N_2O_3$ Mol. Wt. 232.2

Phenobarbitone is 5-ethyl-5-phenylbarbituric acid.

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Phenobarbitone contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{12}H_{12}N_2O_3$, calculated on the dried basis.

Category. Sedative; anticonvulsant.

Description. Colourless crystals or a white, crystalline powder.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests C, D and E may be omitted if tests A and B are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenobarbitone IPRS* or with the reference spectrum of phenobarbitone.

B. Determine the melting point (2.4.21) of the substance under examination and of a mixture of equal quantities of the substance under examination and *phenobarbitone IPRS*. The difference between the melting points, which are about 175°, is not greater than 2°.

C. Complies with the test for identification of barbiturates (2.3.2).

D. Dissolve about 20 mg in 5 ml of *ethanol*, add a drop of *cobalt chloride solution* and a drop of *dilute ammonia solution*; a violet colour is produced.

E. It gives the reaction of non-nitrogen substituted barbiturates (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution in a mixture of 20 volumes of *2 M sodium hydroxide* and 30 volumes of *water* is clear (2.4.1), and not more intensely coloured than reference solution YS6 (2.4.1).

Acidity. Mix 1.0 g with 50 ml of *water*, boil for 2 minutes, allow to cool, filter and adjust the volume to 50 ml. To 10 ml of the filtrate add 0.15 ml of *methyl red solution*; not more than 0.1 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution from orange-yellow to pure yellow.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.125 g of the substance under examination in 5 ml of *methanol* and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Mix 1.0 ml of the test solution and 20.0 ml of *methanol*, dilute to 100.0 ml with the mobile phase. Mix 1.0 ml of the solution with 2.0 ml of *methanol* and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg each of *phenobarbitone impurity A IPRS* and *phenobarbitone impurity B IPRS* in 2.0 ml of *methanol* and dilute to 10.0 ml with the mobile phase. Mix 1.0 ml of the solution with 20.0 ml of *methanol* and dilute to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a solution prepared by dissolving 6.6 g of *sodium acetate* in 1000 ml of *water*, add 3 ml of *glacial acetic acid*, adjusted to pH 4.5 with *glacial acetic acid* and 40 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Name	Relative retention time
Phenobarbitone impurity A[1]	0.2
Phenobarbitone impurity B[2]	0.3
Phenobarbitone (Retention time: about 14 minutes)	1.0

[1](5*RS*)-5-ethyl-2,6-diimino-5-phenyltetrahydropyrimidin-4(1*H*)-one,

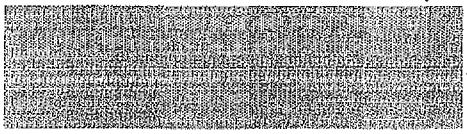
[2](5*RS*)-5-ethyl-6-imino-5-phenyldihydropyrimidine-2,4(1*H*,3*H*)-dione.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to phenobarbitone impurities A and B is not less than 1.5.

For identification of impurities, use the chromatogram obtained with reference solution (b) to identify the peaks due to phenobarbitone impurities A and B.

Inject reference solution (a), (b) and the test solution. Run the chromatogram 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak corresponding to phenobarbitone impurity A is not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent), the area of any other secondary peak corresponding to phenobarbitone impurity B is not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent). The area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.



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Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Assay. Dissolve 0.1 g in 5 ml of *pyridine*, add 0.25 ml of *thymolphthalein solution* and 10 ml of *silver nitrate-pyridine reagent* and titrate with *0.1 M ethanolic sodium hydroxide* until a pure blue colour is obtained. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of sodium hydroxide required.

1 ml of *0.1 M ethanolic sodium hydroxide* is equivalent to 0.01161 g of $C_{12}H_{12}N_2O_3$.

Storage. Store protected from moisture.

Phenobarbitone Tablets

Phenobarbital Tablets

Phenobarbitone Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of phenobarbitone, $C_{12}H_{12}N_2O_3$.

Usual strengths. 15 mg; 30 mg; 60 mg; 100 mg.

Identification

Extract a quantity of the powdered tablets containing about 0.5 g of Phenobarbitone with 50 ml of *ether*, filter through *anhydrous sodium sulphate* and evaporate the ether to dryness on a water-bath. The residue complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenobarbitone IPRS* or with the reference spectrum of phenobarbitone.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium: 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Dilute the filtrate with *alkaline borate buffer pH 9.6* and measure the absorbance at the maximum at about 240 nm(2.4.7). Calculate the content of phenobarbitone, $C_{12}H_{12}N_2O_3$ from the absorbance obtained from a solution of known concentration of *phenobarbitone IPRS* in the same medium.

Q. Not less than 75 per cent of the stated amount of $C_{12}H_{12}N_2O_3$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 0.1 g of Phenobarbitone in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.0005 per cent w/v solution of *phenobarbitone impurity A IPRS* and 0.0005 per cent w/v solution of *phenobarbitone impurity B IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Spherisorb ODS 2),
- mobile phase: a mixture of 25 volumes of *acetonitrile* and 75 volumes of a solution of 0.66 per cent w/v of *sodium acetate* in *water*, adjusted to pH 4.5 with *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Name	Relative retention time
Phenobarbitone impurity A[1]	0.4
Phenobarbitone impurity B[2]	0.5
Phenobarbitone (Retention time; about 9 minutes)	1.0

1(5*RS*)-5-ethyl-2, 6-diimino-5-phenyltetrahydropyrimidin-4(1*H*)-one, 2(5*RS*)-5-ethyl-6-imino-5-phenyldihydropyrimidine-2, 4 (1*H*, 3*H*)-dione.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to phenobarbitone impurity A and impurity B is not less than 1.5.

Inject reference solution (a) and the test solution. Run the chromatogram twice the retention time of phenobarbitone peak for the test solution. The area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.1 g of phenobarbitone in 80 ml of

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the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution. A 0.01 per cent w/v solution of *phenobarbitone IPRS* in the mobile phase.

Use chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

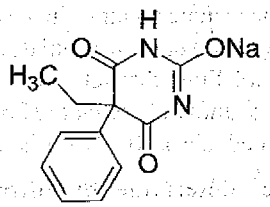
Inject the reference solution and the test solution.

Calculate the content of $C_{12}H_{12}N_2O_3$ in the tablets.

Storage. Store protected from moisture.

Phenobarbitone Sodium

Phenobarbital Sodium; Soluble Phenobarbitone; Soluble Phenobarbital



$C_{12}H_{11}N_2NaO_3$ Mol. Wt. 254.2

Phenobarbitone Sodium is sodium 5-ethyl-5-phenylbarbiturate.

Phenobarbitone Sodium contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{12}H_{11}N_2NaO_3$, calculated on the dried basis.

Category. Sedative; anticonvulsant.

Description. A white powder or crystalline granules or flaky crystals; hygroscopic.

Identification

Test A may be omitted if tests B, C, D, E and F are carried out. Tests C, D and E may be omitted if tests A, B and F are carried out.

A. Dissolve 0.2 g in 20 ml of *ethanol (50 per cent)*, acidify with *dilute hydrochloric acid* and extract with 50 ml of *ether*. Wash the ether layer with 10 ml of *water*, dry over *anhydrous sodium sulphate* and filter. Evaporate the filtrate to dryness and dry the residue at 105°.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenobarbitone IPRS* or with the reference spectrum of phenobarbitone.

B. Determine the melting point (2.4.21), of the residue obtained in test A and of a mixture of equal quantities of the residue and *phenobarbitone IPRS*. The difference between the melting points, which are about 175°, is not greater than 2°.

C. Complies with the test for identification of barbiturates (2.3.2), but using the following solutions.

Test solution. A 0.1 per cent w/v solution of the substance under examination in *ethanol (50 per cent)*.

Reference solution. A 0.09 per cent w/v solution of *phenobarbitone IPRS* in *ethanol (50 per cent)*.

D. 1 g dissolves completely in 20 ml of *ethanol (90 per cent)* (distinction from barbitone sodium).

E. It gives the reaction of non-nitrogen substituted barbiturates (2.3.1).

F. Ignite about 0.1 g; the residue gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution in *ethanol (50 per cent)* is clear (2.4.1), and not more intensely coloured than reference solution YS7 (2.4.1).

pH (2.4.24). Not more than 10.2, determined in a 10.0 per cent w/v solution.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 5 volumes of *concentrated ammonia*, 15 volumes of *ethanol (95 per cent)* and 80 volumes of *chloroform*.

Test solution. Dissolve 1 g of the substance under examination in 100 ml of *ethanol (95 per cent)*.

Reference solution. Dilute 0.5 ml of the test solution to 100.0 ml with *ethanol (95 per cent)*.

Apply 20 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. Spray with *diphenylcarbazone mercuric reagent*. Allow the plate to dry in air and spray with freshly prepared *alcoholic potassium hydroxide solution* (20 per cent w/v). Heat at 105° for 5 minutes. Any secondary spot in the chromatogram obtained with the test solution, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Loss on drying (2.4.19). Not more than 7.0 per cent, determined on 0.5 g by drying in an oven at 150° for 4 hours.

Assay. Dissolve 0.15 g in 2 ml of *water* and add 8 ml of *0.05 M sulphuric acid*. Heat to boiling and cool. Add 30 ml of

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methanol and shake until dissolution is complete. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25). After the first inflection, stop the addition of the *sodium hydroxide*, add 10 ml of *pyridine*, mix and continue the titration until the second inflection is reached. The difference between the volumes represents the amount of sodium hydroxide required.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.02542 g of $C_{12}H_{11}N_2NaO_3$.

Storage. Store protected from moisture.

Phenobarbitone Injection

Phenobarbital Sodium Injection; Phenobarbitone Sodium Injection; Soluble Phenobarbitone Injection

Phenobarbitone Injection is a sterile solution of Phenobarbitone Sodium in a mixture of nine volumes of Propylene Glycol and one volume of Water for Injections.

Phenobarbitone Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of phenobarbitone sodium, $C_{12}H_{11}N_2NaO_3$.

Usual strengths. 30 mg, 60 mg, 65 mg and 130 mg per ml.

Identification

To a volume containing 1 g of Phenobarbitone Sodium add 15 ml of *water* if necessary, make slightly acidic with *1 M sulphuric acid* and filter. The residue, after washing with *water* and drying at 105°, complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenobarbitone IPRS* or with the reference spectrum of phenobarbitone.

B. Dissolve 50 mg in 2 ml of a 0.2 per cent w/v solution of *cobalt acetate* in *methanol*, warm, add 50 mg of powdered *borax* and heat to boiling; a bluish violet colour is produced.

Tests

pH (2.4.24). 10.0 to 11.0.

Bacterial endotoxins (2.2.3). Not more than 0.3 Endotoxin Unit per mg of phenobarbitone sodium.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Weigh 2 g, add 30 ml of *water* and 3 g of *sodium carbonate*, stir to dissolve and titrate with *0.1 M silver nitrate* until a distinct turbidity is observed when viewed against a black background, the solution being stirred vigorously throughout the titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.02542 g of $C_{12}H_{11}N_2NaO_3$.

Determine the weight per ml of the injection (2.4.29) and calculate the weight in volume of $C_{12}H_{11}N_2NaO_3$.

Storage. Store in single dose containers.

Labelling. The label states that the injection should not be used if the solution is discoloured or if it contains a precipitate.

Phenobarbitone Sodium Tablets

Phenobarbital Sodium Tablets; Soluble Phenobarbitone Tablets; Soluble Phenobarbital Tablets

Phenobarbitone Sodium Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of phenobarbitone sodium, $C_{12}H_{11}N_2NaO_3$.

Usual strengths. 30 mg; 60 mg.

Identification

A. Heat 0.1 g of the residue obtained in Assay on a water-bath with 15 ml of *ethanol (25 per cent)* until dissolved, filter while hot and allow to cool. Filter through a sintered-glass crucible, wash with a small quantity of *ethanol (25 per cent)* and dry at 105°. Heat in a sealed tube at 105° for 1 hour.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenobarbitone IPRS* or with the reference spectrum of phenobarbitone.

B. The residue obtained in test A melts at about 175° (2.4.21).

C. Dissolve 50 mg of the residue obtained in Assay in 2 ml of a 0.2 per cent w/v solution of *cobaltous acetate* in *methanol*, warm, add 50 mg of powdered *borax* and heat to boiling; a bluish violet colour is produced.

D. Triturate a quantity of the powdered tablets containing 0.2 g of Phenobarbitone Sodium with 5 ml of *water* and filter; the filtrate is alkaline to *litmus solution* and yields a white precipitate on the addition of *dilute hydrochloric acid*.

E. The powdered tablets, when moistened with *hydrochloric acid* and introduced on a platinum wire into a flame, impart a yellow colour to the flame.

Tests

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.3 g of Phenobarbitone Sodium, dissolve as completely as possible in 10 ml of a 2 per cent

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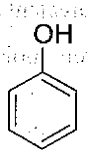
w/v solution of *sodium hydroxide*, saturate with *sodium chloride*, acidify with *hydrochloric acid* and extract with successive quantities, each of 15 ml, of *ether* until complete extraction is effected. Wash the combined extracts with two quantities, each of 2 ml, of *water* and extract the combined washings with 10 ml of *ether*. Add the ether to the main ether layer and dry the residue to constant weight at 105°.

1 g of the residue is equivalent to 1.095 g of $C_{12}H_{11}N_2NaO_3$.

Storage. Store protected from moisture.

Phenol

Carbolic acid



C_6H_6O Mol. Wt. 94.1

Phenol contains not less than 99.0 per cent and not more than 100.5 per cent of C_6H_6O.

Category. Antiseptic; antipruritic; pharmaceutical aid (antimicrobial preservative).

Description. Colourless or faintly pink or faintly yellowish crystals or crystalline masses; deliquescent.

Identification

A. Dissolve 0.5 g in 2 ml of *strong ammonia solution*. Dilute the solution to about 100 ml with *water* and to 2 ml of the resulting solution, add 0.05 ml of 3 per cent w/v solution of *sodium hypochlorite*; a blue colour develops which becomes progressively more intense.

B. Dissolve 1.0 g in sufficient *water* to produce 15 ml (solution A) and to 1 ml, add 10 ml of *water* and 0.1 ml of *ferric chloride solution*; a violet colour is produced which disappears on the addition of 5 ml of *2-propanol*.

C. To 1 ml of solution A add 10 ml of *water* and 1 ml of *bromine solution*; a pale yellow precipitate is produced.

Tests

Appearance of solution. Solution A is clear (2.4.1), and not more intensely coloured than reference solution BS6 (2.4.1).

Acidity. To 2 ml of solution A add 0.05 ml of *methyl orange solution*; the solution is yellow.

Freezing point (2.4.11). Not less than 39.5°.

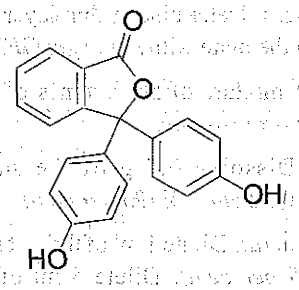
Non-volatile matter. Not more than 0.05 per cent, when 5.0 g is volatilised on a water-bath and dried to constant weight at 105°.

Assay. Weigh 0.5 g and dissolve in sufficient *water* to produce 250.0 ml. Transfer 25.0 ml to a ground-glass-stoppered flask, add 50.0 ml of *0.05 M bromine* and 5 ml of *hydrochloric acid*, stopper, allow to stand for 30 minutes, swirling occasionally, and allow to stand for a further 15 minutes. Add 5 ml of a 20 per cent w/v solution of *potassium iodide*, shake and titrate with *0.1 M sodium thiosulphate* until a faint yellow colour remains. Add 0.5 ml of *starch solution* and 10 ml of *chloroform* and continue the titration with vigorous shaking. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of bromine required.

1 ml of *0.05 M bromine* is equivalent to 0.001569 g of C_6H_6O.

Storage. Store protected from light and moisture.

Phenolphthalein



$C_{20}H_{14}O_4$ Mol. Wt. 318.3

Phenolphthalein is 3,3-bis(4-hydroxyphenyl)phthalide.

Phenolphthalein contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{20}H_{14}O_4$, calculated on the dried basis.

Category. Laxative.

Description. A white or yellowish white, crystalline or amorphous powder.

Identification

A. Dissolves in dilute solutions of alkali hydroxides and in hot solutions of alkali carbonates forming a red solution which is decolorised by dilute acids.

B. Dissolve 25 mg in 100 ml of *ethanol (95 per cent)* (solution A). To 2.0 ml of solution A add 5.0 ml of *1 M hydrochloric acid* and dilute to 50.0 ml with *ethanol (95 per cent)* (solution A_1). To 10.0 ml of solution A add 5.0 ml of *1 M hydrochloric acid*

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and dilute to 50.0 ml with *ethanol (95 per cent)* (solution A_2). To 2.0 ml of solution A add 5.0 ml of *1 M sodium hydroxide* and dilute to 50.0 ml with *ethanol (95 per cent)* (solution B). Examined between 220 nm and 250 nm (2.4.7), solution A_1 shows an absorption maximum at 229 nm. The specific absorbance at the maximum at 229 nm is 922 to 1018. Examined between 250 nm and 300 nm, solution A_2 shows an absorption maximum at 276 nm. The specific absorbance at the maximum at 276 nm is 142 to 158. Examined between 230 nm and 270 nm, solution B shows an absorption maximum at 249 nm. The specific absorbance at the maximum at 249 nm is 744 to 822.

Tests

Solution A. To 2.0 g add 40 ml of *distilled water*, boil, cool and filter.

Appearance of solution. A 4 per cent w/v solution in *ethanol (95 per cent)* is clear (2.4.1) and not more intensely coloured than reference solution YS7 (2.4.1).

Acidity or alkalinity. To 10 ml of solution A, add 0.15 ml of *bromothymol blue solution*. Add 0.05 ml of *0.01 M hydrochloric acid*, the solution is yellow. Add 0.1 ml of *0.01 M sodium hydroxide*, the solution is blue.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 50 volumes of *acetone* and 50 volumes of *dichloromethane*.

Test solution. Dissolve 0.5 g of the substance under examination in 10 ml *ethanol (95 per cent)*.

Reference solution (a). Dilute 1 ml of the test solution to 10 ml with *ethanol (95 per cent)*. Dilute 5 ml of the solution to 100 ml with *ethanol (95 per cent)*.

Reference solution (b). Dissolve 25 mg of *fluorene* in *ethanol (95 per cent)*, add 0.5 ml of the test solution and dilute to 10 ml with *ethanol (95 per cent)*.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air and examine at 254 nm and re-examine after exposure to ammonia vapour. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Chlorides (2.3.12). To 2.5 g, add 40 ml of *water* and heat to boiling. Cool and filter. This solution complies with the limit test for chlorides (100 ppm).

Heavy metals (2.3.13). Heat 5 g with 50 ml of *dilute hydrochloric acid* on a water-bath for 5 min and filter. Evaporate the filtrate almost to dryness and dissolve the residue in 50 ml of *water*. 12 ml of the solution complies with the limit test for heavy metals, Method D (20 ppm). Use 10 ml of *lead standard solution (2 ppm Pb)* to prepare the standard.

Sulphates (2.3.17). 15 ml of solution A complies with the limit test for sulphates (200 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

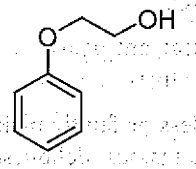
Assay. Dissolve 0.1 g in 5 ml of *dimethylformamide*. Add 5 ml of *sodium carbonate solution*, 10 ml of *sodium hydrogen carbonate solution*, 35 ml of *water* and 50.0 ml of *0.05 M iodine*. Add 10 ml of *dichloromethane* and 20 ml of *dilute sulphuric acid*. Titrate the excess of iodine with *0.1 M sodium thiosulphate*, using 0.3 ml of *starch solution* added towards the end of the titration, as indicator. Carry out a blank titration.

1 ml of *0.05 M iodine* is equivalent to 0.003979 g of $C_{20}H_{14}O_4$.

Storage. Store protected from moisture.

Phenoxyethanol

2-Phenoxyethanol



$C_8H_{10}O_2$ Mol. Wt.138.2

Phenoxyethanol is 1-hydroxy-2-phenoxyethane.

Phenoxyethanol contains not less than 99.0 per cent and not more than 100.5 per cent of $C_8H_{10}O_2$.

Category. Pharmaceutical aid.

Description. A colourless, slightly viscous liquid.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenoxyethanol IPRS* or with the reference spectrum of phenoxyethanol.

B. When examined in the range 240 nm to 350 nm (2.4.7), a 0.008 per cent w/v solution in *water*, shows two absorption maxima at 269 nm and 275 nm and specific absorbance at 269 nm is 95 to 105 and at 275 nm is 75 to 85.

C. Shake 2 ml of Phenoxyethanol with a mixture of 4.0 g of *potassium permanganate*, 5.4 g of *sodium carbonate* and

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75 ml of *water* for 30 minutes. Add 25 g of *sodium chloride* and stir continuously for 60 minutes, filter and adjusted to pH 1.7 with *hydrochloric acid*. The melting point of the precipitate, after recrystallisation from *water* is 96° to 99°.

Tests

Refractive index (2.4.27). 1.537 to 1.539

Relative density (2.4.29). 1.105 to 1.110

Related substances. Determine by gas chromatography (2.4.13).

Test solution. Dissolve 5 g of the substance under examination in *ethanol* and dilute to 20.0 ml with *ethanol*.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *ethanol*.

Reference solution (b). Dissolve 25 mg of *phenol (impurity A)* in 10.0 ml of *ethanol*.

Reference solution (c). Dilute 1.0 ml, each of, reference solution (a) and reference solution (b) to 10.0 ml with *ethanol*.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 10.0 ml with *ethanol*.

Chromatographic system

- a fused-silica column 30 m x 0.25 mm, packed with macrogol 20000 (0.25 µm),
- temperature:

column	time (in min)	temperature (°)
	0-13	90 → 220
	13-23	220

- inlet port at 250° and detector at 270°,
- a flame-ionisation detector,
- flow rate: 1 ml per minute, using nitrogen as the carrier gas,
- injection volume: 1 µl,
- split ratio: 1:100.

The retention time of phenoxyethanol is about 13 minutes.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to phenol and phenoxyethanol is not less than 15.

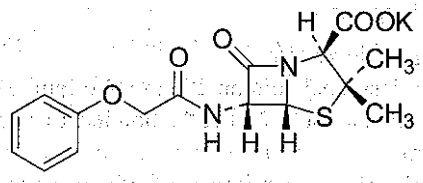
Inject reference solution (d) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of peak in the chromatogram obtained with reference solution (d) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 3 times the area of the peak in the chromatogram obtained with reference solution (d) (0.3 per cent). Ignore any peak with an area less than 0.5 times the area of the peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

Assay. Dissolve 2 g in 10.0 ml of freshly prepared *acetic anhydride solution*, heat in a water bath for 45 minutes. Cool, add 10.0 ml of *water*, further heat for 2 minutes, cool and add 10.0 ml of *butanol*, shake vigorously and titrate excess of *acetic acid* with *1 M sodium hydroxide* using 0.2 ml of *phenolphthalein* solution as indicator. Carry out a blank titration.

1 ml of *1 M sodium hydroxide* is equivalent to 0.1382 g of $C_8H_{10}O_2$.

Phenoxymethylpenicillin Potassium

Penicillin V Potassium



$C_{16}H_{17}KN_2O_5S$ Mol. Wt. 388.5

Phenoxymethylpenicillin Potassium is potassium (6*R*)-6-(2-phenoxyacetamido)penicillinate, produced by the growth of certain strains of *Penicillium notatum* or related organisms on a culture medium containing an appropriate precursor, or obtained by any other means.

Phenoxymethylpenicillin Potassium contains not less than 86.0 per cent of total penicillins $C_{16}H_{17}N_2O_5S$, calculated on the anhydrous basis.

Category. Antibacterial.

Description. A white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Test B may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenoxymethylpenicillin potassium IPRS* or with the reference spectrum of phenoxymethylpenicillin potassium.

B. It gives reaction (B) of penicillins and cephalosporins (2.3.1).

C. It gives reaction (A) of potassium salts (2.3.1).

Tests

pH (2.4.24). 5.5 to 7.5, determined in a 0.5 per cent w/v solution.

Specific optical rotation (2.4.22). + 215.0° to + 230.0°, determined in a 1.0 per cent w/v solution in *carbon dioxide-free water*.

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Light absorption (2.4.7). Absorbance of a 0.1 per cent w/v solution in *0.1 M sodium hydroxide* at the maximum at about 306 nm, not more than 0.33. Absorbance of a 0.02 per cent w/v solution in *0.1M sodium hydroxide* at the maximum at about 274 nm, not less than 0.50.

Phenoxyacetic acid. Not more than 0.5 per cent.

Determine by liquid chromatography (2.4.14).

Test solution. Weigh a suitable quantity of the substance under examination, dissolve in *phosphate buffer pH 6.6* and dilute to obtain a solution having a known concentration of about 20 mg per ml.

Reference solution. Weigh a suitable quantity of phenoxyacetic acid, dissolve in the *phosphate buffer pH 6.6* and dilute to obtain a solution having a known concentration of about 0.1 mg per ml.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane chemically bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of *water*, 35 volumes of *acetonitrile* and 1 volume of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of phenoxyacetic acid.

Limit of *p*-hydroxyphenoxymethylpenicillin. Not more than 5.0 per cent.

Using the chromatograms obtained with the test solution in the Assay, calculate the content of *p*-hydroxyphenoxymethylpenicillin from the peak response of *p*-hydroxyphenoxymethylpenicillin and the sum of the peak responses of *p*-hydroxyphenoxymethylpenicillin and phenoxymethylpenicillin.

Water (2.3.43). Not more than 1.0 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 125 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve a weighed quantity of *phenoxymethylpenicillin potassium IPRS* in the mobile phase and dilute to obtain a solution having a known concentration of about 2.5 mg per ml.

Reference solution (b). A solution in the mobile phase containing 0.25 per cent w/v each of benzylpenicillin potassium and phenoxymethylpenicillin potassium.

Chromatographic system

- a stainless steel column 30 cm x 4 mm, packed with octadecylsilane chemically bonded to porous silica (3 to 10 µm),
- mobile phase: a mixture of 65 volumes of *water*, 35 volumes of *acetonitrile* and 5.75 volumes of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Inject reference solution (b).The relative retention times are about 0.8 for benzylpenicillin and 1.0 for phenoxymethylpenicillin. The column efficiency determined from the phenoxymethylpenicillin peak is not less than 1800 theoretical plates and the resolution between benzylpenicillin and phenoxymethylpenicillin is not less than 3.0.

Inject reference solution (a). The relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject reference solution (a) and the test solution. Record the chromatograms and measure the responses for the phenoxymethylpenicillin peak and any *p*-hydroxyphenoxymethylpenicillin peak with a retention time of about 0.4 relative to that of the main phenoxymethylpenicillin peak.

Calculate the content of $C_{16}H_{17}N_2O_5S$, from the sum of the peak responses of the *p*-hydroxyphenoxymethylpenicillin and phenoxymethylpenicillin peaks in the chromatograms obtained with the test solution and reference solution (a).

Storage. Store protected from moisture.

Phenoxymethylpenicillin Potassium Tablets

Phenoxymethylpenicillin Tablets; Penicillin V Potassium Tablets; Penicillin V Tablets

Phenoxymethylpenicillin Potassium Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of phenoxymethylpenicillin, $C_{16}H_{18}N_2O_5S$.

Usual strengths. The equivalent of 62.5 mg, 125 mg, 250 mg and 500 mg of phenoxymethylpenicillin.

Identification

A. Shake a quantity of the powdered tablets containing 80 mg of phenoxymethylpenicillin with *water*, dilute to 250 ml with *water* and filter. When examined between 230 and 360 nm

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(2.4.7), the filtrate shows absorption maxima at about 268 nm and 274 nm and a minimum at about 272 nm.

B. Shake a quantity of the powdered tablets containing 10 mg of phenoxymethylpenicillin with 10 ml of *water*, filter and add 0.5 ml of *neutral red solution*. Add sufficient *0.01 M sodium hydroxide* to produce a permanent orange colour and then add 1.0 ml of *penicillinase solution*; the solution changes rapidly to red.

C. Ignite 0.5 g of the powdered tablets, add 5 ml of *2 M hydrochloric acid*, boil, cool and filter. The filtrate gives reaction (B) of potassium salts (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted if necessary, at the maximum at about 268 nm (2.4.7). At the same time measure the absorbance of a solution of known concentration of *phenoxymethylpenicillin potassium IPRS* at the maximum at about 268 nm. Calculate the content of $C_{16}H_{18}N_2O_5S$, in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{16}H_{18}N_2O_5S$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and finely powder 20 tablets. Dissolve a weighed quantity of the powder containing about 0.25 g of phenoxymethylpenicillin in the mobile phase by shaking for 5 minutes and dilute to 100.0 ml with the mobile phase. Filter through a 0.5 µm or finer filter and use the filtrate.

Reference solution (a). Dissolve a weighed quantity of *phenoxymethylpenicillin potassium IPRS* in the mobile phase and dilute to obtain a solution having a known concentration of about 2.5 mg per ml.

Reference solution (b). A solution in the mobile phase containing 0.25 per cent w/v each of benzylpenicillin potassium and phenoxymethylpenicillin potassium.

Chromatographic system
- a stainless steel column 30 cm x 4 mm, packed with octadecylsilane chemically bonded to porous silica,
- mobile phase: a mixture of 650 volumes of *water*, 350 volumes of *acetonitrile* and 5.75 volumes of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

The relative retention times are about 0.8 for benzylpenicillin and 1.0 for phenoxymethyl-penicillin.

Inject reference solution (b). The test is not valid unless the column efficiency determined from the phenoxymethylpenicillin peak is not less than 1800 theoretical plates and the resolution between the benzylpenicillin and phenoxymethylpenicillin peaks is not less than 3.0.

Inject reference solution (a). The relative standard deviation for replicate injections is not more than 1.0 per cent.

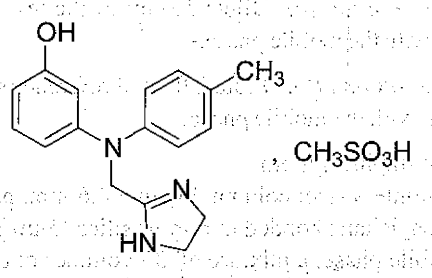
Inject reference solution (a) and the test solution. Record the chromatograms and measure the responses for the phenoxymethylpenicillin peak and any *p*-hydroxyphenoxymethylpenicillin peak with a retention time of about 0.4 relative to that of the main phenoxymethylpenicillin peak.

Calculate the content of $C_{16}H_{17}N_2O_5S$, from the sum of the peak responses of the *p*-hydroxyphenoxymethylpenicillin and phenoxymethylpenicillin peaks in the chromatograms obtained with the test solution and reference solution (a).

Storage. Store protected from moisture.

Labelling. The label states the strength in terms of the equivalent amount of phenoxymethylpenicillin.

Phentolamine Mesylate



$C_{17}H_{19}N_3O,CH_4O_3S$ Mol. Wt. 377.5

Phentolamine Mesylate is 3-[[4,5-dihydro-1*H*-imidazol-2-yl)methyl](4-methylphenyl)aminophenol methanesulphonate.

Phentolamine Mesylate contains not less than 99.0 per cent and not more than 100.5 per cent of $C_{17}H_{19}N_3O$, CH_4O_3S, calculated on the dried basis.

Category. Alpha-adrenoceptor antagonist.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phentolamine mesylate IPRS* or with the reference spectrum of phentolamine mesylate.

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B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution shows an absorption maximum only at about 278 nm; absorbance at about 278 nm, about 0.5.

C. Dissolve 0.5 g in 5 ml of *ethanol (95 per cent)* and 5 ml of *0.1 M hydrochloric acid* and add 2 ml of a 0.5 per cent w/v solution of *ammonium metavanadate*; a light green precipitate is produced.

D. Mix 50 mg with 0.2 g of powdered *sodium hydroxide*, heat to fusion and continue the heating for a few seconds longer. Cool, add 0.5 ml of *water* and a slight excess of *2 M hydrochloric acid* and warm; *sulphur dioxide* is evolved, which turns moistened starch iodate paper blue.

Tests

Acidity or alkalinity. Dissolve 0.1 g in 10 ml of *carbon dioxide-free water* and add 0.1 ml of *methyl red solution*. The solution is not red and not more than 0.05 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Prepare the solutions immediately before use.

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with phenylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 33 volumes of *acetonitrile* and 67 volumes of a 0.05 per cent w/v solution of *ammonium acetate* previously adjusted to pH 5.9 with *dilute acetic acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 10 µl.

Name	Relative retention time	Correction factor
Phentolamine impurity A[1]	0.7	1.7
Phentolamine (Retention time: about 15 minutes)	1.0	—
Phentolamine impurity C[2]	1.2	—

[1] *N*-(2-aminoethyl)-2-[(3-hydroxyphenyl)(4-methylphenyl)amino] acetamide,

[2] 3-[(4-methylphenyl)amino]phenol.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to phentolamine impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any other secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.3 g in 100 ml of *anhydrous 2-propanol* with the aid of ultrasound if necessary. Titrate with *0.1 M tetrabutylammonium hydroxide* in *2-propanol*. Determine the end-point potentiometrically (2.4.25), using a glass electrode and a calomel electrode containing a saturated solution of *tetramethylammonium chloride* in *2-propanol*. Carry out a blank titration.

1 ml of *0.1 M tetrabutylammonium hydroxide* is equivalent to 0.03775 g of $C_{17}H_{19}N_3O, CH_4O_3S$.

Storage. Store protected from light and moisture.

Phentolamine Injection

Phentolamine Mesylate Injection; Phentolamine Mesilate Injection

Phentolamine Injection is a sterile solution of Phentolamine Mesylate in Water for Injections containing anhydrous Glucose.

Phentolamine Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of phentolamine mesylate, $C_{17}H_{19}N_3O, CH_4O_3S$.

Usual strength. 10 mg per ml.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

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Tests

pH (2.4.24). 3.5 to 5.0.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dilute the injection with the mobile phase to obtain a solution containing 0.05 per cent w/v of Phentolamine Mesylate.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 20.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with phenylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 33 volumes of *acetonitrile* and 67 volumes of a 0.05 per cent w/v solution of *ammonium acetate* previously adjusted to pH 5.9 with *dilute acetic acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 10 µl.

Name	Relative retention time	Correction factor
Phentolamine impurity A[1]	0.7	1.7
Phentolamine (Retention time: about 15 minutes)	1.0	—
Phentolamine impurity C[2]	1.2	—

[1]*N*-(2-aminoethyl)-2-[(3-hydroxyphenyl)(4-methylphenyl)amino] acetamide,

[2]3-[(4-methylphenyl)amino]phenol.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (a), (b) and the test solution. Run the chromatogram 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to phentolamine impurity A is not more than 2.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.2 per cent), the area of any other secondary peak is not more than 1.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.2 per cent) and the sum of areas of all the secondary peaks is not more than 4.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (4.2 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Bacterial endotoxins (2.2.3). Not more than 5.8 Endotoxin Units per mg of phentolamine mesylate.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute the injection with the mobile phase to obtain a solution containing 0.01 per cent w/v of Phentolamine Mesilate.

Reference solution. A 0.01 per cent w/v solution of *phentolamine mesilate IPRS* in the mobile phase.

Use chromatographic system as described under Related substances.

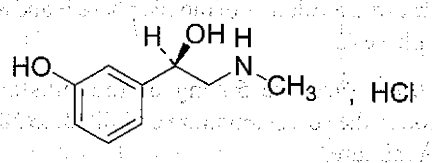
Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{19}N_3O,CH_4SO_3$.

Storage. Store protected from light.

Phenylephrine Hydrochloride



$C_9H_{13}NO_2,HCl$ Mol. Wt. 203.7

Phenylephrine Hydrochloride is (*R*)-1-(3-hydroxyphenyl)-2-methylaminoethanol hydrochloride.

Phenylephrine Hydrochloride contains not less than 98.5 per cent and not more than 101.0 per cent of $C_9H_{13}NO_2$, HCl, calculated on the dried basis.

Category. Sympathomimetic.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenylephrine hydrochloride IPRS* or with the reference spectrum of phenylephrine hydrochloride.

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B. Dissolve about 10 mg in 1 ml of *water* and add 0.05 ml of *cupric sulphate solution* and 1 ml of *5 M sodium hydroxide*; a violet colour is produced. Add 1 ml of *ether* and shake; the ether layer remains colourless.

C. Dissolve 0.3 g in 3 ml of *water*, add 1 ml of *6 M ammonia* and initiate crystallisation by scratching the side of the tube with a glass rod. The melting range of the crystals, after washing with iced *water* and drying at 105° for 2 hours, is 171° to 176° (2.4.21).

D. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. Dissolve 2.0 g in 100 ml of *carbon dioxide-free water* prepared from *distilled water* (solution A). Solution A is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. To 10 ml of solution A add 0.1 ml of *methyl red solution* and 0.2 ml of *0.01 M sodium hydroxide*. The solution is yellow and not more than 0.4 ml of *0.01 M hydrochloric acid* is required to change the colour of the solution to red.

Specific optical rotation (2.4.22). −47.0° to −43.0°, determined in solution A.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 20 volumes of mobile phase B and 80 volumes of mobile phase A.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution. Dilute 5.0 ml of the test solution to 100.0 ml with the solvent mixture. Further dilute 2.0 ml of the solution to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 5.5 cm x 4.0 mm, packed with endcapped octadecylsilane bonded to porous silica (3 µm),
- column temperature: 45°,
- mobile phase: A. a mixture of 10 volumes of *acetonitrile* and 90 volumes of buffer solution prepared by dissolving 3.25 g of *sodium octanesulphonate monohydrate* in 1000 ml of *water* by stirring for 30 minutes, adjusted to pH 2.8 with *dilute orthophosphoric acid*,
  B. a mixture of 10 volumes of buffer solution and 90 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	93	7
3	93	7
13	70	30
14	93	7

Name	Relative retention time	Correction factor
Phenylephrine (Retention time: about 2.8 minutes)	1.0	—
Phenylephrine impurity C[1]	1.3	0.5
Phenylephrine impurity E[2]	3.6	0.5

[1]phenylephrone,

[2]benzylphenylephrone.

Inject the test solution. The test is not valid unless the tailing factor is not more than 1.9 for the principal peak.

Inject the reference solution and the test solution. In the chromatogram obtained the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Sulphates (2.3.17). 15 ml of solution A complies with the limit test for sulphates (500 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.15 g in a mixture of 0.5 ml of *0.1 M hydrochloric acid* and 80 ml of *ethanol (95 per cent)* and titrate with *0.1 M ethanolic sodium hydroxide* determining the end-point potentiometrically (2.4.25). Record the volume added between the two inflections.

1 ml of *0.1 M ethanolic sodium hydroxide* is equivalent to 0.02037 g of $C_9H_{13}NO_2,HCl$.

Storage. Store protected from light and moisture.

Phenylephrine Eye Drops

Phenylephrine Hydrochloride Eye Drops

Phenylephrine Eye Drops is a sterile solution of Phenylephrine Hydrochloride in purified water.

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Phenylephrine Eye Drops Contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of phenylephrine hydrochloride, $C_9H_{13}NO_2$,HCl.

Usual strengths. 2.5 per cent w/v; 5 per cent w/v; 10 per cent w/v.

Identification

A. In the test for Related substances, the principal spot in the chromatogram obtained with reference solution (a) corresponds to that in the chromatogram obtained with reference solution (c).

B. Dissolve about 10 mg in 1 ml of *water* and add 0.05 ml of *cupric sulphate solution* and 1 ml of *5 M sodium hydroxide*; a violet colour is produced. Add 1 ml of *ether* and shake; the ether layer remains colourless.

Tests

pH (2.4.24). 3.0 to 7.5.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H*.

Mobile phase. A mixture of 5 volumes of *chloroform*, 15 volumes of *10 M ammonia solution* and 80 volumes of *propan-2-ol*.

Test solution. Dilute the eye drop with *methanol* to obtained a solution containing 2.0 per cent w/v of Phenylephrine Hydrochloride.

Reference solution (a). Dilute 1 volume of the eye drops to 40 volumes with *methanol*.

Reference solution (b). Dilute 1 volume of the eye drops to 100 volumes with *methanol*.

Reference solution (c). A 0.05 per cent w/v solution of *phenylephrine hydrochloride IPRS* in *methanol*.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 15 cm. After development, dry the plate in a current of cold air and spray with *ninhydrin solution*. Heat the plate at 100° to 105° for 5 to 10 minutes and examine in daylight. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent).

Other tests. Comply with the tests stated under Eye Drops.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute suitable volume of the eye drops with *water* to obtain a solution containing 0.1 per cent w/v of Phenylephrine Hydrochloride.

Reference solution. A 0.1 per cent w/v solution of *phenylephrine hydrochloride IPRS* in the *water*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with end capped octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil BDS C18),
- mobile phase: a solution prepared by dissolving 0.1 per cent w/v of *sodium heptanesulphonate* in a mixture of 2 volumes of *glacial acetic acid*, 400 volumes of *methanol* and 600 volumes of *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 273 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_9H_{13}NO_2$,HCl in the eye drop.

Storage. Store protected from light.

Phenylephrine Injection

Phenylephrine Hydrochloride Injection

Phenylephrine Injection is a sterile solution of Phenylephrine Hydrochloride in Water for Injections.

Phenylephrine Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of phenylephrine hydrochloride, $C_9H_{13}NO_2$,HCl.

Usual strength. 10 mg per ml.

Identification

A. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (b).

B. To a volume containing 10 mg of Phenylephrine Hydrochloride add, if necessary, sufficient *water* to produce 1 ml and then add 0.05 ml of *cupric sulphate solution* and 1 ml of *5 M sodium hydroxide*; a violet colour is produced. Add 1 ml of *ether* and shake; the ether layer remains colourless.

C. It gives reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 4.5 to 6.5.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H*.

Mobile phase. A mixture of 80 volumes of *2-propanol*, 15 volumes of *10 M ammonia* and 5 volumes of *chloroform*.

Test solution (a). Evaporate a volume of the injection containing 20 mg of Phenylephrine Hydrochloride to dryness and dissolve the residue in 1 ml of *methanol*.

Test solution (b). Dilute 1 volume of test solution (a) to 200 volumes with *methanol*.

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Reference solution (a). Dilute 1 volume of test solution (b) to 2.5 volumes with *methanol*.

Reference solution (b). A 0.01 per cent w/v solution of *phenylephrine hydrochloride IPRS* in *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in cold air, spray with *ninhydrin solution*, heat at 105° for 10 minutes and examine in daylight. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with test solution (b) and not more than two such spots are more intense than the spot in the chromatogram obtained with reference solution (a).

Bacterial endotoxins (2.2.3). Not more than 25.0 Endotoxin Units per mg of phenylephrine.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. To a measured volume containing about 50 mg of Phenylephrine Hydrochloride add sufficient *0.5 M sulphuric acid* to produce 100.0 ml. Dilute 10.0 ml of the solution to 100.0 ml with *0.5 M sulphuric acid* and measure the absorbance of the resulting solution at the maximum at about 273 nm (2.4.7). Calculate the content of $C_9H_{13}NO_2$,HCl taking 90 as the specific absorbance at 273 nm.

Storage. Store protected from light.

Phenylephrine Hydrochloride and Chlorpheniramine Maleate Drops

Phenylephrine Hydrochloride and Chlorpheniramine Maleate Oral Drops contains phenylephrine hydrochloride and chlorpheniramine maleate, in a suitable vehicle.

Phenylephrine Hydrochloride and Chlorpheniramine Maleate Oral Drops contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of phenylephrine hydrochloride, $C_9H_{13}NO_2$,HCl and chlorpheniramine Maleate, $C_{16}H_{19}ClN_2$,$C_4H_4O_4$.

Usual Strength. Phenylephrine Hydrochloride 5 mg and Chlorpheniramine Maleate 2 mg per ml.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.0 to 5.0.

Other tests. Comply with the tests stated under Oral liquids.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Transfer a volume of oral drops containing 25 mg of Phenylephrine Hydrochloride to a 250-ml volumetric flask and dilute to volume with the mobile phase and filter.

Reference solution. A 0.01 per cent w/v solution of *phenylephrine hydrochloride IPRS* and 0.004 per cent w/v solution of *chlorpheniramine maleate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with nitrile groups bonded to porous silica (5 µm) (Such as Phenomenex CN),
- mobile phase: a mixture of 45 volumes of a buffer solution prepared by dissolving 0.462 g of *diammonium hydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 4.5 with *orthophosphoric acid solution* and 55 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Storage. Store protected from light.

Phenylephrine Hydrochloride and Chlorpheniramine Maleate Syrup

Phenylephrine Hydrochloride and Chlorpheniramine Maleate Syrup contains phenylephrine hydrochloride and chlorpheniramine maleate in a suitable flavoured vehicle.

Phenylephrine Hydrochloride and Chlorpheniramine Maleate Syrup contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of phenylephrine hydrochloride, $C_9H_{13}NO_2$,HCl and chlorpheniramine Maleate, $C_{16}H_{19}ClN_2$,$C_4H_4O_4$.

Usual Strength. Phenylephrine Hydrochloride 5 mg and Chlorpheniramine Maleate 2 mg per 5 ml.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.0 to 5.0.

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Other tests. Comply with the tests stated under Oral liquids.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of syrup containing 5 mg of Phenylephrine Hydrochloride in 40 ml of the mobile phase with the aid of ultrasound for 15 minutes, further dilute to 50.0 ml with the mobile phase and filter.

Reference solution. A 0.01 per cent w/v solution of *phenylephrine hydrochloride IPRS* and 0.004 per cent w/v solution of *chlorpheniramine maleate IPRS* in the mobile phase.

Chromatographic system

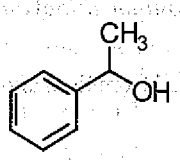
- a stainless steel column 25 cm x 4.6 mm, packed with nitrile groups bonded to porous silica (5 µm) (Such as Phenomenex CN),
- mobile phase: a mixture of 45 volumes of a buffer solution prepared by dissolving 0.462 g of *diammonium hydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 4.5 with *orthophosphoric acid solution* and 55 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Storage. Store protected from light.

Phenylethyl Alcohol



$C_8H_{10}O$ Mol. Wt. 122.2

Phenylethyl Alcohol is 1-Phenylethanol.

Category. Pharmaceutical aid.

Description. A clear, colourless liquid.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenylethyl alcohol IPRS* or with reference spectrum of phenylethyl alcohol.

Tests

Specific gravity (2.4.29). 1.017 to 1.02.

Refractive index (2.4.27). 1.531 to 1.534 at 20°.

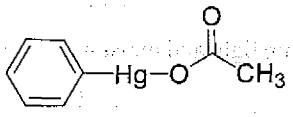
Sulphated ash (2.3.18). Not more than 0.005 per cent, by evaporating 10 ml in a suitable crucible and ignite to constant weight.

Aldehyde. Shake 5 ml with 5 ml of *1 M sodium hydroxide* and allow to stand for 1 hour; no yellow colour apprears in the organic layer.

Chlorinated compounds. Wind a 1.5 cm x 5 cm strip of 20-mesh copper gauze around the end of a copper wire. Heat the gauze in the nonluminous flame of a bunsen burner until it glows without colouring the flame green. Permit the gauze to cool, and heat several times until a good coat of oxide has formed. Apply with a medicine dropper 2 drops of *phenylethyl alcohol* to the cooled gauze, ignite, and permit it to burn freely in the air. Again cool the gauze, add 2 more drops of *phenylethyl alcohol* and burn as before. Continue this process until a total of 6 drops has been added and ignited, and then hold the gauze in the outer edge of the bunsen flame, adjusted to a height of about 4 cm; no transient green colour or other colour is imparted to the flame.

Storage. Store protected from light and moisture, at a temperature between 8° to 25°.

Phenylmercuric Acetate



$C_8H_8HgO_2$ Mol. Wt. 336.7

Phenylmercuric Acetate is (acetato)phenylmercury.

Phenylmercuric Acetate contains not less than 98.0 per cent and not more than 100.5 per cent of $C_8H_8HgO_2$.

Category. Pharmaceutical aid (antimicrobial preservative).

Description. A white to creamy white, crystalline powder, or small white prisms or leaflets.

Identification

A. To 100 mg add 0.5 ml of *nitric acid*, warm gently until a dark brown colour is produced and dilute with *water* to 10 ml; the characteristic odour of *nitrobenzene* is produced.

B. To 100 mg add 0.5 ml of *sulphuric acid* and 1 ml of *ethanol (95 per cent)* and warm; the characteristic odour of *ethyl acetate* is produced.

C. To 5 ml of a saturated solution in *water*, add a few drops of a freshly prepared 10 per cent w/v solution of *sodium sulphide*;

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a white precipitate is formed which darkens slowly on boiling and allowing to stand.

Tests

Mercuric salts and heavy metals. Heat about 100 mg with 15 ml of *water*, cool and filter. To the filtrate add a few drops of a freshly prepared 10 per cent w/v solution of *sodium sulphide*; the precipitate formed shows no immediate colour.

Polymercurated benzene compounds. Shake 2.0 g with 100 ml of *acetone*. Filter, wash the residue with successive portions of *acetone* until a total of 50 ml is used, dry the residue at 105° for 1 hour and weigh. The weight of the residue does not exceed 30 mg (1.5 per cent).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Assay. Weigh 0.4 g, transfer to a 100-ml flask, add 15 ml of *water*, 5 ml of *formic acid* and 1 g of *zinc dust* and reflux for 30 minutes. Cool, filter and wash the filter paper and the amalgam with *water* until the washings are no longer acidic to litmus. Dissolve the amalgam in 40 ml of *8 M nitric acid*. Heat on a *water*-bath for 3 minutes and add 0.5 g *urea* and sufficient *0.02 M potassium permanganate* to produce a permanent pink colour. Cool and add *hydrogen peroxide solution* to decolorise the solution. Add 1 ml of *ferric ammonium sulphate solution* and titrate with *0.1 M ammonium thiocyanate*. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of ammonium thiocyanate required.

1 ml of *0.1 M ammonium thiocyanate* is equivalent to 0.01684 g of $C_8H_8HgO_2$.

Storage. Store protected from light and moisture.

Phenylmercuric Nitrate

Phenylmercuric Nitrate is a mixture of phenylmercuric nitrate, $C_6H_5HgNO_3$ and phenylmercuric hydroxide, C_6H_5HgOH.

Phenylmercuric Nitrate contains not less than 62.5 per cent and not more than 63.5 per cent of mercury, Hg, calculated on the dried basis.

Category. Antiseptic; pharmaceutical aid (antimicrobial preservative).

Description. A white or pale yellow powder.

Identification

A. To 0.1 g add 3 ml of *sulphuric acid*; the mixture becomes yellow and the characteristic odour of *nitrobenzene* is produced.

B. To 0.1 g add 45 ml of *water* and heat to boiling with shaking. Cool, filter and add sufficient *water* to produce 50 ml (solution A). To 1 ml of solution A add 1 ml of *2 M hydrochloric acid*; a white, flocculent precipitate is produced.

C. To 5 ml of solution A add 8 ml of *water* and 0.1 ml of a freshly prepared 10 per cent w/v solution of *sodium sulphide*; a white precipitate is formed which darkens slowly on boiling and allowing to stand.

D. To 5 ml of solution A add 1 ml of *2 M hydrochloric acid*, 2 ml of *dichloromethane* and 0.2 ml of *dithizone solution* and shake; the lower layer is orange-yellow.

E. Solution A gives reaction of nitrates (2.3.1).

Tests

Appearance of solution. Solution A is colourless (2.4.1).

Inorganic mercuric compounds. To a 10 ml of solution A add 2 ml of *potassium iodide solution* and 3 ml of *2 M hydrochloric acid* and filter; the filtrate is colourless. Wash the precipitate with 2 ml of *water*, combine the filtrate and washings and add 2 ml of *2 M sodium hydroxide* and sufficient *water* to produce 20 ml. 12 ml of the solution complies with the limit test for heavy metals, Method D (2.3.13). Use 10 ml of *lead standard solution (1 ppm Pb)* to prepare the standard (0.1 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying over *phosphorus pentoxide* at a pressure of 1.5 to 2.5 kPa for 24 hours.

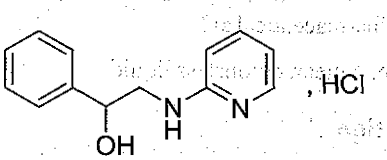
Assay. Weigh 0.2 g and dissolve in a mixture of 90 ml of *water* and 10 ml of *nitric acid*. Add 2 ml of *ferric ammonium sulphate solution* and titrate with *0.1 M ammonium thiocyanate* until a persistent reddish yellow colour is obtained. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of *ammonium thiocyanate* required.

1 ml of *0.1 M ammonium thiocyanate* is equivalent to 0.02006 g of Hg.

Storage. Store protected from light and moisture.

Phenyramidol Hydrochloride

Fenyramidol Hydrochloride



$C_{13}H_{14}N_2O,HCl$ Mol. Wt. 250.7

Phenyramidol Hydrochloride is 1-Phenyl-2-(2-pyridylamino) ethanol hydrochloride.

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Phenyramidol Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{13}H_{14}N_2O$,HCl.

Category. Muscle Relaxant; Analgesic.

Description. A white to cream coloured crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with the reference spectrum of *phenyramidol hydrochloride IPRS* or with the reference spectrum of phenyramidol hydrochloride.

B. A 0.04 per cent w/v solution of the substance under examination in *water*. To 5 ml of the solution, add 20 ml of *1 M hydrochloric acid* and dilute to 200 ml with *water* and filter. When examined in the range 200 nm to 400 nm (2.4.7), shows two maxima at 238 nm and 310 nm.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution. A 0.001 per cent w/v solution of *phenyramidol hydrochloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Zorbax C18),
- column temperature: 35°,
- mobile phase: a mixture of 900 volumes of *water*, 1 volume of *orthophosphoric acid* and 100 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 306 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the sum of areas of all the secondary peaks is not more than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent).

Light absorption. The absorbance of 10 per cent w/v solution at 450 nm (2.4.7) is not more than 0.02.

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.4.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 2.0 g by drying in an oven for 3 hours at 105°.

Assay. Dissolve 0.24 g in 50 ml *acetic acid*, add 10 ml of *mercuric acetate* solution. Titrate with *0.1 M perchloric acid*. Determine the end point potentiometrically (2.4.25). Carry out the blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02507 g of $C_{13}H_{14}N_2O$,HCl.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Phenyramidol Tablets

Phenyramidol Hydrochloride Tablets

Phenyramidol Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of phenyramidol hydrochloride, $C_{13}H_{14}N_2O$,HCl.

Usual strength. 400 mg.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Dissolve a weighed quantity of the powdered tablet containing 0.1 g of Phenyramidol Hydrochloride in *water* and dilute to 250 ml with *water*. To 5 ml of the solution, add 20 ml of *1 M hydrochloric acid* and dilute to 200 ml with *water* and filter. When examined in the range 200 nm to 400 nm (2.4.7), shows two maxima at 238 nm and 310 nm.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Dilute 5.0 ml of the filtrate to 50.0 ml with the mobile phase.

Reference solution. A 0.04 per cent w/v solution of *phenyramidol hydrochloride IPRS* in the dissolution medium. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

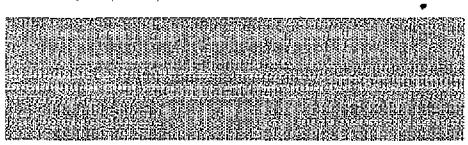
Use the chromatographic system as described under Assay.

Calculate the content of $C_{13}H_{14}N_2O$,HCl in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{13}H_{14}N_2O$.HCl.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).



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Test solution. Weigh and powder 20 tablets. Dissolve a quantity of powder containing 0.05 g of Phenyramidol Hydrochloride in *water* and dilute to 50.0 ml of *water*. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.1 per cent w/v solution of *phenyramidol hydrochloride IPRS* in *water*. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 1 volume of *triethylamine* to 600 volumes of *methanol* and 400 volumes of *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 309 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

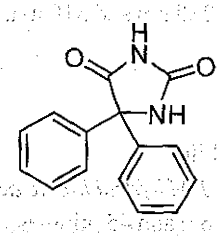
Inject the reference solution and the test solution.

Calculate the content of $C_{13}H_{14}N_2O,HCl$ in the tablets.

Storage. Store protected from light and moisture.

Phenytoin

Diphenylhydantoin



$C_{15}H_{12}N_2O_2$ Mol.Wt. 252.3

Phenytoin is 5,5-diphenylimidazolidine-2,4-dione.

Phenytoin contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{15}H_{12}N_2O_2$, calculated on the dried basis.

Category. Anticonvulsant.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenytoin IPRS* or with the reference spectrum of phenytoin.

B. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a).

C. To about 10 mg, add 1 ml of *water* and 0.05 ml of *ammonia*. Heat until boiling begins. Add 0.05 ml of a 5 per cent w/v solution of *copper sulphate* in *dilute ammonia* and shake; a pink crystalline precipitate is formed.

Tests

Appearance of solution. Dissolve 1.0 g in a mixture of 5 ml of *1 M sodium hydroxide* and 20 ml of *water*. The solution is clear (2.4.1) and not more intensely coloured than reference solution BYS5 (2.4.1).

Acidity or alkalinity. To 1.0 g, add 45 ml of *water* and boil for 2 minutes. Allow to cool and filter. Wash the filter with *carbon dioxide-free water* and dilute the combined filtrate and washings to 50 ml with the same solvent. To 10 ml of the solution add 0.15 ml of *methyl red solution*; not more than 0.5 ml of *0.01 M hydrochloric acid* is required to change the colour of the solution to red. To 10 ml of the solution add 0.15 ml of *bromothymol blue solution*; not more than 0.5 ml of *0.01 M sodium hydroxide* is required to change the colour of the solution to blue.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). A 0.01 per cent w/v solution of *phenytoin IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 20 volumes of *methanol*, 35 volumes of *acetonitrile* and 45 volumes of a 0.58 per cent w/v solution of *ammonium dihydrogen phosphate*, adjusted to pH 2.5 with *orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Phenytoin impurity C[1]	0.5	---
Phenytoin impurity D[2]	0.6	1.7
Phenytoin impurity E[3]	0.8	1.4
Phenytoin (Retention time: about 4 minutes)	1.0	—

[1]2,2-diphenylglycine,

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²3a,6a-diphenyltetrahydroimidazo[4,5-*d*]imidazole-2,5(1*H*,3*H*)-dione, ²(carbamoylamino)(diphenyl)acetic acid.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram 4 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to phenytoin impurity E is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent), the area of any peak corresponding to phenytoin impurity C is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any peak corresponding to phenytoin impurity D is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

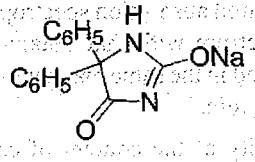
Assay. Dissolve 0.2 g in 50 ml of *dimethylformamide*. Titrate with *0.1 M sodium methoxide*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M sodium methoxide* is equivalent to 0.02523 g of $C_{15}H_{12}N_2O_2$.

Storage. Store protected from light.

Phenytoin Sodium

Diphenylhydantoin Sodium



$C_{15}H_{11}N_2NaO_2$ Mol. Wt. 274.3

Phenytoin Sodium is 4-oxo-5,5-diphenyl-2-imidazolidin-2-olate.

Phenytoin Sodium contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{15}H_{11}N_2NaO_2$, calculated on the anhydrous basis.

Category. Anticonvulsant; antiarrhythmic.

Description. A white powder; somewhat hygroscopic.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenytoin sodium IPRS* or with the reference spectrum of phenytoin sodium.

B. Dissolve 0.25 g in 5 ml of *water* and acidify with *dilute hydrochloric acid*; a white precipitate is produced.

C. Dissolve 0.1 g in 10 ml of a 10 per cent w/v solution of *pyridine*, add 1 ml of *cupric sulphate with pyridine solution* and allow to stand for 10 minutes; a blue precipitate is produced.

D. Incinerate 0.1 g; the residue after neutralisation with *hydrochloric acid* and addition of 2 ml of *water* gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. Suspend 1.0 g in 5 ml of *water* and dilute to 20 ml with *0.1 M sodium hydroxide*; the solution is clear (2.4.1), and not more intensely coloured than reference solution BYS6 (2.4.1).

Free phenytoin. Dissolve 0.3 g in 10 ml of a mixture of equal volumes of *pyridine* and *water* and add 0.5 ml of *dilute phenolphthalein solution* and 3 ml of *silver nitrate-pyridine reagent*. Not more than 1.0 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution to pink.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system

– a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),

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- mobile phase: a mixture of 20 volumes of *methanol*, 35 volumes of *acetonitrile* and 45 volumes of a 0.58 per cent w/v solution of *ammonium dihydrogen phosphate*, adjusted to pH 2.5 with *orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Phenytoin impurity C[1]	0.5	---
Phenytoin impurity D[2]	0.6	1.7
Phenytoin impurity E[3]	0.8	1.4
Phenytoin (Retention time: about 4 minutes)	1.0	—

[1]2,2-diphenylglycine,

[2]3a,6a-diphenyltetrahydroimidazo[4,5-*d*]imidazole-2,5(1*H*,3*H*)-dione,

[3](carbamoylamino)(diphenyl)acetic acid.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram 4 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to phenytoin impurity E is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent), the area of any peak corresponding to phenytoin impurity C is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any peak corresponding to phenytoin impurity D is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not more than 3.0 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Buffer solution. A *0.05 M monobasic ammonium phosphate*, adjusted to pH 2.5 with *orthophosphoric acid*.

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (a). A solution containing 0.01 per cent w/v of *phenytoin sodium IPRS* and 0.015 per cent w/v of *benzoin* in the mobile phase.

Reference solution (b). A 0.005 per cent w/v solution of *phenytoin sodium IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 45 volumes of buffer solution, 20 volumes of *methanol* and 35 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to phenytoin and benzoin is not less than 1.5 in the chromatogram obtained with reference solution (a), the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution.

Calculate the content of $C_{15}H_{11}N_2NaO_2$

Storage. Store protected from moisture.

Phenytoin Capsules

Phenytoin Sodium Capsules; Diphenylhydantoin Sodium Capsules

Phenytoin Capsules contain not less than 92.7 per cent and not more than 107.5 per cent of the stated amount of phenytoin sodium, $C_{15}H_{11}N_2NaO_2$.

Usual strengths. 25 mg, 50 mg and 100 mg.

Identification

A. Centrifuge the precipitated mixture obtained in test B, dissolve the residue in *methanol*, evaporate and dry at 105° for 30 minutes. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenytoin sodium IPRS* treated in the same manner or with the reference spectrum of phenytoin.

B. Shake a quantity of the content of capsules containing about 0.5 g of Phenytoin Sodium with 10 ml of *water* and filter. The filtrate yields a white precipitate on the addition of *2 M hydrochloric acid*.

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C. To 0.1 g of the residue obtained in test A, add 0.5 ml of *1 M sodium hydroxide*, 10 ml of a 10 per cent w/v solution of *pyridine* and 1 ml of *copper sulphate-pyridine reagent* and allow to stand for 10 minutes. A blue precipitate is produced.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 10 volumes of *13.5 M ammonia*, 45 volumes of *chloroform* and 45 volumes of *propan-2-ol*.

Test solution. Shake a quantity of the content of capsules containing about 0.2 g of Phenytoin Sodium with 5 ml of *methanol*, warm on a water-bath with shaking and filter.

Reference solution (a). Dilute 1 ml of the test solution to 200 ml with *methanol*.

Reference solution (b). A 0.02 per cent w/v solution of *benzophenone* in *methanol*.

Apply to the plate 10 µl of each solution. After development, dry at 80° for 5 minutes and examine under ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution corresponding to benzophenone is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Shake a quantity of the mixed contents of 20 capsules containing about 0.25 g of Phenytoin Sodium with 50 ml of *0.01 M sodium hydroxide*. Centrifuge, acidify 25 ml of the clear liquid with 10 ml of *0.1 M hydrochloric acid* and extract with successive quantities of 50, 40 and 25 ml of *ether*. Wash the combined extracts with 10 ml of *water*, evaporate to dryness and dry the residue at 105°. Dissolve in 50 ml of *anhydrous pyridine*. Titrate with *0.1 M tetrabutylammonium hydroxide* using 0.3 per cent w/v solution of *thymol blue* in *anhydrous pyridine* as indicator.

1 ml of *0.1 M tetrabutylammonium hydroxide* is equivalent to 0.02743 g of $C_{15}H_{11}N_2NaO_2$.

Phenytoin Injection

Phenytoin Sodium Injection; Diphenylhydantoin Sodium injection

Phenytoin Injection is a sterile material consisting of Phenytoin Sodium with or without buffering agents and other excipients. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injection or other suitable solvent, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Phenytoin Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of phenytoin sodium, $C_{15}H_{11}N_2NaO_2$.

Usual strengths. 100 mg; 250 mg.

Description. A white powder; somewhat hygroscopic.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenytoin sodium IPRS* or with the reference spectrum of phenytoin sodium.

B. Dissolve 0.25 g in 5 ml of *water* and acidify with *dilute hydrochloric acid*; a white precipitate is produced.

C. Dissolve 0.1 g in 10 ml of a 10 per cent w/v solution of *pyridine*, add 1 ml of *cupric sulphate with pyridine solution* and allow to stand for 10 minutes; a blue precipitate is produced.

D. Incinerate 0.1 g; the residue after neutralisation with *hydrochloric acid* and addition of 2 ml of *water* gives the reactions of sodium salts (2.3.1).

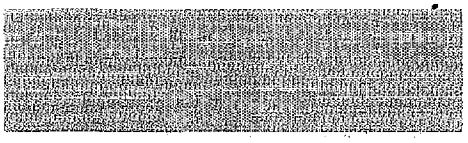
Tests

Appearance of solution. Suspend 1.0 g in 5 ml of *water* and dilute to 20 ml with *0.1 M sodium hydroxide*; the solution is clear (2.4.1), and not more intensely coloured than reference solution BYS6 (2.4.1).

Completeness of solution. The contents dissolve in the quantity of the solvent recommended on the label and give a clear solution.

pH (2.4.24). 10.0 to 12.0, determined in a 5.0 per cent w/v solution in the stated solvent.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.



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Mobile phase. A mixture of 45 volumes of *chloroform*, 45 volumes of *2-propanol* and 10 volumes of *strong ammonia solution*.

Test solution. Dissolve 0.4 g of the substance under examination in 10 ml of *methanol*.

Reference solution (a). A 0.04 per cent w/v solution of the substance under examination in *methanol*.

Reference solution (b). A 0.02 per cent w/v solution of *benzophenone* in *methanol*.

Apply to the plate 10 µl of each solution. After development, dry the plate at 80° for 5 minutes and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and any spot corresponding to benzophenone is not more intense than the spot in the chromatogram obtained with reference solution (b).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not more than 3.0 per cent, determined on 1.0 g.

Bacterial endotoxins (2.2.3). Not more than 0.3 Endotoxin Unit per mg of phenytoin sodium.

Assay. Weigh 0.18 g of the mixed contents of 10 containers, suspend in 2 ml of *water*, add 8 ml of *0.05 M sulphuric acid* and heat gently for 1 minute. Add 30 ml of *methanol*, cool. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25). After the first inflection, stop the addition of *sodium hydroxide*, add 5 ml of *silver nitrate solution* in *pyridine*, mix and continue the titration until a second inflection is reached. Record the volume of *0.1 M sodium hydroxide* added between the two inflections.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.02743 g of $C_{15}H_{11}N_2NaO_2$.

Storage. Store protected from moisture at a temperature not exceeding 30°.

Labelling. The label states (1) the quantity of Phenytoin Sodium contained in it; (2) the directions for preparing the Injection.

Phenytoin Oral Suspension

Diphenylhydantoin Oral Suspension

Phenytoin Oral Suspension is a suspension of phenytoin in a suitable flavoured vehicle.

Phenytoin Oral Suspension contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of phenytoin, $C_{15}H_{12}N_2O_2$.

Usual strength. 25 mg per ml.

Identification

Disperse a quantity of the suspension containing 0.1 g of Phenytoin with 20 ml of *water*, filter, acidify with *dilute hydrochloric acid* and extract with 10 ml of *chloroform*. Wash the chloroform extract with *water*, dry with *anhydrous sodium sulphate* and evaporate to dryness and dry the residue at 105°. The residue complies with the following test. Determine by infrared absorption spectrohotometry(2.4.6). Compare the spectrum with that obtained with *phenytoin sodium IPRS* treated in the same manner or with the reference spectrum of phenytoin.

Tests

pH (2.4.24). 4.5 to 5.5.

Benzil and benzophenone. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 30 volumes of *1, 4-dioxan* and 75 volumes of *hexane*.

Test solution. Disperse a quantity of suspension containing about 30 mg of Phenytoin in 5 ml of *water* add 2 ml of *2 M hydrochloric acid*, mix well and extract with five 20 ml quantities of *ether*. Combine the ether extracts, wash with three 10 ml quantities of *water*, evaporate to dryness and dissolve the residue in 1.5 ml of a mixture of 1 volume of *glacial acetic acid* and 9 volumes of *acetone*.

Reference solution (a). A 0.004 per cent w/v solution of *benzophenone* in *ethanol (95 per cent)*.

Reference solution (b). A 0.004 per cent w/v solution of *benzil* in *ethanol (95 per cent)*.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution corresponding to benzophenone and benzyl respectively is not more intense than the spot in the chromatogram obtain with reference solution (a) and reference solution (b) (0.2 per cent).

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Disperse a quantity of the suspension containing about 0.2 g of Phenytoin, add 10 ml of *2 M hydrochloric acid* and 15 ml of *water*, extract with three 50 ml quantities of a mixture of 3 volumes of *chloroform* and 1 volume of *propan-2-ol* and evaporate the combined extract to dryness. Dry the residue at 105° for 1 hour, cool and dissolve in a mixture of 3 ml of *1 M sodium hydroxide* and 50 ml of *water* with the aid of gentle heat. Cool and pass a current of *carbon dioxide* through the solution until precipitation of phenytoin is complete, filter and wash the residue with two 10 ml quantities of *water* and dry at 105° for 2 hours.

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Determine the weight per ml (2.4.29) of the oral suspension.

Calculate the content of $C_{15}H_{12}N_2O_2$, weight in volume.

Storage. Store at a temperature not exceeding 30°. Do not freeze.

Phenytoin Tablets

Phenytoin Sodium Tablets; Diphenylhydantoin Sodium Tablets

Phenytoin Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of phenytoin sodium, $C_{15}H_{11}N_2NaO_2$. The tablets are coated.

Usual strengths. 50 mg; 100 mg.

Identification

A. Shake a quantity of the powdered tablets containing 0.1 g of Phenytoin Sodium with 20 ml of *water*, filter, acidify with *dilute hydrochloric acid* and extract with 10 ml of *chloroform*. Wash the chloroform extract with *water*, dry with *anhydrous sodium sulphate* and evaporate to dryness and dry the residue at 105°. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phenytoin sodium IPRS* treated in the same manner or with the reference spectrum of phenytoin.

B. Triturate a quantity of the powdered tablets containing 0.5 g of Phenytoin Sodium with 10 ml of *water* and filter. Acidify with *dilute hydrochloric acid*; a white precipitate is produced.

C. The powdered tablets, when moistened with *hydrochloric acid* and introduced on a platinum wire into a flame, impart a yellow colour to the flame.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 75 volumes of *hexane* and 30 volumes of *dioxan*.

Test solution. Shake a quantity of the powdered tablets containing 0.1 g of Phenytoin Sodium with 5 ml of *methanol*, warm on a water-bath with shaking and filter.

Reference solution. A 0.01 per cent w/v solution of *benzophenone* in *methanol*.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. In the chromatogram obtained with the test solution any spot corresponding to benzophenone is not more intense than the spot in the chromatogram obtained with the reference solution.

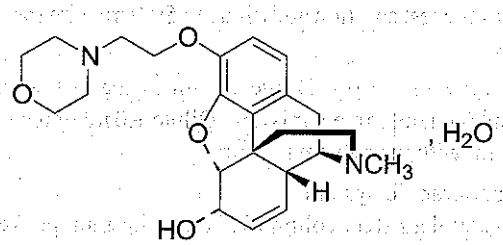
Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.25 g of Phenytoin Sodium, shake with 40 ml of *0.01 M sodium hydroxide* for 5 minutes and add sufficient *0.01 M sodium hydroxide* to produce 50.0 ml. Centrifuge, acidify 25.0 ml of the clear liquid with 10 ml of *0.1 M hydrochloric acid* and extract successively with 50, 40, 25 and 25 ml of *ether*. Wash the combined extracts with 10 ml of *water*, evaporate to dryness and dry the residue at 105°. Dissolve in 50 ml of *anhydrous pyridine* and titrate with *0.1 M tetrabutylammonium hydroxide*, using 0.3 per cent w/v solution of *thymol blue* in *pyridine* as indicator and taking care to prevent absorption of carbon dioxide from the atmosphere. Carry out a blank titration.

1 ml of *0.1 M tetrabutylammonium hydroxide* is equivalent to 0.02743 g of $C_{15}H_{11}N_2NaO_2$.

Storage. Store protected from moisture.

Pholcodine



$C_{23}H_{30}N_2O_4,H_2O$ Mol. Wt. 416.5

Pholcodine is 7,8-didehydro-4,5α-epoxy-17-methyl-3-[2-(morpholin-4-yl)ethoxy]morphinan-6α-ol monohydrate.

Pholcodine contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{23}H_{30}N_2O_4$, calculated on the dried basis.

Category. Opoid cough suppressant.

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pholcodine IPRS* or with the reference spectrum of pholcodine.

B. To 10 ml of a 0.1 per cent w/v solution add 75 ml of *water* and 10 ml of *1 M sodium hydroxide* and dilute to 100 ml with *water*. When examined in the range 230 nm and 360 nm (2.4.7), the resulting solution shows an absorption maximum at about 284 nm; absorbance at about 284 nm, 0.36 to 0.39.

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C. Dissolve 50 mg in 1 ml of *sulphuric acid* and add 0.05 ml of a 10 per cent w/v solution of *ammonium molybdate*; a pale blue colour is produced. Warm gently; the colour changes to deep blue. Add 0.05 ml of *2 M nitric acid*; the colour changes to brownish red.

Tests

Specific optical rotation (2.4.22). −98.0° to −94.0°, determined at 20° in a 2.0 per cent w/v solution in *ethanol (95 per cent)*.

Morphine. Dissolve 0.1 g in sufficient of *0.1 M hydrochloric acid* to produce 5 ml, add 2 ml of a 1 per cent w/v solution of *sodium nitrite*, allow to stand for 15 minutes and add 3 ml of *6 M ammonia*. The solution is not more intensely coloured than reference solution BS4 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dilute 80 ml of *acetonitrile* to 1000 ml with the *0.02 M phosphate buffer solution.*

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). A 0.1 per cent w/v solution of *codeine* with the solvent mixture, dilute to 0.5 ml of the solution, add 0.5 ml of the test solution and dilute to 50.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 7.5 cm x 4.6 mm, packed with endcapped phenylhexylsilane bonded to porous silica (3 µm),
- column temperature: 35°,
- mobile phase: a mixture of 50 ml of *tetrahydrofuran* and 75 ml of *acetonitrile*, diluted to 1000 ml with 0.02 M phosphate buffer solution prepared by dissolving 80.0 ml of *0.2 M sodium hydroxide* and 100.0 ml of *0.2 M potassium dihydrogen phosphate* in *water* and dilute to 1000.0 ml with *water*, adjusted to pH 7.9 with *0.2 M sodium hydroxide*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 20 µl.

Name	Relative retention time
Pholcodine impurity A[1]	0.4
Pholcodine impurity B[2]	0.8
Pholcodine (Retention time: about 10 minutes)	1.0
Pholcodine impurity D[3]	2.3

[1]morphine,

[2]codeine,

[3]unknown.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to pholcodine impurity B and pholcodine is not less than 3.0.

Inject reference solution (b) and the test solution. Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to pholcodine impurities A, B and D, each of, is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent). The area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). 3.9 to 4.5 per cent, determined on 0.5 g by drying in an oven at 105°.

Assay. Dissolve 0.18 g in 50 ml of *anhydrous glacial acetic acid*, warming gently. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically at the second inflection (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01993 g of $C_{23}H_{30}N_2O_4$.

Storage. Store protected from moisture.

Pholcodine Linctus

Pholcodine Linctus is a solution containing 0.1 per cent w/v solution of Pholcodine and 1 per cent w/v solution of Citric Acid Monohydrate in a suitable flavoured vehicle.

Pholcodine Linctus contains not less than 0.090 per cent and not more than 0.110 per cent w/v of pholcodine, $C_{23}H_{30}N_2O_4H_2O$.

Category. Opioid cough suppressant.

Identification

To 20 ml add 20 ml of *water*, make alkaline to *litmus paper* with *5 M ammonia*, extract with two quantities, each of 20 ml, of *chloroform*, washing each extract with 5 ml of *water*, dry the combined extracts with *anhydrous sodium sulphate*, filter and evaporate to dryness. If necessary, add 0.1 ml of ether and scratch the sides of the vessel with a glass rod to induce

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crystallisation. The crystals, dried at a pressure not exceeding 2 kPa, comply with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pholcodine IPRS* or with the reference spectrum of pholcodine.

B. When examined in the range 230 nm and 360 nm (2.4.7), a 0.01 per cent w/v solution in *0.01 M sodium hydroxide* shows an absorption maximum only at about 284 nm.

C. To a portion of the crystals add 0.05 ml of *nitric acid* and mix; a yellow colour is produced.

D. Dissolve the remainder of the crystals in 1 ml of *sulphuric acid* and add 0.05 ml of *ammonium molybdate-sulphuric acid solution*; a pale blue colour is produced. Warm gently; the colour changes to deep blue. Add 0.05 ml of *2 M nitric acid*; the colour changes to brownish red.

Tests

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Weigh 50 g and add sufficient *5 M ammonia* to make the solution alkaline to *litmus paper*, extract with four quantities, each of 25 ml, of *chloroform*, washing each extract with the same 5 ml of *water*. Combine the extracts and evaporate until the volume is reduced to 15 ml. Titrate with *0.02 M perchloric acid*, using *quinaldine red solution* as indicator. Carry out a blank titration.

1 ml of *0.02 M perchloric acid* is equivalent to 0.004165 g of $C_{23}H_{30}N_2O_4,H_2O$.

Determine the weight per ml of the linctus (2.4.29), and calculate the content of $C_{23}H_{30}N_2O_4,H_2O$, weight in volume.

Storage. Store protected from light.

Labelling. The label states the strength in terms of the equivalent amount of pholcodine.

Phosphoric Acid

Orthophosphoric Acid; Concentrated Phosphoric Acid

H_3PO_4 Mol. Wt. 98.0

Phosphoric acid contains not less than 84.0 per cent w/w and not more than 90.0 per cent w/w of H_3PO_4.

Category. Pharmaceutical aid (acidifying agent).

Description. A clear, colourless, syrupy liquid; corrosive. When kept at a low temperature it may solidify, producing a mass of colourless crystals which do not melt until the temperature reaches 28°.

Identification

A. Dilute with *water*; the solution is strongly acidic (2.4.16).

B. Dilute 10.0 g to 150 ml with *water* (solution A). Neutralise with *2 M sodium hydroxide*; the resulting solution gives the reactions of phosphates (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid*; the resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). Dilute 1.2 ml with 10 ml of *water*, neutralise with *dilute ammonia solution*, add sufficient *dilute acetic acid* to render the solution acidic and then dilute to 25 ml with *water*. The resulting solution complies with the limit test for heavy metals, Method A (10 ppm).

Iron (2.3.14). 10 ml of solution A complies with the limit test for iron (60 ppm).

Chlorides (2.3.12). 5 g complies with the limit test for chlorides (50 ppm).

Sulphates (2.3.17). 20 ml of solution A complies with the limit test for sulphates (100 ppm).

Alkali phosphates. To 1.7 g in a graduated cylinder add 6 ml of *ether* and 2 ml of *ethanol (95 per cent)*; no turbidity is produced.

Aluminium and calcium. To 1.7 g add 10 ml of *water* and 8 ml of *dilute ammonia solution*; the solution remains clear.

Hypophosphorus acid and phosphorous acid. To 5 ml of solution A add 2 ml of a 1.7 per cent w/v solution of *silver nitrate* and heat on a water-bath for 5 minutes; the appearance of the solution does not change.

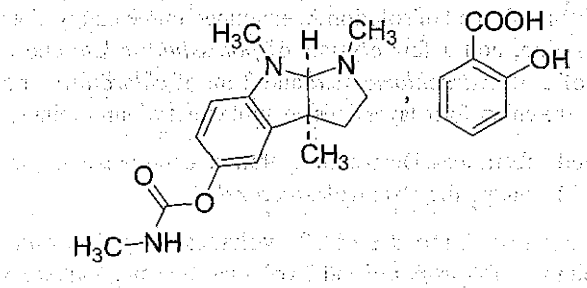
Assay. Weigh 1.0 g, add a solution of 10 g of *sodium chloride* in 30 ml of *water* and titrate with *1 M sodium hydroxide* using *dilute phenolphthalein solution* as indicator.

1 ml of *1 M sodium hydroxide* is equivalent to 0.04900 g of H_3PO_4.

Storage. Store protected from moisture, in glass containers.

Physostigmine Salicylate

Eserine Salicylate



$C_{15}H_{21}N_3O_2,C_7H_6O_3$ Mol. Wt. 413.5

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Physostigmine Salicylate is (3*aS*,8*aR*)-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo[2,3-*b*]indol-5-yl methylcarbamate salicylate.

Physostigmine Salicylate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{15}H_{21}N_3O_2$, $C_7H_6O_3$, calculated on the dried basis.

Category. Anticholinesterase.

Description. A colourless or faintly yellow crystals, turning red gradually under the action of air and light and rapidly in presence of moisture.

Identification

Test A may be omitted if tests B, C and D are carried out. Test C may be omitted if tests A, B and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *physostigmine salicylate IPRS* or with the reference spectrum of physostigmine salicylate.

B. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (a).

C. To a 1 per cent w/v solution add *1 M sodium hydroxide*; a white precipitate which turns pink is produced. The precipitate dissolves in an excess of the reagent, producing a red solution.

D. A 0.9 per cent w/v solution in *carbon dioxide-free water* gives reaction (A) of salicylates (2.3.1).

Tests

Appearance of solution. Dissolve 0.9 g without heating in 95 ml of *carbon dioxide-free water* prepared from *distilled water*, and dilute to 100 ml with the same solvent (solution A). The solution, examined immediately after preparation, is clear (2.4.1), and colourless (2.4.1).

pH (2.4.24). 5.1 to 5.9, determined in solution A immediately after preparation.

Specific optical rotation (2.4.22). – 94.0° to – 90.0°, determined in solution A immediately after preparation.

Eseridine. To 5 ml of solution A, examined immediately after preparation, add a few crystals of *potassium iodate* and a drop of *2 M hydrochloric acid* and 2 ml of *chloroform* and shake; the chloroform layer does not turn violet within 1 minute.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 100 volumes of *cyclohexane*, 23 volumes of *2-propanol* and 2 volumes of *strong ammonia solution*.

Test solution (a). Dissolve 0.2 g of the substance under examination in 10 ml of *ethanol (95 per cent)*.

Test solution (b). Dissolve 0.1 g of the substance under examination in 100 ml of *ethanol (95 per cent)*.

Reference solution (a). A 0.1 per cent w/v solution of *physostigmine salicylate IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 0.01 per cent w/v solution of *physostigmine salicylate IPRS* in *ethanol (95 per cent)*.

Apply to the plate 20 µl of each solution. After development, dry the plate in cold air, carry out a second chromatographic development in same direction, dry the plate in air and spray with freshly prepared *acetic potassium iodobismuthate solution* and then with *hydrogen peroxide solution (10 vol)*. Examine the plate within 2 minutes. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b).

Sulphates (2.3.17). 15 ml of solution A complies with the limit test for sulphates (0.1 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent, determined on the residue obtained in the test for Loss on drying.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.35 g in 50 ml of a mixture of equal volumes of *chloroform* and *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.04135 g of $C_{15}H_{21}N_3O_2$,$C_7H_6O_3$.

Storage. Store protected from light and moisture.

Physostigmine Injection

Physostigmine Salicylate Injection; Eserine Salicylate Injection

Physostigmine Injection is a sterile solution of Physostigmine Salicylate in Water for Injections.

Physostigmine Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of physostigmine salicylate, $C_{15}H_{21}N_3O_2$, $C_7H_6O_3$.

Usual strength. 600 µg per ml.

Identification

A. Warm a volume containing 3 mg of Physostigmine Salicylate with 0.3 ml of *5 M ammonia*; a yellowish red solution is produced which on evaporation gives a bluish residue.

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B. The residue obtained in test A dissolves in *ethanol (95 per cent)* producing a blue solution which, on the addition of *6 M acetic acid*, appears blue by transmitted light and exhibits a red fluorescence which intensifies on dilution with *water*.

C. The residue obtained in test A dissolves in *sulphuric acid* producing a green solution which, on the gradual addition of *ethanol (95 per cent)*, changes to red but reverts to green when the ethanol is evaporated.

Tests

pH (2.4.24). 4.0 to 6.0.

Bacterial endotoxins (2.2.3). Not more than 83.4 Endotoxin Units per mg of physostigmine salicylate.

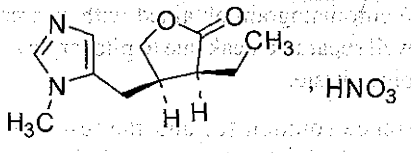
Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Transfer a measured volume containing 30 mg of Physostigmine Salicylate to a separator, add about 0.25 g of *sodium bicarbonate* and extract with six quantities, each of 15 ml, of *chloroform*. Filter the combined chloroform extracts through about 10 g of *anhydrous sodium sulphate*. Add 25 ml of *anhydrous glacial acetic acid* to the filtrate. Titrate with *0.01 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.01 M perchloric acid* is equivalent to 0.004135 g of $C_{15}H_{21}N_3O_2$, $C_7H_6O_3$.

Storage. Store protected from light, in single dose containers. The injection should not be used if it is more than slightly discoloured.

Pilocarpine Nitrate



$C_{11}H_{16}N_2O_2, HNO_3$ Mol. Wt. 271.3

Pilocarpine Nitrate is (3*S*,4*R*)-3-ethyl-4-[(1-methyl-1*H*-imidazol-5-yl)methyl]dihydrofuran-2(3*H*)-one nitrate.

Pilocarpine Nitrate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{11}H_{16}N_2O_2, HNO_3$, calculated on the dried basis.

Category. Cholinergic.

Description. Colourless crystals or a white, crystalline powder; sensitive to light.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pilocarpine nitrate IPRS* or with the reference spectrum of pilocarpine nitrate.

B. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a).

C. Dissolve about 10 mg in 2 ml of *water*, add 2 drops of a 5 per cent w/v solution of *potassium dichromate*, 1 ml of *hydrogen peroxide solution (10 vol)* and 2 ml of *chloroform* and shake; the chloroform layer turns violet.

D. It gives reaction (A) of nitrates (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution YS6 (2.4.1).

pH (2.4.24). 3.5 to 4.5, determined in a 5.0 per cent w/v solution prepared immediately before use in *carbon dioxide-free water*.

Specific optical rotation (2.4.22). +79.5° to +83.0°, determined in a 5.0 per cent w/v solution in *carbon dioxide-free water*, prepared immediately before use.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in *water* and dilute to 100.0 ml with *water*.

Reference solution (a). A 0.05 per cent w/v solution of *pilocarpine nitrate IPRS* in *water*.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 200.0 ml with *water*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 55 volumes of *methanol*, 60 volumes of *acetonitrile* and 885 volumes of a 0.068 per cent w/v solution of *tetrabutylammonium dihydrogen phosphate*, adjusted to pH 7.7 with *diluted ammonia*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

The retention time of the principal peak is about 20 minutes. The elution order is pilocarpine impurity B (pilocarpic acid), pilocarpine impurity C (isopilocarpic acid), pilocarpine impurity A (isopilocarpine) and pilocarpine.

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Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak corresponding to piloarpine impurity A is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), the sum of areas of pilocarpine impurities A and B is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent) and the sum of areas of all the secondary peaks other than pilocarpine impurities A and B is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and any peak due to the nitrate ion with a relative retention time with reference to pilocarpine of about 0.3.

Other alkaloids. To a 1 per cent w/v solution add *dilute ammonia solution*; no turbidity is produced. To a 1 per cent w/v solution add a few drops of *potassium dichromate solution*; no turbidity is produced.

Chlorides (2.3.12). 25 ml of a 10.0 per cent w/v solution complies with the limit test for chlorides (100 ppm).

Iron (2.3.14). 20 ml of a 10.0 per cent w/v solution complies with the limit test for iron (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.25 g in 30 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02713 g of $C_{11}H_{16}N_2O_2, HNO_3$.

Storage. Store protected from light and moisture.

Pilocarpine Eye Drops

Pilocarpine Nitrate Eye Drops

Pilocarpine Eye Drops is a sterile solution of Pilocarpine Nitrate in Purified Water.

Pilocarpine Eye Drops contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pilocarpine nitrate, $C_{11}H_{16}N_2O_2, HNO_3$.

Usual strengths. 1 per cent w/v; 2 per cent w/v; 4 per cent w/v.

Identification

Evaporate to dryness a volume containing about 20 mg of Pilocarpine Nitrate on a water-bath and heat for 1 hour at 105°. On the residue determine by infrared absorption spectrophotometry (2.4.6), compare the spectrum with that obtained with *pilocarpine nitrate IPRS* or with the reference spectrum of pilocapine nitrate.

Tests

pH (2.4.24). 3.0 to 5.5.

Pilocarpic acid. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a suitable volume of solution containing about 80 mg of Pilocarpine Nitrate to 100.0 ml with *water*.

Reference solution (a). Dilute 1.0 ml of the test solution to 25.0 ml with *water*.

Reference solution (b). A 0.08 per cent w/v solution of *pilocarpine nitrate IPRS* in *0.0025 M sodium hydroxide*, heat in a boiling water-bath for 30 minutes and cool.

Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- mobile phase: a mixture of 5.5 volumes of *methanol*, 6.0 volumes of *acetonitrile* and 88.5 volumes of *0.002 M tetrabutylammonium dihydrogen orthophosphate*, adjusted to pH 7.75 using *6 M ammonia*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the peak in the chromatogram obtained with reference solution (b) shows well separated peak due to pilocarpic acid followed by pilocarpine nitrate.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of peak corresponding to pilocarpic acid is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent).

Other tests. Comply with the test stated under Eye Drops.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a suitable volume of Eye drops containing about 80 mg of Pilocarpine Nitrate to 100 ml with *water*.

Reference solution. A 0.08 per cent w/v solution of *pilocarpine nitrate IPRS* in *water*.

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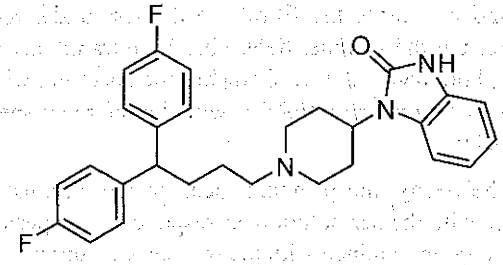
Chromatographic system
- a stainless steel column 15 cm × 4.6 mm packed with endcapped octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- mobile phase: a mixture of 5.5 volumes of *methanol*, 6.0 volumes of *acetonitrile* and 88.5 volumes of *0.002 M tetrabutylammonium dihydrogen orthophosphate*, adjusted to pH 7.75 using *6 M ammonia*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 220 nm.
- injection volume: 20 µl.

Inject the reference solution and the test solution. Two major peaks are obtained with both solutions. Run the chromatogram twice the retention time of the principal peak. Ignore the peak due to nitric acid and has the shorter retention time.

Calculate the content of $C_{11}H_{16}N_2O_2$, HNO_3 in the eye drops.

Pimozide



$C_{28}H_{29}F_2N_3O$ Mol.Wt. 461.6

Pimozide is 1-[1-[4,4-bis(4-fluorophenyl)butyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one.

Pimozide contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{28}H_{29}F_2N_3O$, calculated on the dried basis.

Category. Antipsychotic.

Description. A white to almost white powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pimozide IPRS* or with the reference spectrum of pimozide.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*

Mobile phase. A mixture of 10 volumes of *acetone* and 90 volumes of *methanol.*

Test solution. Dissolve 30 mg of the substance under examination in 10 ml of the mobile phase.

Reference solution (a). A 0.3 per cent w/v solution of *pimozide IPRS* in the mobile phase.

Reference solution (b). A solution containing 0.3 per cent w/v each of *pimozide IPRS* and *benperidol IPRS* in the mobile phase.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in a current of warm air for 15 minutes and expose it to iodine vapour until the spots appear. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

C. Mix about 5 mg of the substance under examination with 45 mg of *heavy magnesium oxide* and ignite in a crucible until an almost white residue is obtained. Allow to cool, add 1 ml of *water*, 0.05 ml of *phenolphthalein solution* and about 1 ml of *dilute hydrochloric acid* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 ml of *alizarin S solution* and 0.1 ml of *zirconyl nitrate solution*, add 1.0 ml of the filtrate. Mix allow to stand for 5 minutes and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

D. Melting point (2.4.21). 216° to 220°.

Tests

Appearance of solution. A 1.0 per cent w/v solution in *methanol* is clear (2.4.1) and not more intensely coloured than reference solution YS7 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in 10 ml of *methanol.*

Reference solution (a). Dissolve 5 mg of *pimozide IPRS* and 2 mg of *mebendazole IPRS* in 100 ml of *methanol.*

Reference solution (b). Dilute 5.0 ml of the test solution to 100 ml with *methanol.* Dilute 1.0 ml of the solution to 10.0 ml with *methanol.*

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- mobile phase: A. a solution containing 0.25 per cent w/v of *ammonium acetate* and 0.85 per cent w/v of *tetrabutylammonium hydrogen sulphate*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,

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– flow rate: 2 ml per minute,
– spectrophotometer set at 280 nm,
– injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
10	70	30
15	70	30
15.1	80	20
20	80	20

Name	Relative retention time
Pimozide impurity A[1]	0.1
Pimozide impurity B[2]	0.9
Pimozide impurity C[3]	0.95
Pimozide impurity D[4]	1.1
Pimozide impurity E[5]	1.3

[1]1-(piperidin-4-yl)-1,3-dihydro-2H-benzimidazol-2-one,

[2]1-[1-[(4RS)-4-(4-fluorophenyl)-4-phenylbutyl]piperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one,

[3]1-[1-[(4RS)-4-(2-fluorophenyl)-4-(4-fluorophenyl)butyl]piperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one,

[4]1-[1-[4,4-bis(4-fluorophenyl)butyl]-1,2,3,6-tetrahydropyridin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one,

[5]1-[1-[4,4-bis(4-fluorophenyl)butyl]piperidin-4-yl 1-oxide]-1,3-dihydro-2H-benzimidazol-2-one.

The relative retention time with respect to pimozide for mebendazole is about 0. 88.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to mebendazole and pimozide is not less than 5.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to pimozide impurity A, pimozide impurity B, pimozide impurity C, pimozide impurity D and pimozide impurity E, each of, is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.75 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.3 g in 50 ml of a mixture of 1 volume of *anhydrous glacial acetic acid* and 7 volumes of *methyl ethyl ketone* and titrate with *0.1 M perchloric acid*, using 0.2 ml of *naphtholbenzein solution* as indicator. Carry out a blank titration.

1 ml *0.1 M perchloric acid* is equivalent to 0.04616 g of $C_{28}H_{29}F_2N_3O$.

Storage. Store protected from light.

Pimozide Tablets

Pimozide Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of pimozide, $C_{28}H_{29}F_2N_3O$.

Usual strengths. 1 mg; 2 mg; 4 mg.

Identification

A. Shake a quantity of the powdered tablets containing about 20 mg of Pimozide with 20 ml of *dichloromethane* for 5 minutes, filter and evaporate the filtrate to dryness under reduced pressure. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pimozide IPRS* or with the reference spectrum of pimozide.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.01 M hydrochloric acid*,
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.0002 per cent w/v solution of *pimozide IPRS* in dissolution medium.

Use chromatographic system as described under Related substances.

Calculate the content of pimozide, $C_{28}H_{29}F_2N_3O$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{28}H_{29}F_2N_3O$.

Related substances. Determine by liquid chromatography (2.4.14).

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Test solution. Shake a quantity of powdered tablets containing about 40 mg of Pimozide with 20 ml of *methanol* for 30 minutes, mix with the aid of ultrasound for 10 minutes, centrifuge and filter the supernatant liquid.

Reference solution (a). Dilute 1 ml of the test solution to 200 ml with *methanol.*

Reference solution (b). A solution containing 0.005 per cent w/v of *pimozide IPRS* and 0.002 per cent w/v of *mebendazole IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm packed with endcapped octadecylsilane bonded to porous silica (3 µm) (Such as Hypersil ODS),
- mobile phase: A. a solution containing 0.25 per cent w/v of *ammonium acetate* and 0.85 per cent w/v of *tetrabutylammonium hydrogen sulphate,*
  B. *acetonitrile,*
- a gradient programme using the conditions given below,
- flow rate: 2 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
10	70	30
15	70	30
16	80	20
30	80	20

Inject reference solution (b). The test is not valid unless the resolution between the peaks obtained with mebendazole and pimozide is not less than 5.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of the areas of all secondary peaks is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.75 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Test solution. Shake one tablet with 7 ml of *methanol* for 30 minutes, dilute with *methanol* to produce a solution containing 0.01 per cent w/v of Pimozide, mix with the aid of ultrasound for 10 minutes, centrifuge and filter the supernatant liquid.

Reference solution (a). A 0.01 per cent w/v solution of *pimozide IPRS* in *methanol.*

Reference solution (b). A solution containing 0.005 per cent w/v of *pimozide IPRS* and 0.002 per cent w/v of *mebendazole IPRS* in *methanol.*

Use chromatographic system as described under Related substances.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to pimozide and mebendazole is not less than 5.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{28}H_{29}F_2N_3O$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of powdered tablets containing about 20 mg of Pimozide with 35 ml of *methanol* for 30 minutes, dilute to 50 ml with *methanol,* mix with the aid of ultrasound for 10 minutes, centrifuge and filter the supernatant liquid.

Reference solution (a). A 0.04 per cent w/v solution of *pimozide IPRS* in *methanol.*

Reference solution (b). A solution containing 0.004 per cent w/v of *pimozide IPRS* and 0.002 per cent w/v of *mebendazole IPRS* in *methanol.*

Use chromatographic system as described under Related substances.

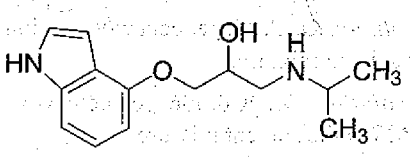
Inject reference solution (b). The test is not valid unless the resolution between the peaks due to pimozide and mebendazole is not less than 5.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{28}H_{29}F_2N_3O$ in the tablets.

Storage. Store protected from light.

Pindolol



$C_{14}H_{20}N_2O_2$ Mol. wt. 248.3

Pindolol is (*RS*)-1-indol-4-yloxy-3-isopropylaminopropan-2-ol.

Pindolol contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{14}H_{20}N_2O_2$, calculated on the dried basis.

Category. Beta-adrenoceptor antagonist.

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Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Test B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pindolol IPRS* or with the reference spectrum of pindolol.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution in a 0.085 per cent v/v solution of *hydrochloric acid* in *methanol* shows absorption maxima at about 264 nm and at about 287 nm, and a shoulder at about 275 nm, absorbances at about 264 nm, 0.66 to 0.70 and at about 287 nm, 0.34 to 0.38.

C. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (a).

Tests

Appearance of solution. A 5.0 per cent w/v solution in *dilute acetic acid* is clear (2.4.1) and not more intensely coloured than reference solution BYS4 (2.4.1).

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

NOTE— Prepare the solutions immediately before use and protected from light.

Solvent mixture. 99 volumes of *methanol* and 1 volume of *anhydrous glacial acetic acid*.

Mobile phase. A freshly prepared mixture of 50 volumes of *ethyl acetate*, 50 volumes of *methanol* and 4 volumes of *strong ammonia solution*.

Test solution (a). Dissolve 0.2 g of the substance under examination in 10 ml of the solvent mixture.

Test solution (b). Dissolve 0.2 g of the substance under examination in 100 ml of the solvent mixture.

Reference solution (a). A 0.2 per cent w/v solution of *pindolol IPRS* in the solvent mixture.

Reference solution (b). A 0.006 per cent w/v solution of *pindolol IPRS* in the solvent mixture.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 10 cm. Dry the plate immediately in a current of cold air and examine under ultraviolet at 254 nm without delay. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.3 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.2 g in 80 ml of *methanol*. Titrate with *0.1 M hydrochloric acid*, determining the end-point potentiometrically (2.4.25).

1 ml of *0.1 M hydrochloric acid* is equivalent to 0.02483 g of $C_{14}H_{20}N_2O_2$.

Storage. Store protected from light and moisture.

Pindolol Tablets

Pindolol Tablets contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pindolol, $C_{14}H_{20}N_2O_2$.

Usual strengths. 5 mg; 15 mg.

Identification

A. Shake a quantity of the powdered tablets containing 50 mg of Pindolol with 80 ml of *ether* for 30 minutes, filter and dry the extract with *anhydrous sodium sulphate*. Filter the extract, remove the *ether* using a rotary evaporator and dry the residue over *phosphorus pentoxide* at 110° at a pressure not exceeding 2 kPa for 1 hour. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pindolol IPRS* or with the reference spectrum of pindolol.

B. When examined in the range 230 nm to 360 nm (2.4.7), the final solution obtained in the Assay shows absorption maxima at about 264 nm and 287 nm.

C. Shake a quantity of the powdered tablets containing 20 mg of Pindolol with 5 ml of a mixture of 99 volumes of *methanol* and 1 volume of *glacial acetic acid* for 45 minutes. Centrifuge and dilute 1 ml of the supernatant liquid to 50 ml with the *acetic acid-methanol* mixture. To 2 ml of the solution add 1 ml of *dimethylaminobenzaldehyde solution*; a violet-blue colour is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium: 500 ml of *0.1 M hydrochloric acid*,

Speed and time: 50 rpm and 15 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the mobile phase.

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Reference solution (a). A 0.002 per cent w/v solution of *pindolol IPRS* in the dissolution medium. Dilute 5.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A 0.0005 per cent w/v solution of *nortriptyline hydrochloride IPRS* in reference solution (a).

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with dimethylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 19 volumes of *acetonitrile*, 19 volumes of *methanol* and 2 volumes of 0.6 per cent w/v solution of *ammonium carbonate*,
- flow rate: 3 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

The relative retention time for pindolol and nortriptyline is about 0.6 and 1.0, respectively.

Inject reference solution (a) and (b). The test is not valid unless the resolution between pindolol and nortriptyline hydrochloride peaks is not less than 1.5 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{14}H_{20}N_2O_2$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{14}H_{20}N_2O_2$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

NOTE— Prepare the solutions immediately before use and protected from light.

Solvent mixture. 99 volumes of *methanol* and 1 volume of *anhydrous glacial acetic acid*.

Mobile phase. A freshly prepared mixture of 50 volumes of *ethyl acetate*, 50 volumes of *methanol* and 4 volumes of *strong ammonia solution*.

Test solution. Shake a quantity of the powdered tablets containing 20 mg of Pindolol with 5 ml of the solvent mixture for 15 minutes, centrifuge and apply the supernatant liquid to the plate as the last solution.

Reference solution (a). Dilute 1.0 ml of the test solution to 10 ml with the solvent mixture and further dilute 7 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of the test solution to 10 ml with the solvent mixture and further dilute 3 ml of the solution to 100.0 ml with the solvent mixture.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 10 cm. Dry the plate in air, spray immediately with *dimethylaminobenzaldehyde solution* and warm at 50° for 20 minutes. Any spot with R_f value of about 0.1 in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.7 per cent). Any other secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.3 per cent). Ignore any spot remaining on the line of application.

Uniformity of content. Complies with the test stated under Tablets.

Disperse one tablet in 5 ml of *water*, add 50 ml of *methanol*, shake for 10 minutes and dilute to 100.0 ml with *methanol*, mix and filter. Dilute 10.0 ml of filtrate with *methanol* to produce a solution containing 0.001 per cent w/v of pindolol and measure the absorbance of the resulting solution at the maximum at about 264 nm (2.4.7). Calculate the content of $C_{14}H_{20}N_2O_2$ taking 338 as the specific absorbance at 264 nm.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14), as described under Dissolution with the following modifications.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 20 mg of pindolol in 4 ml of *water* and add 30 ml of the mobile phase and dilute to 100.0 ml with the mobile phase, filter.

Reference solution (a). A 0.02 per cent w/v solution of *pindolol IPRS* in the mobile phase.

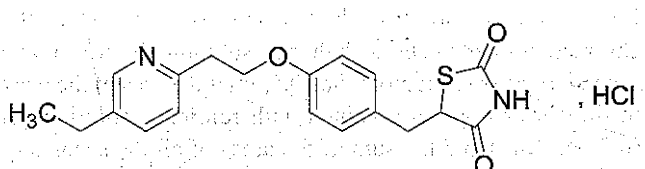
Reference solution (b). A 0.02 per cent w/v solution of *nortriptyline hydrochloride IPRS* in reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{14}H_{20}N_2O_2$ in the tablets.

Storage. Store protected from light and moisture.

Pioglitazone Hydrochloride



$C_{19}H_{20}N_2O_3S,HCl$ Mol.Wt. 392.9

Pioglitazone Hydrochloride is (*RS*)-5-{4-[2-(5-ethyl-2-pyridinyl)ethoxy]benzyl}thiazolidine-2,4-dione hydrochloride.

Pioglitazone Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{19}H_{20}N_2O_3S,HCl$, calculated on the dried basis.

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Category. Antidiabetic.

Description. A white or almost white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pioglitazone hydrochloride IPRS* or with the reference spectrum of pioglitazone hydrochloride.

B. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution in *methanol* is clear (2.4.1) and not more intensely coloured than reference solution BYS7 (2.4.1).

Related Substances. Determine by liquid chromatography (2.4.14)

Test solution. Dissolve 30 mg of the substance under examination in 100.0 ml of *methanol*.

Reference solution (a). A 0.03 per cent w/v solution of *pioglitazone hydrochloride IPRS* in *methanol*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 25°,
- mobile phase: a mixture of 50 volumes of *0.01 M potassium dihydrogen phosphate* and 50 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2500 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than the area of the peak in the chromatogram obtained with reference solution (b) (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Determine by liquid chromatography (2.4.14) as described under the test for Related substances.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{19}H_{20}N_2O_3S,HCl$.

Pioglitazone Tablets

Pioglitazone Hydrochloride Tablets

Pioglitazone Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pioglitazone, $C_{19}H_{20}N_2O_3S$.

Usual strengths. 15 mg; 30 mg; 45 mg.

Identification

A. Shake a quantity of the powdered tablets containing 0.1 g of pioglitazone with 40 ml of *methanol*, filter and evaporate the filtrate to dryness. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pioglitazone hydrochloride IPRS* or with the reference spectrum of pioglitazone hydrochloride.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 75 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtered solution, suitably diluted with the medium if necessary, at the maximum at about 270 nm (2.4.7). Calculate the content of $C_{19}H_{20}N_2O_3S$ in the medium from the absorbance obtained from a solution of known concentration of *pioglitazone hydrochloride IPRS* in the same medium.

Q. Not less than 70 per cent of the stated amount of $C_{19}H_{20}N_2O_3S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Transfer a quantity of the powder equivalent to 30 mg of pioglitazone and add about 150 ml of *methanol*. Mix with the aid of ultrasound at a temperature not exceeding 30° for 15 minutes and shake for

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20 minutes. Dilute to 250 ml with *methanol* and mix. Filter through a membrane filter and discard the first 5 ml. Use the filtrate as the test solution.

Reference solution. A solution of *pioglitazone hydrochloride IPRS* in *methanol* containing 0.00012 per cent w/v of pioglitazone.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of *0.01 M potassium dihydrogen phosphate* and 50 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the peak in the chromatogram obtained with the reference solution (2.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 35 volumes of *water* and 65 volumes of *acetonitrile*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 30 mg of pioglitazone in 20 ml of *water*, with the aid of ultrasound. Add about 150 ml of the solvent mixture and mix with the aid of ultrasound for 15 minutes, dilute to 250.0 ml with the solvent mixture and filter.

Reference solution. A 0.0136 per cent w/v solution of *pioglitazone hydrochloride IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen orthophosphate* and 1.15 g of *diammonium hydrogen phosphate* in 1000 ml of *water*, adding 1.0 ml of *triethylamine* and adjusted to pH 5.0 with *orthophosphoric acid* and 65 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{20}N_2O_3S$ in the tablets.

Labelling. The label states the strength in terms of the equivalent amount of pioglitazone.

Pioglitazone and Metformin Hydrochloride Tablets

Pioglitazone Hydrochloride and Metformin Hydrochloride Tablets; Metformin Hydrochloride and Pioglitazone Hydrochloride Tablets

Pioglitazone and Metformin Hydrochloride Tablets contain an amount of pioglitazone hydrochloride equivalent to not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of pioglitazone, $C_{19}H_{20}N_2O_3S$, and not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of metformin hydrochloride, $C_4H_{11}N_5,HCl$.

Usual strength. Pioglitazone, 15 mg and Metformin Hydrochloride, 500 mg.

Identification

A. *For pioglitazone* — Dissolve a suitable quantity of the powdered tablets containing pioglitazone hydrochloride in *water* to obtain 0.003 per cent w/v of pioglitazone and mix with the aid of ultrasound. Pass 5 ml of this suspension through a filter having an average pore size not more than 0.45 µm, then wash the filter with 10 ml of *water* and discard the filtrate. Wash the filter with 5 ml of *0.1 M hydrochloric acid* and use the filtrate. When examined in the range 230 nm to 350 nm (2.4.7), shows a maxima between 267 nm and 271 nm.

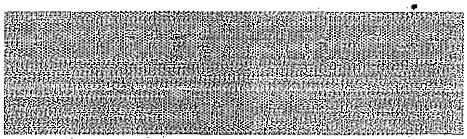
For metformin hydrochloride — Dissolve a suitable quantity of the powdered tablets containing metformin hydrochloride in *water* to obtain 0.04 per cent w/v of metformin hydrochloride and mix with the aid of ultrasound. Pass this suspension through a filter having an average pore size not greater than 0.45 µm, discard the first few ml of filtrate. Dilute the filtrate to obtain a solution of 0.0008 per cent w/v of metformin hydrochloride. When examined in the range 200 nm to 350 nm (2.4.7), shows a maxima between 230 nm and 234 nm.

B. In the Assay, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with reference solution (c).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of buffer, prepared by adjusting *0.1 M citric acid* to pH 2.5 with *0.2 M dibasic sodium phosphate*,
Speed and time. 50 rpm and 30 minutes.



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Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *methanol* and *0.1 M hydrochloric acid*.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution (a). A 0.037 per cent w/v solution of *pioglitazone hydrochloride IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.00185 per cent w/v of *pioglitazone hydrochloride IPRS* obtained by diluting reference solution (a) and 0.05555 per cent w/v of *metformin hydrochloride IPRS in* the medium (for the tablets containing 500 mg of metformin hydrochloride per tablet) or 0.0944 per cent w/v of *metformin hydrochloride IPRS*(for the tablets containing 850 mg of metformin hydrochloride per tablet) in the medium.

Chromatographic system

- a stainless steel column 15 cm x 6.0 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a buffer solution prepared by dissolving 7.2 g of *sodium dodecyl sulphate* in 1000 ml of equal volumes of *0.05 M monobasic ammonium phosphate* and *acetonitrile*,
- flow rate: 1 ml per minute,
- injection volume: 5 µl.

For detection, use 255 nm for metformin and 225 nm for pioglitazone.

Inject reference solution (b). The test is not valid unless the tailing factor is not more than 2.0 for pioglitazone and not more than 2.5 for metformin and the relative standard deviation for replicate injections for each of the peaks corresponding to pioglitazone and metformin is not more than 2.0 per cent.

Inject reference solution (b) and the test solution.

Calculate the content of $C_{19}H_{20}N_2O_3S$ and $C_4H_{11}N_5,HCl$ in the medium.

Q. Not less than 80 per cent of the stated amounts of each of $C_{19}H_{20}N_2O_3S$ and $C_4H_{11}N_5,HCl$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — A volume of methanol not to exceed 20.0 per cent of the total volume of standard solution may be used to dissolve pioglitazone hydrochloride IPRS.

For pioglitazone —

Solvent mixture. Equal volumes of *methanol* and *0.1 M hydrochloric acid*.

Test solution. Weigh and powder 10 tablets. Weigh a quantity of the powder containing 18 mg of Pioglitazone, add 50 ml of the *solvent mixture*, shake for 30 minutes and dilute to 100.0 ml in the mobile phase. Mix, filter and discard first few ml of filtrate.

Reference solution (a). Dissolve a sufficient quantity of *pioglitazone hydrochloride IPRS* in *methanol* and dilute to volume to obtain the concentration equivalent to 0.02 per cent w/v solution of *pioglitazone hydrochloride IPRS* with the mobile phase.

Reference solution (b). A solution equivalent to 0.0001 per cent w/v of *pioglitazone hydrochloride IPRS* obtained by diluting reference solution (a) with the mobile phase.

Reference solution (c).Dissolve 30 mg of benzophenone in *methanol* and dilute to 100.0 ml with *methanol*. To 1.0 ml of the solution add 5.0 ml of reference solution (a) and dilute to 50.0 ml with the mobile phase to obtain a solution equivalent to 0.002 per cent w/v of *pioglitazone hydrochloride IPRS* and 0.0006 per cent w/v of benzophenone.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 250 volumes of *acetonitrile*, 250 volumes of *0.1 M ammonium acetate* and 10 volumes of *glacial acetic acid*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 269 nm,
- injection volume: 40 µl.

NOTE — The flow rate may be adjusted to achieve the retention time of the pioglitazone peak at about 7 minutes.

The elution order is pioglitazone and benzophenone.

Inject reference solution (b) and (c). The test is not valid unless the resolution between pioglitazone and benzophenone peaks is not less than 10.0 and the tailing factor for pioglitazone is not more than 1.5 in the chromatogram obtained with reference solution (c) and the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution. Run the chromatogram at least 4 times the retention time of the principal peak for the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 1.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent). Ignore any peaks due to metformin and its impurities that elute before 4.5 minutes, corresponding to the relative retention time to the pioglitazone peak of about 0.64.

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For metformin hydrochloride —

Test solution. Weigh and powder 10 tablets. Weigh a quantity of the powder containing 100 mg of metformin hydrochloride, add 50 ml of *water*, shake for 30 minutes and dilute 100.0 ml with *water*. Mix, filter and discard first few ml of filtrate.

Reference solution (a). A 0.0005 per cent w/v solution of *metformin hydrochloride IPRS* in *water*.

Reference solution (b). A solution containing 0.0005 per cent w/v of *metformin hydrochloride IPRS*, and 0.0002 per cent w/v of melamine in *water*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with C30 silane bonded to fully porous spherical silica (5 µm),
- mobile phase: A. dissolve 1.74 g of *sodium 1-pentanesulphonate* and 1.15 g of *monobasic ammonium phosphate* in 1000 ml of *water*,
  B. a mixture of 30 volumes of *water* and 70 volumes of *acetonitrile*.
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

NOTE — The flow rate may be adjusted to achieve the retention time of the metformin peak of about 8 minutes.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
15	70	30
15.1	0	100
25	0	100
25.1	100	0
35	100	0

The relative retention time of melamine with reference to metformin is about 0.9.

Inject reference solution (b).The test is not valid unless the resolution between melamine and metformin hydrochloride is not less than 4.0 and the tailing factor for metformin hydrochloride is not more than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *methanol* and *0.1 M hydrochloric acid.*

Test solution. Weigh and powder 10 tablets. Disperse a quantity of the powder containing 15 mg of pioglitazone in *0.1 M hydrochloric acid* to produce a solution containing 0.0075 per cent w/v of pioglitazone. Shake for 30 minutes and disperse with the aid of ultrasound for 5 minutes. Mix, filter and discard first few ml of filtrate. Dilute the filtrate with *0.1 M hydrochloric acid* to produce a solution containing 0.0015 per cent w/v of pioglitazone.

Reference solution (a). A solution containing 0.05 per cent w/v of *p-methoxyacetophenone*, and 0.04 per cent w/v of *butylparaben* in the solvent mixture.

Reference solution (b). A solution containing 0.084 per cent w/v of *pioglitazone hydrochloride IPRS* in the solvent mixture.

Reference solution (c). A solution containing 0.25 per cent w/v of *metformin hydrochloride IPRS* (for the tablets containing 500 mg of metformin hydrochloride per tablet) or 0.425 per cent w/v of *metformin hydrochloride IPRS* (for the tablets containing 850 mg of metformin hydrochloride per tablet)and 0.0084 per cent w/v of *pioglitazone hydrochloride IPRS* prepared by diluting reference solution (b) in *0.1 M hydrochloric acid.*

Reference solution (d). Transfer 10.0 ml of reference solution (c) and 5.0 ml of reference solution (a) to a 50-ml volumetric flask and dilute to the volume with *0.1 M hydrochloric acid.*

Use the chromatographic system as described under Dissolution using injection volume: 10 µl.

NOTE— The flow rate may be adjusted to achieve the retention time of the metformin peak of about 5 minutes.

Name	Relative retention time
Metformin	1.0
p-Methoxyacetophenone	1.2
Pioglitazone	1.8
Butylparaben	2.1

Inject reference solution (d). The test is not valid unless the resolution between metformin and p-methoxyacetophenone peaks and pioglitazone and butylparaben peaks is not less than 2.5 and the relative standard deviation for replicate injections for the peaks corresponding to metformin and pioglitazone is not more than 1.0 per cent.

Inject reference solution (d) and the test solution.

Calculate the contents of $C_{19}H_{20}N_2O_3S$ and $C_4H_{11}N_5,HCl$ in the tablets.

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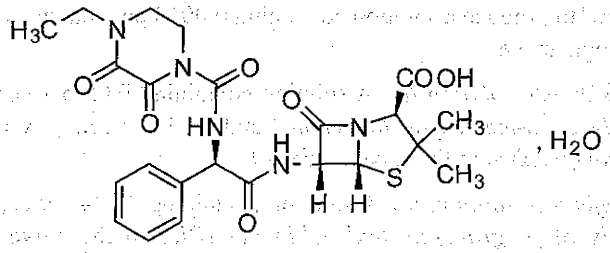
Storage. Store protected from moisture.

Labelling. The label states the strength in terms of the equivalent amount of pioglitazone and metformin hydrochloride.

Piperacillin



$C_{23}H_{27}N_5O_7S,H_2O$ Mol. Wt. 535.6

Piperacillin is (6*R*)-6-[*R*-2-(4-ethyl-2,3-dioxopiperazine-1-carboxamido)-2-phenylacetamido]penicillanic acid monohydrate.

Piperacillin contains not less than 96.0 per cent and not more than 102.0 per cent of $C_{23}H_{27}N_5O_7S$, calculated on the anhydrous basis.

Category. Penicillin antibacterial.

Description. A white or almost white powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *piperacillin IPRS or* with the reference spectrum of piperacillin.

Tests

Appearance of solution. A 10 per cent w/v solution in *sodium carbonate solution* is clear (2.4.1) and not more intensely coloured than the reference solution BS8 (2.4.1).

Specific optical rotation (2.4.22). + 165° to + 175°, determined in a 1.0 per cent w/v solution in *methanol.*

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Use freshly prepared solutions.

Solvent mixture. 25 volumes of *acetonitrile* and 75 volumes of 3.12 per cent w/v solution of *sodium dihydrogen phosphate.*

Test solution (a). Dissolve 25 mg of the substance under examination in 50 ml of the solvent mixture.

Test solution (b). Dissolve 40 mg of the substance under examination in 20 ml of the solvent mixture.

Reference solution (a). A 0.05 per cent w/v solution of *piperacillin IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 25 ml with the solvent mixture.

Reference solution (c). Dissolve 10 mg of *piperacillin IPRS* and 10 mg of *anhydrous ampicillin IPRS* (*piperacillin impurity A IPRS*) in 50 ml solvent mixture.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 100 ml with the solvent mixture. Dilute 1.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 576 volumes of *water,* 200 volumes of 3.12 per cent w/v solution of *sodium dihydrogen phosphate* and 24 volumes of an 8.0 per cent w/v solution of *tetrabutylammonium hydroxide,* adjusted to pH 5.5 with *dilute phosphoric acid* or *dilute sodium hydroxide solution,* add 200 volumes of *acetonitrile,*

  B. a mixture 126 volumes of *water,* 200 volumes of a 3.12 per cent w/v solution of *sodium dihydrogen phosphate* and 24 volumes of an 8.0 per cent w/v solution of *tetrabutylammonium hydroxide,* adjusted to pH 5.5 with *dilute phosphoric acid* or *dilute sodium hydroxide solution*; add 650 volumes of *acetonitrile,*.
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	88	12
t_g	88	12
t_g +30	0	100
t_g + 45	88	12

where, t_g = retention time of piperacillin determined with reference solution (b)

Inject reference solution (b), (c) and (d). The test is not valid unless the resolution between the peaks due to piperacillin impurity A and piperacillin in the chromatogram obtained with reference solution (c) is not less than 10; signal-to-noise ratio is not less than 3 for the principal peak in the chromatogram obtained with reference solution (d); mass distribution ratio is (2:3) for the peak due to piperacillin in the chromatogram obtained with reference solution (c). The area of any secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

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***N,N*-Dimethylaniline** (2.3.21). Not more than 20 ppm.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water. (2.3.43). 2.0 per cent to 4.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14), as described under test for Related substances.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject reference solution (a) and test solution (a).

Calculate the content of $C_{23}H_{27}N_5O_7S$.

Piperacillin intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.07 Endotoxin Unit per mg of piperacillin.

Storage. Store protected from light and moisture.

Piperacillin Intravenous Infusion

Piperacillin Intravenous Infusion is a sterile solution of Piperacillin Sodium in Water for Injections. It is prepared by dissolving Piperacillin for Intravenous Infusion in the requisite amount of Water for Injections before use.

The intravenous infusion complies with the requirements stated under Parenteral Preparations (Injections).

Piperacillin for Intravenous Infusion

Piperacillin for Intravenous Infusion is a sterile material consisting of Piperacillin Sodium or Piperacillin Sodium prepared by the interaction of piperacillin with *sodium bicarbonate*, with or without excipients. It is filled in a sealed container.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parental Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Piperacillin for Intravenous Infusion contains not less than 94.0 per cent and not more than 115.0 per cent of the stated amount of piperacillin, $C_{23}H_{27}N_5O_7S$.

Usual strengths. 1 g per vial; 2 g per vial; 4 g per vial.

The contents of the sealed container comply with the requirements stated under Parental Preparations (Powder for Injections) and with the following requirements.

Identification

A. Dissolve a quantity of the powder containing about 0.25 g of anhydrous piperacillin in 20 ml of *water*, add 0.5 ml of *dilute hydrochloric acid*, mix well, allow to stand until a precipitate appears and filter. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *piperacillin IPRS* or with the reference spectrum of piperacillin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

C. It gives the reaction of sodium salts (2.3.1).

Tests

pH (2.4.24). 4.8 to 6.8, determined in a 10.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powder containing about 39 mg of anhydrous piperacillin in 100 ml of the mobile phase.

Reference solution (a). A 0.0008 per cent w/v solution of *piperacillin IPRS* in the mobile phase.

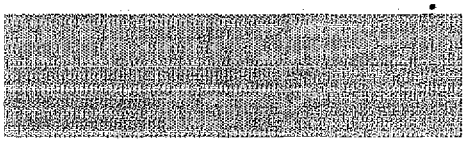
Reference solution (b). A 0.0004 per cent w/v solution of *piperacillin IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 to 10 µm) (Such as Beckman Ultrasphere ODS),
- mobile phase: a mixture of 0.3 volume of *0.4 M tetrabutylammonium hydroxide*, 10 volumes of *0.2 M sodium dihydrogen orthophosphate monohydrate*, 44.7 volumes of *water* and 45 volumes of *methanol*, cool and adjusted to pH 5.5 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution the area of the peak with a relative retention time of about 0.6 is not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.5 per cent). The area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (2 per cent).

Water (2.3.43). Not more than 2.0 per cent, determined on 0.5 g.



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Bacterial endotoxins (2.2.3). Not more than 2.5 Endotoxin Units per ml of a solution prepared in the following manner. Dissolve a quantity in *water BET* to obtain a solution containing 50 mg of piperacillin per ml. Carry out the test using Maximum Valid dilution of the solution calculated from the declared sensitivity of the lysate used in the test.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powder containing about 39 mg of anhydrous piperacillin in 100 ml of the mobile phase.

Reference solution (a). Dissolve 40 mg of *piperacillin IPRS* in *methanol* and dilute to 100 ml with the mobile phase.

Reference solution (b). A solution containing 0.01 per cent w/v of *anhydrous ampicillin IPRS* and 0.02 per cent w/v of *piperacillin IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as Beckman Ultrasphere ODS),
- mobile phase: a mixture of 0.3 volume of *0.4 M tetrabutylammonium hydroxide*, 10 volumes of *0.2 M sodium dihydrogen orthophosphate monohydrate*, 44.7 volumes of *water* and 45 volumes of *methanol*, cool and adjusted to pH 5.5 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

Inject reference solution (b). The resolution between the peaks due to anhydrous ampicillin and piperacillin is not less than 16.

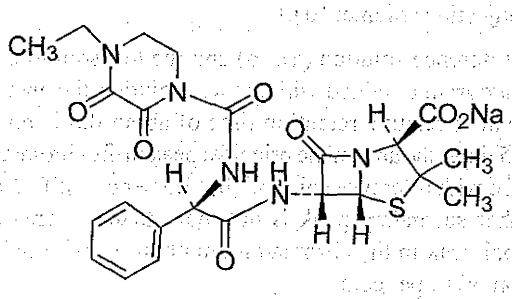
Inject the reference solution and the test solution (a).

Calculate the content of $C_{23}H_{27}N_5O_7S$ in the infusion.

Storage. Store protected from moisture.

Labelling. The label of the sealed container states the quantity of active ingredient contained in it in terms of the equivalent amount of anhydrous piperacillin.

Piperacillin Sodium



$C_{23}H_{26}N_5NaO_7S$ Mol.Wt. 539.5

Piperacillin is Sodium (*2S,5R,6R*)-6-[[(*2R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate.

Piperacillin Sodium contains not less than 95.0 per cent and not more than 102.0 per cent of $C_{23}H_{26}N_5NaO_7S$, calculated on the anhydrous basis.

Category. Antibacterial.

Description. A white or almost white powder, hygroscopic.

Identification

A. Dissolve 0.25 g in *water*, add 0.5 ml of *dilute hydrochloric acid* and 5 ml of *ethyl acetate*; stir and allow to stand for 10 minutes in iced water. Filter the crystals through a small sintered-glass filter (40), applying suction. Wash with 5 ml of *water* and 5 ml of *ethyl acetate*, then dry in an oven at 60° for 60 minutes.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *piperacillin IPRS* or with the reference spectrum of piperacillin.

B. Gives the reaction (A) of sodium salts (2.3.1).

Tests

Appearance of solution. A 10 per cent w/v solution in *water* is clear (2.4.1).

pH (2.4.24). 5.0 to 7.0, determined in 10 per cent w/v solution.

Specific optical rotation (2.4.22). + 175° to + 190°, determined in a 1.0 per cent w/v solution in *water*.

Light absorption (2.4.7). Absorbance of a 10 per cent w/v solution in *water* at the maximum at about 430 nm, not more than 0.10.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Use freshly prepared solutions.

Solvent mixture. 25 volumes of *acetonitrile* and 75 volumes of 3.12 per cent w/v solution of *sodium dihydrogen phosphate*.

Test solution (a). Dissolve 25 mg of the substance under examination in 50.0 ml of the solvent mixture.

Test solution (b). Dissolve 40 mg of the substance under examination in 20.0 ml of the solvent mixture.

Reference solution (a). A 0.05 per cent w/v solution of *piperacillin IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 25.0 ml with the solvent mixture.

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Reference solution (c). Dissolve 10 mg of *piperacillin IPRS* and 10 mg of *anhydrous ampicillin IPRS (piperacillin impurity A IPRS) [(2S,5R,6R)-6-[[(2R)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid (ampicillin) IPRS]* in 50.0 ml of the solvent mixture.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 576 volumes of *water*, 200 volumes of a 3.12 per cent w/v solution of *sodium dihydrogen phosphate* and 24 volumes of an 8.0 per cent w/v solution of *tetrabutylammonium hydroxide*, adjusted to pH 5.5 with *dilute phosphoric acid* or *dilute sodium hydroxide solution*; add 200 volumes of *acetonitrile*,

  B. a mixture of 126 volumes of *water*, 200 volumes of a 3.12 per cent w/v solution of *sodium dihydrogen phosphate* and 24 volumes of an 8.0 per cent w/v solution of *tetrabutylammonium hydroxide*, adjusted to pH 5.5 with *dilute phosphoric acid* or *dilute sodium hydroxide solution*; add 650 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	88	12
tg	88	12
tg + 30	0	100
tg + 45	88	12

where, tg = retention time of piperacillin determined with reference solution (b)

Inject reference solution (b), (c), (d) with isocratic elution at the initial mobile phase composition and test solution (b) according to the gradient programme. The test is not valid unless the resolution between the peaks due to piperacillin impurity A and piperacillin in the chromatogram obtained with reference solution (c) is not less than 10; if necessary, adjust the ratio A:B of the mobile phase; signal-to-noise ratio is not less than 3 for the principal peak in the chromatogram obtained with reference solution (d); mass distribution ratio is 2 to 3 for the peak due to piperacillin in the chromatogram obtained with reference solution (c). The area of any secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

***N,N*-Dimethylaniline.** Not more than 20 ppm. Determine by gas chromatography (2.4.13).

Internal standard solution. Dissolve 50 mg of *N,N-diethylaniline* in 4 ml of *0.1 M hydrochloric acid* and dilute to 50.0 ml with *water*. Dilute 1.0 ml of the solution to 100.0 ml with *water*.

Test solution. Dissolve in a ground-glass-stoppered tube 0.5 g of the substance under examination in 30.0 ml of *water*. Add 1 ml of the internal standard solution. Adjust the solution to a temperature of 26-28°. Add 1 ml of *strong sodium hydroxide solution* and mix until completely dissolved. Add 2.0 ml of *trimethylpentane*. Shake for 2 minutes and allow the phases to separate. Use the upper layer.

Reference solution. Dissolve 50 mg of *N,N-dimethylaniline* in 4.0 ml of *0.1 M hydrochloric acid* and dilute to 50.0 ml with *water*. Dilute 1.0 ml of the solution to 100.0 ml with *water*. Dilute 1.0 ml of the solution to 30.0 ml with *water*. Add 1.0 ml of the internal standard solution and 1.0 ml of *strong sodium hydroxide solution*. Add 2.0 ml of *trimethylpentane*. Shake for 2 minutes and allow the phases to separate. Use the upper layer.

Chromatographic system

- a fused silica capillary column 25 m × 0.32 mm, coated with cross-linked polymethylphenylsiloxane (film thickness 0.52 µm),
- temperature: column:180°,
- temperature:

column	time (min.)	temperature (°)
	0	150
	0-5	150
	5-11.25	150→275
	11.25-14.25	275

- inlet port at 220° and detector at 300°,
- flame ionization detector,
- column head pressure of 50 kPa and a split vent of 20 ml per minutes, using helium as the carrier gas,
- split ratio: 1:20,
- a split-liner consisting of a column about 1 cm long packed with *diatomaceous earth for gas chromatography* impregnated with 10 per cent *m/m* of *poly(dimethyl)siloxane*,

The retention times of *N,N*-dimethylaniline is about 3.6 minutes and *N,N*-diethylaniline is about 5.0 minutes.

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Inject 1 µl each of the reference solution and the test solution. Record the chromatogram and calculate the content of *N,N*-dimethylaniline.

Water. (2.3.43). Not more than 2.0 per cent, determined on 0.5 g.

Piperacillin Sodium intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.07 Endotoxin Unit per mg of piperacillin sodium.

Assay. Determine by liquid chromatography (2.4.14), as described under test for Related substances with the following modifications.

- mobile phase: Initial composition of the mixture of the mobile phase A and B, adjust where applicable.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject reference solution (a) and test solution (a).

Calculate the content of $C_{23}H_{26}N_5NaO_7S$, multiplying the result by 1.042.

Storage. Store protected from moisture.

Piperacillin and Tazobactam Injection

Piperacillin and Tazobactam Injection is a sterile material consisting of Piperacillin Sodium and Tazobactam Sodium with or without excipients. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Piperacillin and Tazobactam Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of piperacillin, $C_{23}H_{27}N_5O_7S$ and tazobactam, $C_{10}H_{12}N_4O_5S$, the labelled amounts representing proportions of piperacillin to tazobactam of 8:1. It may contain small amounts of a suitable buffers and stabilizers.

Usual strengths. Piperacillin, 4 g and Tazobactum, 500 mg per vial; Piperacillin, 2 g and Tazobactum, 250 mg per vial.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 5.0 to 7.0, determined in a 4.0 per cent w/v solution of piperacillin immediately after preparation.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Use freshly prepared solutions, stored between 2° to 8° after preparation.

Solvent mixture A. 25 volumes of *acetonitrile* and 75 volumes of *water.*

Solvent mixture B. 4 volumes *of acetonitrile* and 96 volumes of solvent mixture A.

Test solution. Dissolve a sufficient quantity of injection in the mobile phase to obtain a solution containing 0.2 per cent w/v of Piperacillin.

Reference solution (a). A 0.05 per cent w/v solution of *tazobactam IPRS* in solvent mixture A.

Reference solution (b). A 0.1 per cent w/v solution of *piperacillin IPRS* in solvent mixture B (*NOTE-Dissolve first in acetonitrile, using about 4 per cent of the final volume, and dilute with solvent mixture B to volume).*

Reference solution (c). A solution containing 0.0006 per cent w/v of *tazobactam related compound A IPRS* and 0.0025 per cent w/v of *tazobactam IPRS* in solvent mixture A.

Reference solution (d). Dilute reference solution (a) and (b) with the mobile phase to obtain a solution containing 0.00025 per cent w/v of *tazobactam IPRS* and 0.002 per cent w/v of *piperacillin IPRS.*

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with phenyl group bonded to porous silica (3 µm),
- sample temperature: 5°,
- mobile phase: a mixture of 75 volumes of a buffer solution prepared by dissolving 1 vial of *tetrabutylammonium hydrogen sulphate* in 1000 ml of *water* and 25 volumes of *acetonitrile*, adjusted to pH 3.8 with 20 per cent v/v of *orthophosphoric acid*,

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– flow rate: 1 ml per minute,
– spectrophotometer set at 210 nm,
– injection volume: 20 µl.

Name	Relative retention time	Correction factor
Tazobactam related compound A[1]	0.12	1.33
Tazobactam	0.25	—
Piperacillin impurity B[2]	0.31	—
Piperacillinpenilloic acid[3,4]	0.36	—
Piperacillinpenicilloic acid[3,5]	0.51	1.79
Acetylated penicilloic acid of piperacillin[6]	0.55	—
Piperacillin impurity C[2]	0.62	—
Piperacillin impurity D[2]	0.67	—
Piperacillin	1.0	—

[1](2*S*,3*S*)-2-Amino-3-methyl-3-sulfino-4-(1*H*-1,2,3-triazol-1-yl) butyric acid,

[2]Specified unknown impurities,

[3]This compound has two epimers that usually co -elute but that may be separated as a result of minor changes in the chromatographic conditions,

[4](4*S*)-2-{[2-(4-Ethyl-2,3-dioxopiperazine-1-carboxamido)-2-phenyl-acetamido]methyl}-5,5-dimethylthiazolidine-4-carboxylic acid,

[5](2*R*,4*S*)-2-{(1R)-Carboxy[2-(4-ethyl-2,3-dioxopiperazine-1-carboxamido)-2-phenylacetamido]methyl}-5,5-dimethylthiazolidine-4-carboxylic acid,

[6](2*R*,4*S*)-3-Acetyl-2-{(1*R*)-carboxy[2-(4-ethyl-2,3-dioxopiperazine-1-carboxamido)-2-phenylacetamido]methyl}-5,5-dimethylthiazolidine-4-carboxylic acid.

Inject reference solution (c) and (d). The test is not valid unless the resolution between tazobactam related compound A and tazobactam peak is not less than 3.0 in the chromatogram obtained with reference solution (c), the tailing factor for tazobactam and piperacillin peak is not more than 1.8 and the relative standard deviation for replicate injections is not more than 2.0 per cent for each component in the chromatogram obtained with reference solution (d).

Inject reference solution (d) and the test solution. In the chromatogram obtained with the test solution the area of any peak corresponding to tazobactam related compound A, piperacillin penilloic acid, piperacillin impurity B, C, D and acetylated penicilloic acid of piperacillin, each of, is not more than 0.1 times the area of the piperacillin peak in the chromatogram obtained with reference solution (d) (1.0 per cent), the area of any peak corresponding to piperacillin penicilloic acid is not more than 0.5 times the area of the piperacillin peak in the chromatogram obtained with reference solution (d) (5.0 per cent), the area of any other secondary peak is not more than 0.1 times the area of the piperacillin peak in the chromatogram obtained with reference solution (d) (1.0 per cent) and the sum of the areas of all the secondary peaks other than piperacillin penicilloic acid peak is not more than 0.5 times the area of the piperacillin peak in the chromatogram obtained with reference solution (d) (5.0 per cent).

Water (2.3.43). Not more than 2.5 per cent.

Bacterial endotoxins (2.2.3). Not more than 0.08 Endotoxin Unit per mg in a portion equivalent to 1 mg of a mixture of piperacillin and tazobactam (0.89 mg and 0.11 mg).

Assay. Determine by liquid chromatography (2.4.14)

NOTE—Use freshly prepared solutions, store and inject at 2° to 8°. Analyze within 24 hours of preparation.

Test solution. Determine the weight of the content of 10 containers. Dissolve an accurately weighed quantity of the substance under examination to obtain a solution containing 0.1 per cent w/v of piperacillin and 0.0125 per cent w/v of tazobactam in the mobile phase.

Reference solution. A solution containing 0.1 per cent w/v of *piperacillin IPRS* and 0.0125 per cent w/v of *tazobactam IPRS* in the mobile phase.

Chromatographic system
– a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
– sample temperature: 2° to 8°,
– mobile phase: a mixture of 0.8 volumes of solution prepared by diluting 80.0 ml of 40.0 per cent w/v of *tetrabutylammonium hydroxide* in 100 ml of *water*, 5 volumes of a buffer solution prepared by dissolving 2.76 g of *monobasic sodium phosphate* in 100.0 ml of *water*, 51 volumes of *methanol*, and 43.2 volumes of *water*, adjusted to pH 5.5 with *orthophosphoric acid*.
– flow rate: 1 ml per minute,
– spectrophotometer set at 230 nm,
– injection volume: 10 µl.

The relative retention time for tazobactam with respect to piperacillin is about 0.36.

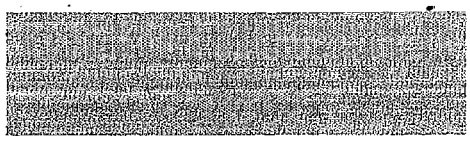
Inject the reference solution. The test is not valid unless the tailing factor for tazobactam and piperacillin peaks is not more than 2.0 and the relative standard deviation of replicate injections is not more than 2.0 per cent for each component.

Inject the reference solution and the test solution.

Calculate the contents of $C_{23}H_{27}N_5O_7S$ and $C_{10}H_{12}N_4O_5S$ in the injection.

Storage. Store at controlled room temperature.

Labelling. The label states the quantity of piperacillins sodium, in terms of the equivalent amount of piperacillin, and the quantity of Tazobactam Sodium, in terms of the equivalent amount of tazobactam.



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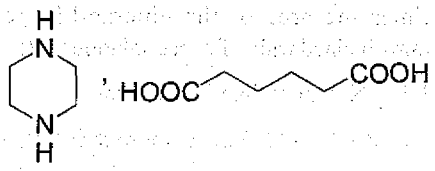
Piperazine Adipate



$C_4H_{10}N_2, C_6H_{10}O_4$ Mol. Wt. 232.3

Piperazine Adipate contains not less than 98.0 per cent and not more than 101.0 per cent of $C_4H_{10}N_2$, $C_6H_{10}O_4$, calculated on the anhydrous basis.

Category. Anthelmintic.

Description. A white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *piperazine adipate IPRS* or with the reference spectrum of piperazine adipate.

B. In the test for Related substances, examine the plate after spraying with both the *ninhydrin* and *iodine* solutions. The principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (a).

C. Dissolve 0.1 g in 5 ml of *water*, add 0.5 g of *sodium bicarbonate*, 0.5 ml of a freshly prepared 5.0 per cent w/v solution of *potassium ferricyanide* and 0.1 ml of *mercury*, shake vigorously for 1 minute and allow to stand for 20 minutes; a reddish colour slowly develops.

Tests

Appearance of solution. A 5.0 per cent w/v solution is clear (2.4.1), and not more intensely coloured than reference solution BS8 (2.4.1).

pH (2.4.24). 5.0 to 6.0, determined in a 5.0 per cent w/v solution.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. 60 volumes of *strong ammonia solution* and 40 volumes of *ethanol*.

Mobile phase. A freshly prepared mixture of 80 volumes of *acetone* and 20 volumes of *strong ammonia solution*.

Test solution (a). Dissolve 1 g of the substance under examination in 10 ml of the solvent mixture.

Test solution (b). Dissolve 1 g of the substance under examination in 100 ml of the solvent mixture.

Reference solution (a). A 1.0 per cent w/v solution of *piperazine adipate IPRS* in the solvent mixture.

Reference solution (b). A 0.025 per cent w/v solution of *ethylenediamine* in the solvent mixture.

Reference solution (c). A 0.025 per cent w/v solution of *triethylenediamine* in the solvent mixture.

Reference solution (d). A solution containing 0.025 per cent w/v of *triethylenediamine* and 1 per cent w/v of the substance under examination in the solvent mixture.

Apply to the plate 5 µl of each solution. After development, dry the plate at 105°, spray with a 0.3 per cent w/v solution of *ninhydrin* in a mixture of 3 volumes of *anhydrous acetic acid* and 100 volumes of *1-butanol* and then with a 0.15 per cent w/v solution of *ninhydrin* in *ethanol* and dry at 105° for 10 minutes. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b). Spray the plate with *0.05 M iodine* and allow to stand for about 10 minutes. Any spot corresponding to *triethylenediamine* in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (c). The test is not valid unless the chromatogram obtained with reference solution (d) shows two separated spots. Ignore any spot remaining on the line of application.

Heavy metals (2.3.13). Dissolve 1.0 g in 20 ml of *water*, 0.5 ml of *0.1 M hydrochloric acid* and add sufficient *water* to produce 25 ml. The resulting solution complies with the limit test for heavy metals, Method A (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.5 per cent, determined on 1.0 g.

Assay. Dissolve 0.2 g in 10 ml of *anhydrous glacial acetic acid* with gentle heating and dilute to 70 ml with the same solvent. Titrate with *0.1 M perchloric acid*, using 0.25 ml of *1-naphtholbenzein solution* as indicator and titrating until the colour of the solution changes from brownish yellow to green. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01161 g of $C_4H_{10}N_2, C_6H_{10}O_4$.

Storage. Store protected from moisture.

Piperazine Adipate Tablets

Piperazine Adipate Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of piperazine hydrate, $C_4H_{10}N_2, 6H_2O$.

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Usual strength. The equivalent of 300 mg of piperazine hydrate. (144 mg of Piperazine Adipate is approximately equivalent to 120 mg of Piperazine Hydrate).

Identification

A. Extract a quantity of the powdered tablets containing 1 g of Piperazine Hydrate with 20 ml of *water* and filter. Dilute 1 ml of the filtrate to 5 ml with *water*, add 0.5 g of *sodium bicarbonate*, 0.5 ml of a freshly prepared 5.0 per cent w/v solution of *potassium ferricyanide* and 0.1 ml of *mercury*, shake vigorously for 1 minute and allow to stand for 20 minutes; a reddish colour slowly develops.

B. To 10 ml of the filtrate obtained in test A add 5 ml of *hydrochloric acid* and extract with three quantities, each of 10 ml, of *ether*, evaporate the combined *ether* extracts to dryness; the residue, after washing with a small volume of *water* and drying at 105°, melts at about 152° (2.4.21).

Tests

Other tests. Comply with the tests stated under Tablets.

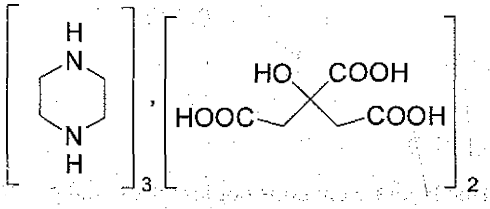
Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.2 g of Piperazine Hydrate, shake with 10 ml of *water* for 1 hour, filter and wash the residue with two quantities, each of 10 ml, of *water*. To the combined extract and washings add 5 ml of *1 M sulphuric acid* and 50 ml of *picric acid solution*, bring to boil, allow to stand for 1 hour and filter through a sintered-glass crucible (porosity No. 4) and wash the residue with successive quantities, each of 10 ml, of a mixture of equal volumes of a saturated solution of *picric acid* and *water* until the washings are free from sulphate. Wash with five quantities, each of 10 ml, of *ethanol* and dry to constant weight at 105°.

1 g of the residue is equivalent to 0.3567 g of $C_4H_{10}N_2, 6H_2O$.

Storage. Store protected from moisture.

Labelling. The label states the strength in terms of the equivalent amount of piperazine hydrate.

Piperazine Citrate



$(C_4H_{10}N_2)_3,2C_6H_8O_7$ Mol. Wt. 642.7 (anhydrous)

Piperazine Citrate is a salt of piperazine with citric acid containing a variable amount of water of crystallisation.

Piperazine Citrate contains not less than 98.0 per cent and not more than 101.0 per cent of $(C_4H_{10}N_2)_3, 2C_6H_8O_7$, calculated on the anhydrous basis.

Category. Anthelmintic.

Description. A white, granular powder.

Identification

Test A may be omitted if tests B and C are carried out. Test B may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *piperazine citrate IPRS* or with the reference spectrum of piperazine citrate.

B. In the test for Related substances, examine the plate after spraying with both the ninhydrin solutions. The principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (a).

C. A 10 per cent w/v solution gives the reactions of citrates (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution is clear (2.4.1), and not more intensely coloured than reference solution BS8 (2.4.1).

pH (2.4.24). 5.0 to 6.0, determined in a 5.0 per cent w/v solution.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. 60 volumes of *strong ammonia solution* and 40 volumes of *ethanol*.

Mobile phase. A freshly prepared mixture of 80 volumes of *acetone* and 20 volumes of *strong ammonia solution*.

Test solution (a). Dissolve 1 g of the substance under examination in 10 ml of the solvent mixture.

Test solution (b). Dissolve 1 g of the substance under examination in 100 ml of the solvent mixture.

Reference solution (a). A 1.0 per cent w/v solution of *piperazine citrate IPRS* in the solvent mixture.

Reference solution (b). A 0.025 per cent w/v solution of *ethylenediamine* in the solvent mixture.

Reference solution (c). A 0.025 per cent w/v solution of *triethylenediamine* in the solvent mixture.

Reference solution (d). A solution containing 0.025 per cent w/v of *triethylenediamine* and 1 per cent w/v of the substance under examination in the solvent mixture.

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Apply to the plate 5 µl of each solution. After development, dry the plate at 105°, spray with a 0.3 per cent w/v solution of *ninhydrin* in a mixture of 3 volumes of *anhydrous acetic acid* and 100 volumes of *1-butanol* and then with a 0.15 per cent w/v solution of *ninhydrin* in *ethanol* and dry at 105° for 10 minutes. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b). Spray the plate with *0.05 M iodine* and allow to stand for about 10 minutes. Any spot corresponding to *triethylenediamine* in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (c). The test is not valid unless the chromatogram obtained with reference solution (d) shows two separated spots. Ignore any spot remaining on the line of application.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method A (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). 10.0 to 14.0 per cent, determined on 0.3 g.

Assay. Dissolve 0.2 g in 10 ml of *anhydrous glacial acetic acid* with gentle heating and dilute to 70 ml with the same solvent. Titrate with *0.1 M perchloric acid*, using 0.25 ml of *1-naphtholbenzein solution* as indicator and titrating until the colour of the solution changes from brownish yellow to green. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01071 g of $(C_4H_{10}N_2)_3, 2C_6H_8O_7$.

Storage. Store protected from light and moisture.

Piperazine Citrate Syrup

Piperazine Citrate Oral Solution; Piperazine Citrate Elixir

Piperazine Citrate Syrup is a solution of Piperazine Citrate in a suitable flavoured Vehicle.

Piperazine Citrate Syrup contains the equivalent of not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of piperazine hydrate, $C_4H_{10}N_2, 6H_2O$.

Usual strength. The equivalent of 750 mg of piperazine hydrate in 5 ml.

(125 mg of Piperazine Citrate or 110 mg of anhydrous piperazine citrate is approximately equivalent to 100 mg of Piperazine Hydrate).

Identification

A. To 1 ml add 5 ml of *2 M hydrochloric acid* and, with stirring, 1 ml of a freshly prepared 50 per cent w/v solution of *sodium nitrite*, cool in ice for 15 minutes, induce crystallisation, wash the crystalline precipitate with *water* and dry at 105°. The crystals melt at about 159° (2.4.21).

B. Warm 10 ml with *activated charcoal* and filter. Boil a portion of the filtrate with an excess of *mercuric sulphate solution*, filter, boil the filtrate and add 0.25 ml of *dilute potassium permanganate solution*; the permanganate solution is decolorised and a white precipitate is produced.

C. Acidify a portion of the filtrate obtained in test B with *1 M sulphuric acid*, add 0.25 ml of *dilute potassium permanganate solution*, warm until the colour is discharged and add an excess of *bromine water*; a white precipitate is produced either immediately or on cooling.

Tests

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Weigh a quantity containing about 0.2 g of Piperazine Hydrate, add 3.5 ml of *0.5 M sulphuric acid* and 10 ml of *water*, add 100 ml of *picric acid solution*, heat on a water-bath for 15 minutes, allow to stand for 1 hour and filter through a sintered-glass crucible (porosity No. 4). Wash the residue with successive quantities, each of 10 ml, of a mixture of equal volumes of a saturated solution of *picric acid* and *water* until the washings are free from sulphate. Wash the residue with five quantities, each of 10 ml, of *ethanol* and dry to constant weight at 105°.

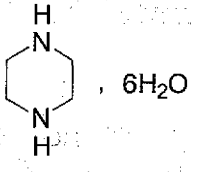
1 g of the residue is equivalent to 0.3567 g of $C_4H_{10}N_2, 6H_2O$.

Determine the weight per ml of the syrup (2.4.29), and calculate the content of $C_4H_{10}N_2, 6H_2O$, weight in volume.

Storage. Store protected from light.

Labelling. The label states the strength in terms of the equivalent amount of piperazine hydrate.

Piperazine Hydrate



$C_4H_{10}N_2, 6H_2O$ Mol. Wt. 194.2

Piperazine Hydrate contains not less than 98.0 per cent and not more than 101.0 per cent of $C_4H_{10}N_2, 6H_2O$.

Category. Anthelmintic.

Description. Colourless, glassy deliquescent crystals.

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Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *piperazine hydrate IPRS* or with the reference spectrum of piperazine hydrate.

B. In the test for Related substances, examine the plate after spraying with both the ninhydrin solutions. The principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (a).

C. Dissolve 0.2 g in 5 ml of *dilute hydrochloric acid*, add 0.5 g of *sodium nitrite* and heat to boiling. Cool in ice for 15 minutes, scratching the walls of the container with a glass rod and filter. The crystals, after washing with 10 ml of ice-cold *water* and drying at 105°, melt at about 159° (2.4.21).

Tests

Appearance of solution. A 5.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution BS8 (2.4.1).

pH (2.4.24). 10.5 to 12.0, determined in a 5.0 per cent w/v solution in *carbon dioxide-free water*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. 60 volumes of *strong ammonia solution* and 40 volumes of *ethanol*.

Mobile phase. A freshly prepared mixture of 80 volumes of *acetone* and 20 volumes of *strong ammonia solution*.

Test solution (a). Dissolve 1 g of the substance under examination in 10 ml of the solvent mixture.

Test solution (b). Dissolve 1 g of the substance under examination in 100 ml of the solvent mixture.

Reference solution (a). A 1 per cent w/v solution of *piperazine hydrate IPRS* in the solvent mixture.

Reference solution (b). A 0.025 per cent w/v solution of *ethylenediamine* in the solvent mixture.

Reference solution (c). A 0.025 per cent w/v solution of *triethylenediamine* in the solvent mixture.

Reference solution (d). A solution containing 0.025 per cent w/v of *triethylenediamine* and 1 per cent w/v of the substance under examination in the solvent mixture.

Apply to the plate 5 µl of each solution. After development, dry the plate at 105°, spray with a 0.3 per cent w/v solution of *ninhydrin* in a mixture of 3 volumes of *anhydrous acetic acid* and 100 volumes of *1-butanol* and then with a 0.15 per cent w/v solution of *ninhydrin* in *ethanol* and dry at 105° for 10 minutes. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b). Spray the plate with *0.05 M iodine* and allow to stand for about 10 minutes. Any spot corresponding to triethylenediamine in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (c). The test is not valid unless the chromatogram obtained with reference solution (d) shows two separated spots. Ignore any spot remaining on the line of application.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method A (20 ppm).

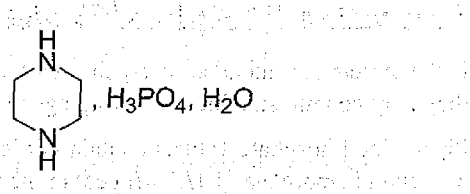
Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Dissolve 0.2 g in 10 ml of *anhydrous glacial acetic acid* with gentle heating and dilute to 70 ml with the same solvent. Titrate with *0.1 M perchloric acid*, using 0.25 ml of *1-naphtholbenzein solution* as indicator and titrating until the colour of the solution changes from brownish yellow to green. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.009705 g of $C_4H_{10}N_2, 6H_2O$.

Storage. Store protected from light and moisture.

Piperazine Phosphate



$C_4H_{10}N_2,H_3PO_4,H_2O$ Mol. Wt. 202.2

Piperazine Phosphate contains not less than 98.5 per cent and not more than 100.5 per cent of $C_4H_{10}N_2,H_3PO_4$, calculated on the anhydrous basis.

Category. Anthelmintic.

Description. A white, crystalline powder.

Identification

A. Dissolve 0.2 g in 5 ml of *dilute hydrochloric acid*, add 0.5 g of *sodium nitrite* and heat to boiling. Cool in ice for 15 minutes, scratching the walls of the container with a glass rod and filter. The crystals, after washing with 10 ml of ice-cold *water* and drying at 105°, melt at about 159° (2.4.21).

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B. Dissolve 0.1 g in 5 ml of *water*, add 0.5 g of *sodium bicarbonate*, 0.5 ml of a freshly prepared 5.0 per cent w/v solution of *potassium ferricyanide* and 0.1 ml of *mercury*, shake vigorously for 1 minute and allow to stand for 20 minutes; a reddish colour slowly develops.

C. It gives the reactions of phosphates (2.3.1).

Tests

pH (2.4.24). 6.0 to 6.5, determined in a 1.0 per cent w/v solution.

Heavy metals (2.3.13). Dissolve 1.0 g in 20 ml of *2 M acetic acid* and add sufficient *water* to produce 25 ml. The solution complies with the limit test for heavy metals, Method A (20 ppm).

Water (2.3.43). 8.0 to 9.5 per cent, determined on 0.25 g.

Assay. Dissolve 0.2 g in a mixture of 3.5 ml of *0.5 M sulphuric acid* and 10 ml of *water*. Add 100 ml of *picric acid solution*, heat on a water-bath for 15 minutes and allow to stand for 1 hour. Filter through a sintered-glass crucible (porosity No. 4) and wash the residue with successive quantities, each of 10 ml, of a mixture of equal volumes of a saturated solution of *picric acid* and *water* until the washings are free from sulphate. Wash the residue with five quantities, each of 10 ml, of *ethanol* and dry to constant weight at 105°.

1 g of the residue is equivalent to 0.3382 g of $C_4H_{10}N_2, H_3PO_4$.

Storage. Store protected from moisture.

Piperazine Phosphate Tablets

If the tablets are intended to be chewed before swallowing they may contain suitable flavouring agents.

Piperazine Phosphate Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of piperazine phosphate, $C_4H_{10}N_2, H_3PO_4, H_2O$.

Usual strengths. The equivalent of 260 mg; 520 mg Piperazine Hydrate (125 mg of piperazine phosphate is approximately equivalent to 120 mg of Piperazine Hydrate).

Identification

Extract a quantity of the powdered tablets containing 1 g of Piperazine Phosphate with 20 ml of *water* and filter. The filtrate complies with the following tests.

A. To 4 ml of the filtrate, add 1 ml of *hydrochloric acid*, 0.5 g of *sodium nitrite* and heat to boiling. Cool in ice for 15 minutes, scratching the walls of the container with a glass rod and filter. The crystals, after washing with 10 ml of ice-cold *water* and drying at 105°, melt at about 159° (2.4.21).

B. Dilute 1 ml of the filtrate to 5 ml with *water*, add 0.5 g of *sodium bicarbonate*, 0.5 ml of a freshly prepared 5.0 per cent w/v solution of *potassium ferricyanide* and 0.1 ml of *mercury*, shake vigorously for 1 minute and allow to stand for 20 minutes; a reddish colour slowly develops.

C. It gives the reactions of phosphates (2.3.1).

Tests

Disintegration. The test does not apply to Piperazine Phosphate Tablets intended to be chewed before swallowing.

Other tests. Comply with the tests stated under Tablets.

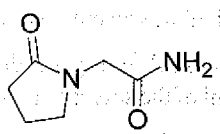
Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.15 g of Piperazine Phosphate, shake with 10 ml of *water* for 1 hour, filter and wash the residue with two quantities, each of 10 ml, of *water*. To the combined extract and washings add 5 ml of *1 M sulphuric acid* and 50 ml of *picric acid solution*, boil, allow the mixture to stand for several hours and filter through a sintered glass crucible (porosity No. 4). Wash the residue with successive quantities, each of 10 ml, of a mixture of equal volumes of a saturated solution of *picric acid* and *water* until the washings are free from sulphate. Wash the residue with five quantities, each of 10 ml, of *ethanol (95 per cent)* and dry to constant weight at 105°.

1 g of the residue is equivalent to 0.3714 g of $C_4H_{10}N_2, H_3PO_4, H_2O$.

Storage. Store protected from moisture.

Labelling. The label states, where applicable, that the tablets are to be chewed before swallowing.

Piracetam



$C_6H_{10}N_2O_2$ Mol.Wt.142.2

Piracetam is 2-(2-oxopyrrolidin-1-yl)acetamide .

Piracetam contains not less than 98.0 per cent and not more than 102.0 per cent of $C_6H_{10}N_2O_2$, calculated on the dried basis.

Category. Nootropic.

Description. A white or almost white powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *piracetam IPRS* or with the reference spectrum of piracetam.

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Tests

Appearance of solution. A 20.0 per cent w/v solution in *water* is clear (2.4.1) and colourless (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 10 volumes of *acetonitrile* and 90 volumes of *water*.

Test solution (a). Dissolve 50 mg of the substance under examination in 100.0 ml of the solvent mixture.

Test solution (b). Dilute 10.0 ml of test solution (a) to 50.0 ml with the solvent mixture.

Reference solution (a). Dissolve 5 mg of the substance under examination and 10 µl of 2-pyrrolidone in sufficient solvent mixture to produce 100.0 ml.

Reference solution (b). Dilute 1.0 ml of test solution (a) to 100.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution (c). Dissolve 50.0 mg of *piracetam IPRS* in 100.0 ml of the solvent mixture. Dilute 10.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with end capped octadecylsilane bonded to silica (5 µm),
- mobile phase: a mixture of 10 volumes of *acetonitrile* and 90 volumes of 0.1 per cent w/v solution of *dipotassium hydrogen phosphate*, adjusted to pH 6.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to 2-pyrrolidone and piracetam is not less than 3.0 and the symmetry factor is not more than 2.0 for the peak due to piracetam.

Inject reference solution (b) and test solution (a). Run the chromatogram 8 times the retention time of piracetam. In the chromatogram obtained with the test solution (a), the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy Metals (2.3.13). 2.0 g complies with limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Determine by liquid chromatography (2.4.14) as described under test for Related substances.

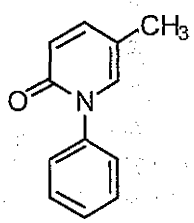
Inject reference solution (c). The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (c) and test solution (b).

Calculate the content of $C_6H_{10}N_2O_2$.

Storage. Store protected from light and moisture.

Pirfenidone



$C_{12}H_{11}NO$ Mol. Wt.185.2

Pirfenidone is 5-methyl-1-phenylpyridin-2-one.

Pirfenidone contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{12}H_{11}NO$, calculated on anhydrous basis.

Category. Antifibrotic and anti-inflammatory.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pirfenidone IPRS* or with the reference spectrum of pirfenidone.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 90 volumes of mobile phase A and 10 volumes of mobile phase B.

Test solution. Dissolve 25 mg of the substance under examination in the solvent mixture and dilute to 25.0 ml with the solvent mixture.

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Reference solution. A mixture of 0.0001 per cent w/v solution of *pirfenidone IPRS* and 0.00015 per cent w/v solution of *2-hydroxy-5-picoline IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Alltima C18),
- mobile phase: A. *0.01M sodium dihydrogen phosphate,* adjusted to pH 3.0 with *orthophosphoric acid,*
  B. *acetonitrile,*
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	75	25
5	75	25
40	30	70
50	30	70
50.1	75	25
60	75	25

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0 for pirfenidone peak.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to 2-hydroxy-5-picoline is not more than the area of the principal peak of 2-hydroxy-5-picoline in the chromatogram obtained with the reference solution (0.15 per cent), the area of any other secondary peak is not more than the area of the principal peak of pirfenidone in the chromatogram obtained with the reference solution (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 10 times the area of the principal peak of pirfenidone in the chromatogram obtained with the reference solution (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.5 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.01 per cent w/v solution of *pirfenidone IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Alltima C18),
- mobile phase: a mixture of 600 volumes of *0.01M sodium dihydrogen phosphate,* adjusted to pH 3.0 with *orthophosphoric acid* and 400 volumes of *acetonitrile,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 8000 theoretical plates, the tailing factor is not more than 2.0, and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{12}H_{11}NO$.

Storage. Store protected from light and moisture.

Pirfenidone Tablets

Pirfenidone Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pirfenidone, $C_{12}H_{11}NO$.

Usual strengths. 200 mg; 400 mg.

Identification

When examined in the range 230 nm to 400 nm (2.4.7), the final solution obtained in the Assay shows absorption maxima at about 314 nm.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium: 900 ml of *0.1 M hydrochloric acid,*
Speed and time. 50 rpm for 45 minutes.

Withdraw a suitable volume of the medium and filter. Dilute the filtrate, if necessary, with the dissolution medium. Measure the absorbance of the solution at the maximum at about 310 nm (2.4.7.) Calculate the content of $C_{12}H_{11}NO$ in the medium from the absorbance of *pirfenidone IPRS* prepared by dissolving in minimum amount of *methanol* and diluting with the dissolution medium.

Q. Not less than 75 per cent of the stated amount of $C_{12}H_{11}NO$.

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Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing 200 mg of pirfenidone in 140 ml of mobile phase with the aid of ultrasound for 10 minutes and dilute to 200.0 ml with the mobile phase and filter.

Reference solution. A 0.0005 per cent w/v solution of *pirfenidone IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS 3V),
- mobile phase: a mixture of 70 volumes of buffer solution containing 0.27 per cent w/v solution of *potassium dihydrogen orthophosphate*, adjusted to pH 3.2 with *orthophosphoric acid* and 30 volumes of *methanol*,
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 310 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

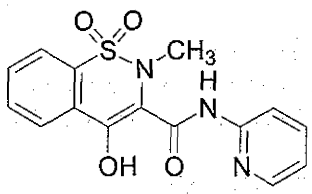
Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Transfer a quantity of the powder containing 2.0 g of Pirfenidone in 10 ml of *water* with the aid of ultrasound for 10 minutes. Add 500 ml of *methanol* and dilute to 1000.0 ml with *methanol*, mix and filter. Dilute 4.0 ml of the solution to 500.0 ml with a mixture of 600 volumes of 0.27 per cent w/v of *potassium dihydrogen orthophosphate* adjusted to pH 3.2 with *orthophosphoric acid* and 400 volumes of *methanol*. Measure the absorbance of the resulting solution at the maximum at about 314 nm (2.4.7). Calculate the content of $C_{12}H_{11}NO$ from the absorbance obtained by dissolving 64 mg of *pirfenidone IPRS* in 100 ml *methanol* and dilute 5 ml of the solution to 200 ml with the solvent mixture.

Storage. Store protected from moisture.

Piroxicam



$C_{15}H_{13}N_3O_4S$ Mol. Wt. 331.4

Piroxicam is 4-hydroxy-2-methyl-*N*-(2-pyridyl)-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide.

Piroxicam contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{15}H_{13}N_3O_4S$, calculated on the anhydrous basis.

Category. Analgesic; antiinflammatory; antipyretic.

Description. An off-white to light tan or light yellow powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *piroxicam IPRS* or with the reference spectrum of piroxicam.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *0.01 M methanolic hydrochloric acid* shows absorption maxima at about 242 nm and 334 nm and a minimum at about 270 nm; absorbance at about 334 nm, about 0.87.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 95 volumes of *toluene* and 5 volumes of *acetic acid*.

Test solution. Dissolve 0.1 g of the substance under examination in 100 ml of a mixture of equal volumes of *chloroform* and *methanol*.

Reference solution. A 0.1 per cent w/v solution of *piroxicam IPRS* in a mixture of equal volumes of *chloroform* and *methanol*.

Apply to the plate 20 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 75 mg of the substance under examination in 50.0 ml of *acetonitrile*.

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Reference solution. Dilute 1.0 ml of the test solution to 10.0 ml with *acetonitrile*. Dilute 1.0 ml of the solution to 50.0 ml with *acetonitrile*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 40 volumes of *acetonitrile* and 60 volumes of a 0.7 per cent w/v solution of *potassium dihydrogen phosphate*, adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

The relative retention time with reference to piroxicam for piroxicam impurity B is about 0.85.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates.

Inject the reference solution and the test solution. Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent), the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (0.4 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.02 per cent).

Heavy metals (2.3.13). 0.4 g complies with the limit test for heavy metals, Method B (50 ppm).

Sulphated ash (2.3.18). Not more than 0.3 per cent.

Water (2.3.43). Not more than 0.5 per cent , determined on 2.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. A 0.005 per cent w/v solution of the substance under examination in *0.1 M methanolic hydrochloric acid*.

Reference solution. A 0.005 per cent w/v solution of *piroxicam IPRS* in *0.1 M methanolic hydrochloric acid*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 55 volumes of a buffer solution prepared by diluting a mixture of 7.72 g of *anhydrous citric acid* in 400 ml of *water* and 5.35 g of *sodium phosphate* in 100 ml of *water* to 1000 ml with *water* and 45 volumes of *methanol*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{13}N_3O_4S$.

Storage. Store protected from light and moisture.

Piroxicam Capsules

Piroxicam Capsules contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of piroxicam, $C_{15}H_{13}N_3O_4S$.

Usual strengths. 10 mg; 20 mg.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 95 volumes of *toluene* and 5 volumes of *acetic acid*.

Test solution. Dissolve a portion of the contents of the capsules in sufficient of a mixture of equal volumes of *chloroform* and *methanol* to obtain a solution containing about 0.1 per cent w/v of Piroxicam. Shake for 10 minutes, filter and use the filtrate.

Reference solution. A 0.1 per cent w/v solution of *piroxicam IPRS* in a mixture of equal volumes of *chloroform* and *methanol*.

Apply to the plate 20 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 100 rpm and 45 minutes.

Withdraw 10 ml of the medium and filter. Measure the absorbance of the filtrate (2.4.7), suitably diluted if necessary, at the maximum at about 242 nm. Calculate the content of $C_{15}H_{13}N_3O_4S$, in the medium taking 352 as the specific absorbance at 242 nm.

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Q. Not less than 75 per cent of the stated amount of $C_{15}H_{13}N_3O_4S$.

Uniformity of content. Complies with the test stated under Capsules.

Test solution. Dissolve the contents of a capsule in 100.0 ml of *0.1 M methanolic hydrochloric acid* and filter. Dilute further if necessary.

Determine by liquid chromatography (2.4.14) using the chromatographic system and the reference solution described under Assay.

Calculate the content of $C_{15}H_{13}N_3O_4S$ in the capsules.

Water (2.3.43). Not more than 8.0 per cent, determined on 0.25 g.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve a weighed quantity of the mixed contents of the capsules containing about 50 mg of Piroxicam in 100.0 ml of *0.1 M methanolic hydrochloric acid*. Dilute 1.0 ml of the solution to 10.0 ml with the same solvent.

Reference solution. A 0.005 per cent w/v solution of *piroxicam IPRS* in *0.1 M methanolic hydrochloric acid*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 45 volumes of *methanol* and 55 volumes of a buffer solution prepared by diluting a mixture of 7.72 g of *anhydrous citric acid* in 400 ml of *water* and 5.35 g of *sodium phosphate* in 100 ml of *water* to 1000 ml with *water*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{13}N_3O_4S$ in the capsules.

Storage. Store protected from light and moisture.

Piroxicam Gel

Piroxicam Gel contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of piroxicam, $C_{15}H_{13}N_3O_4S$.

Usual strength. 0.5 per cent w/w.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Test solution. Disperse a quantity of gel containing 10 mg of piroxicam, with 0.1 ml of a saturated solution of *sodium chloride* until the mixture becomes turbid. Dilute to 5.0 ml with *0.01 M methanolic hydrochloric acid*, shake well, centrifuge and use the clear supernatant solution.

Reference solution. A 0.2 per cent w/v solution of *piroxicam IPRS* in *0.01 M methanolic hydrochloric acid*.

Mobile phase. A mixture of 1 volume of *glacial acetic acid* 10 volumes of *methanol* and 80 volumes of *ethyl acetate*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 7.2 to 8.2 determined in a 10 per cent w/v solution.

2-Pyridylamine. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the gel containing 5 mg of Piroxicam in 5 ml of *0.01 M methanolic hydrochloric acid* add 50 ml of the mobile phase and shake vigorously for 30 minutes, dilute to 100.0 ml with mobile phase, mix and filter through a glass fibre membrane filter (1µm).

Reference solution. Dilute 5.0 ml of a 0.0005 per cent w/v solution of 2-pyridylamine in *0.01 M methanolic hydrochloric acid* to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (such as zorbax stable bond C 8) and stainless steel guard column 12.5 mm × 4.6 mm, packed with octylsilane bonded to porous silica (5µm),
- column temperature: 40°,
- mobile phase: a mixture of 15 volumes of *methanol*, 30 volumes of *acetonitrile* 55 volumes of *0.05 M sodium dihydrogen orthoposphate*, adjusted to pH 3.5 with *5 M orthophosphoric acid*,
- flow rate: 1 ml per minute,
- fluorimetric detector set at excitation wavelength of 313 nm and an emission wavelength of 380 nm,
- injection volume: 20 µl.

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Inject the reference solution and the test solution. The area of any peak corresponding to 2- pyridylamine is not more than that of the principal peak in the chromatogram obtained with reference solution (0.5 per cent).

Other tests. Comply with the tests stated under Gel.

Assay. Determine by liquid chromatography (2.4.14).

Use chromatographic system and test solution as described as under 2-pyridylamine except detector.

Reference solution. A 0.1 per cent w/v solution of *piroxicam IPRS* in *0.01 M methanolic hydrochloric acid,* dissolve with the aid of ultrasound, if necessary. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

Chromatographic system

- spectrophotometer set at 248 nm.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{13}N_3O_4S$ in gel.

Storage. Store at a temperature not exceeding 30°. Do not freeze.

Piroxicam Tablets

Piroxicam Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of piroxicam, $C_{15}H_{13}N_3O_4S$.

Usual strength. 20 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution correspond to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of *0.1 M hydrochloric acid,*
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate at the maximum at about 242 nm (2.4.7). Calculate the content of $C_{15}H_{13}N_3O_4S$ in the medium from the absorbance obtained from a solution of known concentration of *piroxicam IPRS* in the same medium.

Q. Not less than 75 per cent of the stated amount of $C_{15}H_{13}N_3O_4S$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Dissolve a quantity of the powder containing about 50 mg of Piroxicam in 100.0 ml of *0.1 M methanolic hydrochloric acid.* Dilute 1.0 ml of the solution to 10.0 ml with the same solvent.

Reference solution. A 0.005 per cent w/v solution of *piroxicam IPRS* in *0.1 M methanolic hydrochloric acid.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 45 volumes of *methanol* and 55 volumes of a buffer solution prepared by diluting a mixture of 7.72 g of *anhydrous citric acid* in 400 ml of *water* and 5.35 g of *sodium phosphate* in 100 ml of *water* to produce 1000 ml with *water;*
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

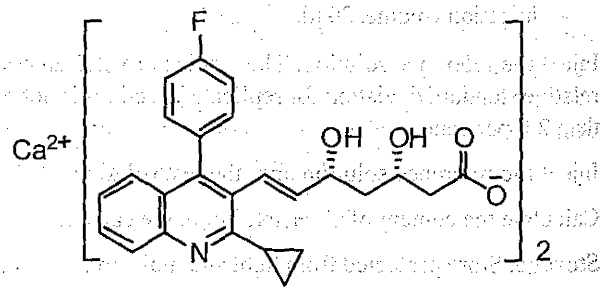
Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{13}N_3O_4S$ in the tablets.

Storage. Store protected from light and moisture.

Pitavastatin Calcium



$C_{50}H_{46}CaF_2N_2O_8$ Mol. Wt. 880.9

Pitavastatin Calcium is Monocalcium bis{(3*R*,5*S*,6*E*)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate}.

Pitavastatin Calcium contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{50}H_{46}CaF_2N_2O_8$, calculated on the anhydrous basis.

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Category. Antihyperlipidemic.

Description. An off-white to yellowish, powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pitavastatin calcium IPRS* or with the reference spectrum of pitavastatin calcium.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). + 9.0° to + 13.0°, determined in a 1.0 per cent w/v solution in *dimethylsulphoxide*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 365 volumes of *acetonitrile*, 50 volumes of *tetrahydrofuran* and 585 volumes of buffer solution prepared by dissolving 1.54 per cent w/v solution of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 4.0 with *glacial acetic acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 20 µl.

Inject the test solution. The area of any secondary peak is not more than 0.2 per cent and the sum of the areas of all the secondary peaks is not more than 1.0 per cent, calculated by area normalization.

Calcium. 4.0 to 5.0 per cent.

Weigh 0.2 g and dissolve in equal volumes of *methanol* and *ammonia-ammonium chloride buffer*. Titrate with *0.05 M ethylenediamine tetraacetate* and determine the end point using *erichrome black T* indicator.

1 ml of *0.05 M ethylenediamine tetraacetate* is equivalent to 0.002 g of *calcium*.

Water (2.3.43). Not more than 5.0 per cent, determined on 0.1 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.01 per cent w/v solution of *pitavastatin calcium IPRS* in the mobile phase.

Use chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3500 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{50}H_{46}CaF_2N_2O_8$.

Plaster of Paris

Dried Calcium Sulphate; Exsiccated Calcium Sulphate

$CaSO_4, ½H_2O$ Mol. Wt. 145.1

Plaster of Paris is prepared by heating powdered gypsum, $CaSO_4,2H_2O$, at about 150° in a controlled manner such that it is substantially converted into the hemihydrate, $CaSO_4,½H_2O$, with minimum production of the anhydrous phases of calcium sulphate. It may contain suitable accelerators or decelerators.

Category. Surgical aid.

Description. A white or almost white powder; hygroscopic.

Identification

It gives the reactions of calcium salts and of sulphates (2.3.1).

Tests

pH (2.4.24). 6.5 to 9.0, determined in a 20.0 per cent w/v slurry in *water*.

Acid insoluble matter. Dissolve 0.5 g in 30 ml of a mixture of 1 volume of *hydrochloric acid* and 2 volumes of *water* and evaporate to dryness in a dish on a water-bath. Heat for 2 hours at 120° and again add 20 ml of the acid mixture. Warm for a few minutes and filter. Wash the residue with warm *water* until free from chlorides, dry, ignite and weigh. The residue weighs not more than 5 mg (1.0 per cent).

Setting properties. 20 g mixed with 10 ml of *water* at 15° to 20° in a cylindrical mould about 2.4 cm in diameter sets in not less than 4 minutes and not more than 6 minutes. The mass thus formed, after standing for 3 hours, possesses sufficient hardness to resist pressure of the fingers at the edges, which retain their sharpness of outline and do not crumble under pressure.

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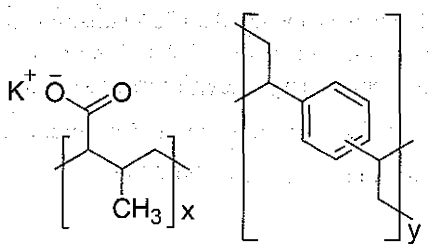
Loss on ignition (2.4.20). 4.5 to 8.0 per cent, determined by igniting to constant weight at red heat.

Storage. Store protected from moisture.

Polacrilin Potassium



Polacrillin Potassium is 2-propenoic acid, 2-methyl-, potassium salt, polymer with diethenylbenzene.

Polacrilin Potassium is the potassium salt of a unifunctional low-cross-linked carboxylic cation-exchange resin prepared from methacrylic acid and divinylbenzene.

Polacrilin Potassium contains not less than 20.6 per cent and not more than 25.1 per cent of potassium, calculated on the dried basis.

Category. Pharmaceutical aid.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *polacrilin potassium IPRS* or with the reference spectrum of polacrilin potassium.

B. Shake 1 g with 10 ml of *water:* the aqueous phase does not respond to the tests for potassium salts (2.3.1). Shake 1 g with 10 ml of *0.1 M hydrochloric acid*; the aqueous phase responds to the tests for potassium salts (2.3.1).

Tests

Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 1 g by drying at 105°, for 6 hours.

Iron (2.3.14). Not more than 0.01 per cent, using the following test solution.

Transfer 0.4 g to a suitable crucible, and ignite at a low heat until thoroughly ashed. Add to the carbonized mass 2 ml of *nitric acid* and 5 drops of *sulphuric acid*, and heat cautiously until white fumes are no longer evolved. Ignite, preferably in a muffle furnace, at 500° to 600°, until the carbon is completely burned off. Cool, add 4 ml of *6 M hydrochloric acid*, cover, digest on a steam bath for 15 minutes, uncover, and slowly evaporate on a steam-bath to dryness. Moisten the residue with 1 drop of *hydrochloric acid*, add 10 ml of hot *water*, and digest for 2 minutes. Dilute with *water* to 25 ml. Filter, if necessary, rinse the crucible and the filter with 10 ml of *water*, combining the filtrate and rinsing in a 50-ml colour-comparison tube, add 2 ml of *hydrochloric acid*, dilute with water to 45 ml, and mix.

Sodium. Not more than 0.2 per cent.

Test solution. Transfer 2 g, weighed, to a 400-ml borosilicate beaker, add 20 ml of *sulphuric acid*, cover with a borosilicate watch glass, and heat until charring is complete. While continuing to heat the beaker, add 20 ml of *nitric acid* dropwise. Continue to heat and add *nitric acid* until all of the organic material has been destroyed as indicated by the contents of the beaker turning from brown to a very pale straw-coloured or colourless solution. Continue to evaporate the solution, and if it turns brown during the evaporation, add *nitric acid* dropwise until the brown colour disappears. Evaporate just to dryness, cool, and dissolve the residue in 40 ml of *water* and 10 ml of *6 M hydrochloric acid*. Heat to boiling, cool and dilute to 100.0 ml with *water*.

To three separate 100-ml volumetric flasks add, respectively, 1.0 ml, 2.0 ml, and 3.0 ml of a solution containing 254.2 mg of *sodium chloride* in 1000 ml of *water*. Add water to volume, and mix to obtain sodium chloride solutions having concentrations equivalent to 1 µg of Na per ml, 2 µg of Na per ml, and 3 µg of Na per ml, respectively. Adjust the settings of a suitable flame photometer so that the emission of the solution containing 3.0 ml of the sodium chloride solution reads close to 100 per cent at 589 nm. Determine the readings of the three solutions at 589 nm. Readjust the wavelength setting to 580 nm, and determine the background emission reading for one of these standards. Pipet 5 ml of the test solution and dilute to 100 ml with *water*. Observe the emission reading of the solution at 589 nm, using the same instrument settings, then readjust the wavelength setting to 580 nm, and observe the background emission reading. Subtract the corresponding background readings from the standard and test specimen readings. Prepare a standard curve by plotting the corrected standard readings versus the square root of the sodium concentration. From this standard curve, determine the sodium content of the test solultion.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Potassium

Sodium stock solution. Dissolve 7.306 g of *sodium chloride* (previously dried at 125° for 30 minutes) in *water* and dilute to 500.0 ml with *water* (5.76 g of Na per 1000 ml).

Potassium stock solution. Dissolve 745.5 mg of *potassium chloride* (previously dried at 125° for 30 minutes) and dilute to 1000 ml with *water* (391 mg of K per 1000 ml).

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Surfactant solution. Transfer 5.0 g of a suitable nonionic surfactant to a 250-ml beaker, add 200 ml of *water*, and stir to dissolve. Transfer this solution to a 500-ml volumetric flask and dilute with *water* to volume.

(NOTE — To prevent foaming when using this solution, gently run the solution down the sides of the vessel, and use gentle action when mixing).

Diluted sodium solution. Dilute a mixture of 50.0 ml of sodium stock solution and 10.0 ml of surfactant solution to 100.0 ml with *water*.

Reference solutions. To three separate 500-ml volumetric flasks transfer, respectively, 3.0 ml, 4.0 ml, and 5.0 ml portions of potassium stock solutions. To each flask, add 50.0 ml of sodium stock solution and 10.0 ml of surfactant solution and dilute to 500.0 ml with *water*. 1 ml of these solutions contains 2.346 µg, 3.128 µg, and 3.910 µg of K, respectively.

Test solution. Transfer 1.4 g of Polacrilin Potassium (previously dried) to a 50-ml silica crucible, moisten with 4 ml of *sulphuric acid*, heat over a small flame until the acid has fumed off, moisten the residue with a few drops of *sulphuric acid*, and ignite strongly. Allow to cool, transfer, with the aid of *water* and dilute to 1000 ml with *water*. To 1.0 ml of the solution, add 20 ml of *diluted sodium solution* and dilute to 100.0 ml with *water*, mix gently to avoid foaming.

Determine the emittances of the reference solutions and the test solution at 766 nm, with a flame photometer. Prepare a standard curve by plotting the standard readings versus the square root of the potassium concentrations. From the curve, determine the concentration, C_U, in µg per ml, of potassium in the test solution.

Calculate the content of potassium in the Polacrilin Potassium by the formula:

$$100C_U$$

Storage. Store protected from moisture.

Poloxamers

$(CH_2CH_2O)_a\,(CH(CH_3)CH_2OH)_b\,(CH_2CH_2O)_c\,H$

b is at least 15 and $(CH_2CH_2O)_{a+c}$ is varied from 20 to 90% by weight

Mol. Wt. ranges from 1000 to >16000

Poloxamers are polyethylenepropylpropyleneglycols.

Synthetic block copolymer of ethylene oxide and propylene oxide represented by the following general formula:

Poloxamer Type	Ethylene oxide units (a)	Propylene oxide units (b)	Content of oxyethylene (per cent)	Average relative molecular mass
124	10-15	18-23	44.8-48.6	2090-2360
188	75-85	25-30	79.9-83.7	7680-9510
237	60-68	35-40	70.5-74.3	6840-8830
338	137-146	42-47	81.4-84.9	12700-17400
407	95-105	54.60	71.5-74.9	9840-14600

A suitable antioxidant may be added.

Category. Pharmaceutical aid.

Description. Poloxamer 124 is a colourless or almost colourless liquid and poloxamers 188, 237, 338, 407 are a white or almost white, waxy powder, microbeads or flakes.

Identification

Test A may be omitted if tests B and C are carried out. Test C may be omitted if tests A and B are carried out.

A. Compare the spectrum with that obtained with corresponding type of *poloxamer IPRS* or with the reference spectrum of corresponding type of poloxamer.

B. Average relative molecular mass.

C. Oxypropylene: oxyethylene ratio.

Tests

Melting point (2.4.21). About 50° for poloxamers 188, 237, 338 and 407.

Appearance of solution. A 10.0 per cent w/v solution in *carbon dioxide-free water* (solution A) is not more intensely coloured than reference solution BYS7 (2.4.1).

pH (2.4.24). 5.0 to 7.5, determined in solution A.

Ethylene oxide, propylene oxide and dioxan. Determine by gas chromatography (2.4.13).

Ethylene oxide stock solution. Disperse about 0.5 g of *ethylene oxide IPRS* with 50.0 ml of *dimethyl sulphoxide*.

Ethylene oxide solution. Dilute 1.0 ml of the ethylene oxide stock solution to 250.0 ml with *dimethyl sulphoxide*.

Propylene oxide stock solution. Introduce about 7 ml of *dichloromethane* into a volumetric flask, add 0.5 g of *propylene oxide* and dilute to 10.0 ml with *dichloromethane*. Dilute 0.5 ml of the solution to 50.0 ml with *dimethyl sulphoxide*.

Propylene oxide solution. Dilute 1.0 ml of the propylene oxide stock solution to 50.0 ml with *dimethyl sulphoxide*.

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Dioxan solution. Introduce 0.1 g of *dioxan* into a flask and dilute to 50.0 ml with *dimethyl sulphoxide*. Dilute 2.5 ml of the solution to 100.0 ml with *dimethyl sulphoxide*.

Mixture solution. Dilute a mixture of 6.0 ml of the ethylene oxide solution, 6.0 ml of the propylene oxide solution and 2.5 ml of the dioxan solution to 25.0 ml with *dimethyl sulphoxide*.

Test solution. To 1.0 g of the substance under examination in a head-space vial, add 4.0 ml of *dimethyl sulphoxide* and close the vial immediately.

Reference solution. To 1.0 g of the substance under examination in a head-space vial, add 2.0 ml of *dimethyl sulphoxide* and 2.0 ml of the mixture solution. Close the vial immediately.

Chromatographic system
- a fused silica column 50 m x 0.32 mm, packed with poly(dimethyl)(diphenyl)siloxane (film thickness 5 µm),
- temperature :
  column 70° to 240°,
  inlet port and detector at 250°,
- a flame ionisation detector,
- flow rate: 1.4 ml per minute using nitrogen as the carrier gas,

Head-space conditions
- equilibration temperature 110°,
- equilibration time 30 minutes,
- transfer line temperature 140°,
- pressurisation time 1 minute,
- injection time 0.05 minute.

Inject 1 µl of the gaseous phase of the test solution and the reference solution. The relative retention time with reference to ethylene oxide is about 6 minutes, for propylene oxide is about 1.3, for dichloromethane is about 1.6, for dioxan is about 3.0, for dimethyl sulphoxide is about 3.7.

The ethylene oxide is not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (1 ppm); propylene oxide is not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (5 ppm) and dioxan is not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (10 ppm).

Average relative molecular mass. Weigh 15 g of the substance under examination into a 250-ml ground glass stoppered flask, add 25.0 ml of *phthalic anhydride solution* and a few glass beads and swirl to dissolve. Boil gently under a reflux condenser for 60 minutes, cool and add 2 quantities, each of 10 ml, of *pyridine*, through the condenser. Add 10 ml of *water*, mix and allow to stand for 10 minutes. Add 40.0 ml of *0.5 M sodium hydroxide* and 0.5 ml of a 1.0 per cent w/v solution of *phenolphthalein* in *pyridine*. Titrate with *0.5 M sodium hydroxide* to a light pink endpoint that persists for 15 second and record the volume of sodium hydroxide used (S). Prepare a blank. Record the volume of sodium hydroxide used (B). Calculate the average relative molecular mass using the following expression:

$$\frac{4000m}{B-S}$$

Oxypropylene:oxyethylene ratio. Nuclear magnetic resonance spectrometry (2.4.34).

Use a 10.0 per cent w/v solution of the substance under examination in *deuterated chloroform*. Record the average area of the doublet appearing at about 1.08 ppm due to the methyl groups of the oxypropylene units (A_1) and the average area of the composite band from 3.2 ppm to 3.8 ppm due to CH_2O groups of both the oxyethylene and oxypropylene units and the CHO groups of the oxypropylene units (A_2) with reference to the internal standard. Calculate the percentage of oxyethylene, by weight, in the sample under examination using the following expression:

$$\frac{3300\alpha}{33\alpha + 58}$$

$$\text{where } \alpha = \frac{A_2}{A_1} - 1$$

Total ash (2.3.19). Not more than 0.4 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 1.0 g.

Storage. Store protected from moisture.

Labelling. The label states the type of poloxamer.

Polyethylene Glycol 1500

Macrogol 1500

Polyethylene Glycol 1500 is a mixture of the polycondensation products of ethylene oxide and water obtained under controlled conditions. It is represented by the formula $HOCH_2[CH_2OCH_2]_nCH_2OH$, where n is between 28 and 36.

Category. Pharmaceutical aid (ointment base).

Description. A white or creamy white, soft, wax-like solid.

Tests

Appearance of solution (2.4.1). A 25.0 per cent w/v solution is not more intensely coloured than reference solution BYS6.

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pH (2.4.24). 4.0 to 7.0, determined in a 5.0 per cent w/v solution.

Freezing point (2.4.11). 42° to 46°.

Hydroxyl value (2.3.27). 70 to 86, determined on 5.0 g.

Viscosity (2.4.28). 25 mm^2s^{-1} to 32 mm^2s^{-1}, determined at 100° by Method A using a U-tube viscometer (size D).

Arsenic (2.3.10). Mix 3.3 g with 3 g of *anhydrous sodium carbonate*, add 10 ml of *bromine solution* and mix thoroughly. Evaporate to dryness on a water-bath, gently ignite and dissolve the cooled residue in 16 ml of *brominated hydrochloric acid* and 45 ml of *water*. Remove the excess of bromine with 2 ml of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (3 ppm).

Heavy metals (2.3.13). Dissolve 4.0 g in 5 ml of a 1.0 per cent w/v solution of *hydrochloric acid* and sufficient *water* to produce 25 ml. The solution complies with the limit test for heavy metals, Method A (5 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Storage. Store protected from moisture.

Polyethylene Glycol 4000

Macrogol 4000

Polyethylene Glycol 4000 is a mixture of the polycondensation products of ethylene oxide and water obtained under controlled conditions. It is represented by the formula $HOCH_2[CH_2OCH_2]_nCH_2OH$, where n is between 69 and 84.

Category. Pharmaceutical aid (ointment base).

Description. A creamy white, hard, wax-like solid, powder or flakes.

Tests

Appearance of solution (2.4.1). A 20.0 per cent w/v solution is not more intensely coloured than reference solution BYS6.

pH (2.4.24). 4.5 to 7.5, determined in a 5.0 per cent w/v solution.

Freezing point (2.4.11). 53° to 56°.

Hydroxyl value (2.3.27). 25 to 32, determined on 14.0 g.

Viscosity (2.4.28). 110 mm^2s^{-1} to 158 mm^2s^{-1}, determined at 100° by Method A using a U-tube viscometer (size E).

Arsenic (2.3.10). Mix 3.3 g with 3 g of *anhydrous sodium carbonate*, add 10 ml of *bromine solution* and mix thoroughly. Evaporate to dryness on a water-bath, gently ignite and dissolve the cooled residue in 16 ml of *brominated hydrochloric acid* and 45 ml of *water*. Remove the excess of bromine with 2 ml of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (3 ppm).

Heavy metals (2.3.13). Dissolve 4.0 g in 5 ml of a 1.0 per cent w/v solution of *hydrochloric acid* and sufficient *water* to produce 25 ml. The solution complies with the limit test for heavy metals, Method A (5 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Storage. Store protected from moisture.

Polyethylene Glycol 6000

Macrogol 6000

Polyethylene Glycol 6000 is a mixture of the polycondensation products of ethylene oxide and water obtained under controlled conditions. It is represented by the formula $HOCH_2[CH_2OCH_2]nCH_2OH$, where n is between 112 and 158.

Category. Pharmaceutical aid (ointment base and tablet excipient).

Description. A white to creamy white, wax-like solid or flakes.

Tests

Appearance of solution (2.4.1). A 15.0 per cent w/v solution is not more intensely coloured than reference solution BYS6.

pH (2.4.24). 4.5 to 7.5, determined in a 5.0 per cent w/v solution.

Freezing point (2.4.11). 56° to 60°.

Viscosity (2.4.28). 250 mm^2s^{-1} to 390 mm^2s^{-1}, determined at 100° by Method A using a U-tube viscometer (size F).

Arsenic (2.3.10). Mix 3.3 g with 3 g of *anhydrous sodium carbonate*, add 10 ml of *bromine solution* and mix thoroughly. Evaporate to dryness on a water-bath, gently ignite and dissolve the cooled residue in 16 ml of *brominated hydrochloric acid* and 45 ml of *water*. Remove the excess of bromine with 2 ml of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (3 ppm).

Heavy metals (2.3.13). Dissolve 4.0 g in 5 ml of a 1.0 per cent w/v solution of *hydrochloric acid* and sufficient *water* to produce 25 ml. The resulting solution complies with the limit test for heavy metals, Method A (5 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Storage. Store protected from moisture.

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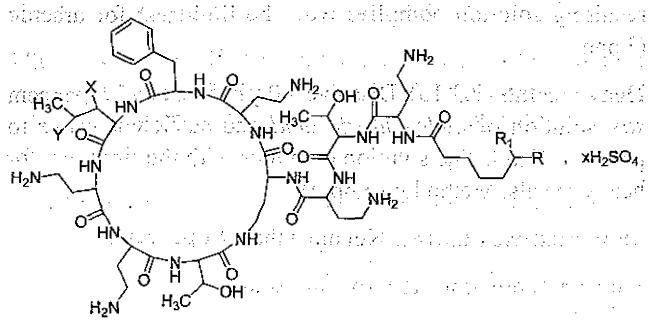
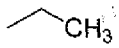
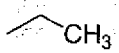
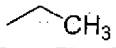
Polymyxin B Sulphate



Polymyxin	R	R_1	X	Y
B1		—CH_3	—H	—CH_3
B2	—CH_3	—CH_3	—H	—CH_3
B3		—H	—H	—CH_3
B1-I		—CH_3	—CH_3	—H

Polymyxin B1

$C_{56}H_{98}N_{16}O_{13}$ Mol. Wt. 1203.5

N-[(*S*)-4-Amino-1-{[(2*S*,3*R*)-1-{[(*S*)-4-amino-1-oxo-1-({(3*S*,6*S*,9*S*,12*S*,15*S*,18*S*,21*S*)-6,9,18-tris(2-aminoethyl)-15-benzyl-3-[(*R*)-1-hydroxyethyl]-12-isobutyl-2,5,8,11,14,17,20-heptaoxo-1,4,7,10,13,16,19-heptaazacyclotricosan-21-yl}amino)butan-2-yl]amino}-3-hydroxy-1-oxobutan-2-yl]amino}-1-oxobutan-2-yl]-6-methyloctanamide.

Polymyxin B2

$C_{55}H_{96}N_{16}O_{13}$ Mol. Wt. 1189.5

N-[(*S*)-4-Amino-1-{[(2*S*,3*R*)-1-{[(*S*)-4-amino-1-oxo-1-({(3*S*,6*S*,9*S*,12*S*,15*S*,18*S*,21*S*)-6,9,18-tris(2-aminoethyl)-15-benzyl-3-[(*R*)-1-hydroxyethyl]-12-isobutyl-2,5,8,11,14,17,20-heptaoxo-1,4,7,10,13,16,19-heptaazacyclotricosan-21-yl}amino)butan-2-yl]amino}-3-hydroxy-1-oxobutan-2-yl]amino}-1-oxobutan-2-yl]-6-methylheptanamide.

Polymyxin B3

$C_{55}H_{96}N_{16}O_{13}$ Mol. Wt. 1189.5

N-[(*S*)-4-Amino-1-{[(2*S*,3*R*)-1-{[(*S*)-4-amino-1-oxo-1-({(3*S*,6*S*,9*S*,12*S*,15*S*,18*S*,21*S*)-6,9,18-tris(2-aminoethyl)-15-benzyl-3-[(*R*)-1-hydroxyethyl]-12-isobutyl-2,5,8,11,14,17,20-heptaoxo-1,4,7,10,13,16,19-heptaazacyclotricosan-21-yl}amino)butan-2-yl]amino}-3-hydroxy-1-oxobutan-2-yl]amino}-1-oxobutan-2-yl]octanamide.

Polymyxin B1-I

$C_{56}H_{98}N_{16}O_{13}$ Mol. Wt. 1203.5

N-[(*S*)-4-Amino-1-{[(2*S*,3*R*)-1-{[(*S*)-4-amino-1-oxo-1-({(3*S*,6*S*,9*S*,12*S*,15*S*,18*S*,21*S*)-6,9,18-tris(2-aminoethyl)-15-benzyl-3-[(*R*)-1-hydroxyethyl]-12-[(*S*)-sec-butyl]-2,5,8,11,14,17,20-heptaoxo-1,4,7,10,13,16,19-heptaazacyclotricosan-21-yl}amino)butan-2-yl]amino}-3-hydroxy-1-oxobutan-2-yl]amino}-1-oxobutan-2-yl]-6-methyloctanamide.

Polymyxin B Sulphate is the sulphate salt of a kind of polymyxin, a substance produced by the growth of *Bacillus polymyxa* (Prazmowski) Migula (Fam. Bacillaceae), or a mixture of two or more such salts. It has a potency of not less than 6000 Polymyxin B units per mg, calculated on the dried basis.

Category. Antibacterial.

Description. A white to buff-coloured powder.

Identification

A. In the test for Composition of Polymyxins, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

B. Dissolve about 2 mg in 5 ml of *water*, add 5 ml of *2.5 M sodium hydroxide* and 5 drops of 1 per cent w/v solution of *cupric sulphate*, shake after addition of each drop; a reddish-violet colour is produced.

C. A 5 per cent w/v solution gives the reactions of sulphates (2.3.1).

Tests

pH (2.4.24). 5.0 to 7.5, determined in a 0.5 per cent w/v solution.

Composition of Polymyxins. Determine by liquid chromatography (2.4.14).

Solvent mixture. 20 volumes of *acetonitrile* and 80 volumes of *water*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution. A 0.05 per cent w/v solution of *polymyxin B sluphate IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm),
- mobile phase: a mixture of 80 volumes of a buffer solution prepared by dissolving 4.5 g of *sodium sulphate anhydrous* in 1000 ml of *water*, adjusted to pH 2.3 with *dilute orthophosphoric acid* and 20 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,

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– spectrophotometer set at 215 nm,
– injection volume: 20 µl.

Name	Relative retention time
Polymyxin B2[1]	0.5
Polymyxin B3	0.6
Polymyxin B1-I	0.8
Polymyxin B1[1]	1.0

[1]These components are not reported individually. They are only reported in the sum of polymyxins.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to polymyxin B2 and polymyxin B3 is not less than 3.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent for the polymyxin B1 peak.

Inject the reference solution and the test solution. Run the chromatogram 1.4 times the retention time of the principal peak, the area of any peak corresponding to polymyxin B3 is not more than 0.06 times the area of the corresponding peak in the chromatogram obtained with the reference solution (6.0 per cent), the area of any peak corresponding to polymyxin B1-I is not more than 0.15 times the area of the corresponding peak in the chromatogram obtained with the reference solution (15.0 per cent) and the sum of polymyxin B1, polymyxin B1-I, polymyxin B2, and polymyxin B3 is not less than 80 per cent.

Related substances. Determine by liquid chromatography (2.4.14), as described under Composition of Polymyxins.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 3.0 per cent and the sum of all the secondary peaks is not more than 17.0 per cent, calculated by area normalisation. Ignore any peak with an area less than 0.007 times the area of polymyxin B1 peak in the chromatogram obtained with the reference solution.

Sulphated ash (2.3.18). Not more than 5.0 per cent, moistening the charred residue with 2 ml of *nitric acid* and 5 drops of *sulphuric acid*.

Loss on drying (2.4.19). Not more than 7.0 per cent, determined on 0.1 g in a capillary-stoppered bottle by drying under vacuum at 60° for 3 hours.

Assay. Determine by the microbiological assay of antibiotics, Method A (2.2.10).

Polymyxin B sulphate intended for use in the manufacture of parenteral preparations complies with the following additional tests.

Sterility. Comply with the test for sterility (2.2.11).

Pyrogens. Complies with the test for pyrogens (2.2.8), using per kg of the rabbit's weight, 1.0 ml of a 20×10^3 polymyxin B units per ml solution of the substance under examination in pyrogen free 0.9 per cent w/v solution of *sodium chloride*.

Storage. Store protected from light and moisture.

Labelling. Where it is intended for use in preparing injectable or other sterile dosage forms, the label states that it is sterile or must be subjected to further processing during the preparation of injectable or other sterile dosage forms.

Polymyxin B for Injection

Polymyxin B for Injection is a sterile material consisting of polymyxin B sulphate. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, or 0.9 per cent w/v sodium chloride injection or 1 per cent w/v procaine hydrochloride injection, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, with in the period recommended by the manufacturer.

Polymyxin B for Injection contains not less than 90.0 per cent and not more than 120.0 per cent of the stated amount of polymyxin B.

Usual strength. 500000 units per vial.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with silica gel G.

Mobile phase. A mixture of 4 volumes of *methanol*, 2 volumes of *isopropyl alcohol*, 2 volumes of *methylene chloride*, 2 volumes of *ammonia solution* and 1.5 volumes of *water*.

Test solution. Dissolve a suitable quantity of Polymyxin B for injection with *0.1M hydrochloric acid* to obtain a solution containing about 10000 polymyxin B units per ml.

Reference solution. Dissolve a suitable quantity of *polymyxin B sulphate IPRS* in *0.1M hydrochloric acid* to obtain a solution containing about 10000 polymyxin B units per ml.

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Apply to the plate 10 µl of each solution. Place the plate in a presaturated chromatographic chamber and allow the mobile phase to rise about three-fourth of the length of the plate. Dry the plate at 105° for 10 minutes. Spray with 0.2 per cent w/v solution of *ninhydrin* in *butyl alcohol* and heat at 105° for 5 minutes. The principal spot in the chromatogram obtained with the test solution corresponds to spot in the chromatogram obtained with the reference solution.

NOTE — If the chromatogram of the test solution yield excessive streaking, proceed as directed for modified procedure.

Modified procedure. Transfer the test solution to a centrifuge tube, add 10 ml of 1.2 per cent w/v solution of *picric acid*, vortex for 1 minute, centrifuge for 10 minutes, and discard the supernatant. Wash the residue with 1 ml portion of *water* until no yellow colour is observed in the washing. Discard the washings and dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 1 ml of *acetone*, add 1 ml of freshly prepared 1 per cent v/v solution of *sulphuric acid* in *acetone*, shake, centrifuge for 5 minutes, and discard the supernatant. Rinse the residue with 1 ml of *acetone*, centrifuge and discard the washing. Repeat the washing until no yellow colour is observed. Dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 0.5 ml of *0.1 M hydrochloric acid*. Repeat the procedure using this modified test solution instead of the test solution.

Tests

pH (2.4.24). 5.0 to 7.5, determined in a 0.5 per cent w/v solution.

Sulphated ash (2.3.18). Not more than 5.0 per cent, charred the residue being moistened with 2 ml of *nitric acid* and 5 drops of *sulphuric acid*.

Loss on drying (2.4.19). Not more than 7.0 per cent, determined on 0.1 g in a capillary-stoppered bottle under vaccum at 60° for 3 hours.

Sterility (2.2.11). Complies with the test for sterility.

Pyrogens. Complies with the test for pyrogens (2.2.8), using the test dose being 1.0 ml per kg of a solution in pyrogen free 0.9 per cent w/v of sodium chloride injection containing 20000 polymyxin B units per ml.

Assay. Determine by the microbiological assay of antibiotics, Method A (2.2.10), and express the results in Units per ml.

Storage. Store protected from light, at a temperature between 20° to 25°.

Labelling. Label it to include that where it is administered intramuscularly and /or intrathecally. it is to be given to patients hospitalized so as to provide constant supervision by a physician.

Polyoxyl 35 Castor Oil

Polyoxyl 35 Castor Oil contains mainly the tri-ricinoleate ester of ethoxylated glycerol, with smaller amounts of polyethylene glycol ricinoleate and the corresponding free glycols. It results from the reaction of glycerol ricinoleate with about 35 moles of ethylene oxide.

Category. Pharmaceutical aid.

Description. A yellow, oily liquid, having a faint.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *polyoxyl 35 castor oil IPRS* or with the reference spectrum of polyoxyl 35 castor oil.

B. Dissolve about 0.1 g in 10 ml of *ethanolic potassium hydroxide solution*, boil for about 3 minutes and evaporate to dryness. The residue dissolve with 5 ml of *water*, yielding a clear solution. Add a few drops of *glacial acetic acid*; a white precipitate is formed.

C. To a 5 .0 per cent w/v solution, add *bromine solution*, dropwise, the *bromine* is decolorized.

Tests

Weight per ml (2.4.29). 1.05 to 1.06.

Viscosity (2.4.28). 600 to 850 centipoises at 25°, a capillary viscometer being used.

Acid value (2.3.23). Not more than 2.0.

Hydroxyl value (2.3.27). 65 to 80.

Saponification value (2.3.37). 60 to 75.

Iodine value (2.3.28). 25 to 35.

Heavy metals (2.3.13). Weigh about 2 g of the substance under examination in a crucible and add sufficient *sulphuric acid* to wet the substance, carefully ignite at a low temperature until thoroughly charred. Add to the carbonized mass 2 ml of *nitric acid* and 5 drops of *sulphuric acid*, and heat cautiously until white fumes no longer are evolved. Ignite, preferably in a muffle furnace, at 500° to 600°, until the carbon is completely burned off. Cool, add 4 ml of *dilute hydrochloric acid*, cover, digest on a steam-bath for 15 minutes, and slowly evaporate on a steam-bath to dryness. Moisten the residue with 1 drop of *hydrochloric acid*, add 10 ml of hot *water*, and digest for 2 minutes. Add *dilute ammonium hydroxide* dropwise until the solution is just alkaline to litmus paper, dilute with *water* to 25 ml, and adjusted to pH 3.0 with *dilute acetic acid*, filter. Rinse the crucible and the filter with 10 ml of *water*, combine the filtrate and rinsing in a 50-ml Nessler cylinder, dilute with *water* to 40 ml, and mix.

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To each of the cylinder containing the standard solution and the test solution, add 2 ml of *acetate buffer pH 3.5*, then add 1.2 ml of *thioacetamide- glycerine* base , dilute with *water* to 50 ml, mix, allow to stand for 2 minutes, and view downward over a white surface: the colour of the solution obtained from the test solution is not more intense than that of the solution obtained from the standard solution, produced by treating 2 ml of *lead standard solution* (10 ppm) in a similar manner.

Sulphated ash (2.3.18). Not more than 0.3 per cent.

Water (2.3.43). Not more than 3.0 per cent, determined on 1 g.

Storage. Store protected from moisture.

Polyoxyl 40 Hydrogenated Castor Oil

Polyoxyl 40 Hydrogenated Castor Oil contains mainly the tri-hydroxystearate ester of ethoxylated glycerol, with smaller amounts of polyethylene glycol tri-hydroxystearate and of the corresponding free glycols. It results from the reaction of glycerol tri-hydroxystearate with about 40 to 45 moles of ethylene oxide.

Category. Pharmaceutical aid.

Identification

A. Dissolve 0.1 g in 1 ml of *water*, add 9 ml of 5.0 per cent w/v solution of *sodium chloride* and heat on a water-bath, the solution becomes turbid at a temperature between 70° and 85°.

B. Dissolve about 0.1 g in 10 ml of *ethanolic potassium hydroxide solution*, boil for about 3 minutes, and evaporate to dryness. Dissolve the residue with 5 ml of *water*, yielding a clear solution. Add a few drops of *glacial acetic acid*; a white precipitate is formed.

Tests

Congealing temperature (2.4.10). 16° to 26°.

Acid value (2.3.23). Not more than 2.0.

Hydroxyl value (2.3.27). 60 to 80.

Saponification value (2.3.37). 45 to 69.

Iodine value (2.3.28). Not more than 2.0.

Heavy metals (2.3.13). Weigh 2 g of the substance under examination in a crucible and add sufficient *sulphuric acid* to wet the substance, carefully ignite at a low temperature until thoroughly charred. Add to the carbonized mass 2 ml of *nitric acid* and 5 drops of *sulphuric acid*, and heat cautiously until white fumes no longer are evolved. Ignite, preferably in a muffle furnace, at 500° to 600°, until the carbon is completely burned off. Cool, add 4 ml of *dilute hydrochloric acid*, cover, digest on a steam bath for 15 minutes, and slowly evaporate on a steam bath to dryness. Moisten the residue with 1 drop of *hydrochloric acid*, add 10 ml of hot *water*, and digest for 2 minutes. Add *dilute ammonium hydroxide* dropwise until the solution is just alkaline to litmus paper, dilute with *water* to 25 ml, and adjust with *dilute acetic acid* to a pH between 3.0 and 4.0, using short-range pH indicator paper as an external indicator. Filter if necessary, rinse the crucible and the filter with 10 ml of *water*, combine the filtrate and rinsing in a 50-ml Nessler cylinder, dilute with *water* to 40 ml and mix.

To each of the cylinder containing the standard solution and the test solution , add 2 ml of *acetate buffer* pH 3.5, then add 1.2 ml of *thioacetamide- glycerine* base , dilute with *water* to 50 ml, mix, allow to stand for 2 minutes, and view downward over a white surface: the colour of the solution obtained from the test solution is not more intense than that of the solution obtained from the standard solution, produced by treating 2 ml of *lead standard solution* (10 ppm) in a similar manner.

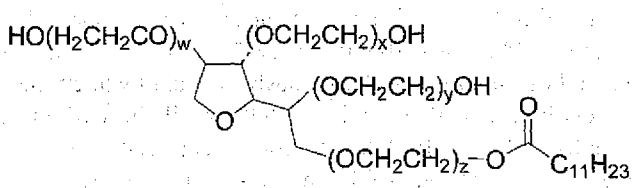
Sulphated ash (2.3.18). Not more than 0.3 per cent.

Water (2.3.43). Not more than 3.0 per cent, determined on 1 g.

Storage. Store protected from moisture.

Polysorbate 20

Polyoxyethylene 20 Sorbitan Monolaurate



w + x + y + z = 20

Polyoxyethylene 20 Sorbitan Monolaurate

Polysorbate 20 is a mixture of partial lauric esters of D-glucitol and its anhydrides copolymerised with approximately 20 moles of ethylene oxide for each mole of D-glucitol and its anhydrides. The lauric acid used for the esterification may contain other fatty acids.

Category. Pharmaceutical aid (non-ionic surfactant).

Description. A clear or slightly opalescent, oily, yellowish or brownish yellow liquid.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *polysorbate 20 IPRS* or with the reference spectrum of polysorbate 20.

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B. Dissolve 0.1 g in 5 ml of *chloroform*, add 0.1 g of *potassium thiocyanate* and 0.1 g of *cobalt nitrate* and stir with a glass rod; a blue colour is produced.

Tests

pH (2.4.24). 5.0 to 7.0, determined in a 5.0 per cent w/v solution in *carbon dioxide-free water*.

Acid value (2.3.23). Not more than 2.0, determined on 5.0 g.

Hydroxyl value (2.3.27). 96 to 108, determined on 2.0 g.

Saponification value (2.3.37). 40 to 50, using 15 ml of *0.5 M ethanolic potassium hydroxide* and diluting with 50 ml of *water* before carrying out the titration.

Iodine value (2.3.28). Not more than 5.0, determined by the iodine bromide method.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Reducing impurities. Dissolve 2.0 g in 25 ml of hot *water*, add 25 ml of *1 M sulphuric acid* and 0.1 ml of *ferroin solution* and titrate with *0.01 M ceric ammonium sulphate* shaking continuously until the colour changes from red to greenish blue persists for 30 seconds. Carry out a blank titration. Not more than 2.0 ml of *0.01 M ceric ammonium sulphate* is required.

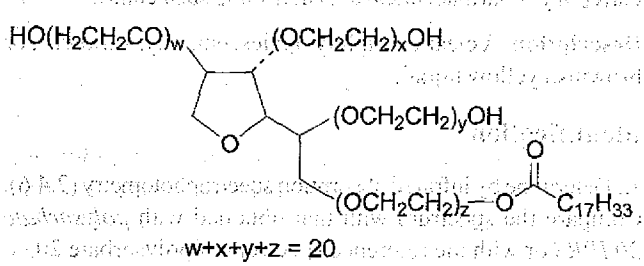
Sulphated ash. Not more than 0.2 per cent, determined by the following method. Weigh 2.0 g in a silica or platinum crucible, add 0.5 ml of *sulphuric acid* and heat on a water-bath for 2 hours. Carefully ignite at a low temperature until thoroughly charred. Add to the carbonised mass 2 ml of *nitric acid* and 0.25 ml of *sulphuric acid*, cautiously heat until white fumes are evolved and then ignite at 600° until the carbon is completely burnt off. Allow to cool, weigh and repeat the operation for periods of 15 minutes until two successive weighings do not differ by more than 0.5 mg.

Water (2.3.43). Not more than 3.0 per cent, determined on 1.0 g.

Storage. Store protected from light and moisture.

Polysorbate 80

Polyoxyethylene 80 Sorbitan Monooleate



Polysorbate 80 is a mixture of partial oleic esters of D-glucitol and its anhydrides copolymerised with approximately 20 moles of ethylene oxide for each mole of D-glucitol and its anhydrides.

Category. Pharmaceutical aid (non-ionic surfactant).

Description. A clear or almost clear, oily, yellowish or brownish yellow liquid.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *polysorbate 80 IPRS* or with the reference spectrum of polysorbate 80.

B. Dissolve 0.1 g in 5 ml of *chloroform*, add 0.1 g of *potassium thiocyanate* and 0.1 g of *cobalt nitrate* and stir with a glass rod; a blue colour is produced.

C. To 2 ml of a 5 per cent w/v solution add 0.5 ml of *bromine water*; the bromine is decolourised.

Tests

Acid value (2.3.23). Not more than 2.0, determined on 5 g.

Hydroxyl value (2.3.27). 65 to 80, determined on 2.0 g.

Saponification value (2.3.37). 45 to 55, using 15 ml of *0.5 M ethanolic potassium hydroxide* and diluting with 50 ml of *water* before carrying out the titration.

Iodine value (2.3.28). 18 to 24, determined by the iodine bromide method.

Reducing impurities. Dissolve 2.0 g in 25 ml of hot *water*, add 25 ml of *1 M sulphuric acid* and 0.1 ml of *ferroin solution* and titrate with *0.01 M ceric ammonium sulphate* shaking continuously until the colour changes from red to greenish blue persists for 30 seconds. Carry out a blank titration. Not more than 5.0 ml of *0.01 M ceric ammonium sulphate* is required.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash. Not more than 0.2 per cent, determined by the following method. Weigh 2.0 g in a silica or platinum crucible, add 0.5 ml of *sulphuric acid* and heat on a water-bath for 2 hours. Carefully ignite at a low temperature until thoroughly charred. Add to the carbonised mass 2 ml of *nitric acid* and 0.25 ml of *sulphuric acid*, cautiously heat until white fumes are evolved and then ignite at 600° until the carbon is completely burnt off. Allow to cool, weigh and repeat the operation for periods of 15 minutes until two successive weighings do not differ by more than 0.5 mg.

Water (2.3.43). Not more than 3.0 per cent, determined on 1.0 g.

Storage. Store protected from light and moisture.

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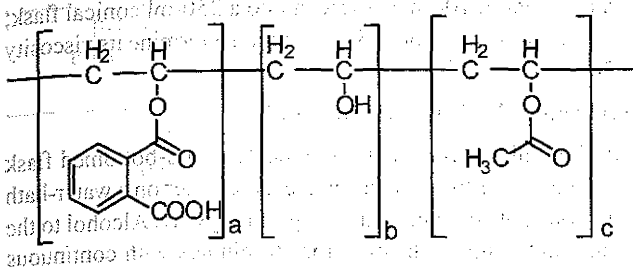
Polyvinyl Acetate Phthalate



Polyvinyl Acetate Phthalate is a reaction product of phthalic anhydride and a partially hydrolyzed polyvinyl acetate.

Polyvinyl Acetate Phthalate contains not less than 55.0 per cent and not more than 62.0 per cent of phthalyl (o-carboxybenzoyl, $C_8H_5O_3$) groups, calculated on the anhydrous acid-free basis.

Category. Pharmaceutical aid.

Identification

A. The test solution obtained in the Assay shows absorption maximum at about 277 nm (2.4.7).

B. To 10 mg contained in a small test-tube add 10 mg of *resorcinol*, mix, add 0.5 ml of *sulphuric acid*, and heat in a liquid-bath at 160° for 3 minutes. Cool, and pour the solution into a mixture of 25 ml of *1 M sodium hydroxide* and 200 ml of *water*, the solution shows a vivid green fluorescence.

C. Dissolve 2 g in 20 ml of *methanol*, and pour 1 ml of the solution onto a clear glass plate; a film is deposited as the methanol evaporates.

Tests

Viscosity (2.4.28). 7 to 11 centipoises, determined by dissolving 15 g in 85 g of *methanol*.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 5.0 per cent, determined on 0.5 g.

Free phthalic acid. Not more than 0.6 per cent, on the anhydrous basis.

Reference solution. Dissolve 50 mg of *phthalic anhydride* in *water* and dilute to 1000 ml with *water*.

Test solution. Dissolve 1.5 g in a mixture of 40 volumes of *dichloromethane* and 10 volumes of *methanol* and dilute to 50 ml with same solvent mixture. Transfer the solution to a separator with the aid of 75 ml of *water*, and swirl, taking care not to shake. Add 100 ml of *hexane*, shake, and allow the mixture to stand until it separates into 2 layers. Transfer the water layer to a 250-ml volumetric flask. Add 100 ml of *water* to the separator, shake, and allow to stand until the layers separate. Transfer the water layer to the same volumetric flask, dilute to 250 ml with *water*. If the solution is cloudy, centrifuge a portion until clear. Determine the absorbance at 277 nm.

Calculate the content of free phthalic acid by using the formula:

$$25 \times \frac{166.13}{148.12} \times \frac{W_S}{W_U} \times \frac{A_U}{A_S}$$

Where, 166.13 and 148.12 are the molecular weights of phthalic acid and phthalic anhydride, respectively,

W_S and W_U are the weights, in mg, of phthalic anhydride and Polyvinyl Acetate Phthalate, respectively,

A_U and A_S are the absorbances of the test solution and the reference solution, respectively.

Free acid other than phthalic. Not more than 0.6 per cent on the anhydrous basis.

Proceed as directed for test solution under Free phthalic acid, but instead of transferring the water extracts to a volumetric flask, transfer them to a 400-ml beaker, and titrate with *0.1 M sodium hydroxide*, using *phenolphthalein* as indicator.

Calculate the volume, V_p, in ml, of *0.1 M sodium hydroxide* consumed by the free phthalic acid in the sample taken by the formula:

$$V_P = \frac{1}{830.6} \times pW$$

in which p is the percentage of free phthalic acid, previously determined, and W is the weight, in mg, of the sample, on the anhydrous basis.

Calculate the percentage of free acid other than phthalic, as acetic acid, taken by the formula:

$$0.05 \times \frac{10}{W} \times (V - V_P)$$

in which V is the total volume, in ml, of *0.1 M sodium hydroxide* used.

Assay.

Test solution. Dissolve 100 mg of the substance under examination, in *ethanol (95 per cent)* and dilute to 1000.0 ml with *ethanol (95 per cent)*.

Reference solution. Dissolve 50 mg of *phthalic anhydride* with heat in 100 ml of *ethanol (95 per cent)* and dilute to 1000 ml with *ethanol (95 per cent)*.

Determine the absorbance at about 275 nm.

Calculate the content of phthalyl, on the acid-free basis, taken by the formula:

$$100 \times \frac{W_S}{W_U} \times \frac{A_U}{A_S}$$

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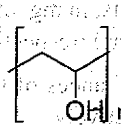
Where, W_S and W_U are the weights, in mg, of phthalic anhydride and Polyvinyl Acetate Phthalate on the anhydrous basis, respectively,

A_U and A_S are the absorbances of the test solution and the reference solution, respectively.

Storage. Store protected from moisture.

Polyvinyl Alcohol



$(C_2H_4O)_n$

Polyvinyl Alcohol is a water soluble synthetic polymer represented by the formula $(C_2H_4O)_n$, in which the average value of n lies between 500 and 5000.

Polyvinyl Alcohol is prepared by 85 per cent to 89 per cent hydrolysis of polyvinyl acetate. The apparent viscosity, in mPa.s at 20° of 4 per cent w/w aqueous solution is not less than 85 per cent and not more than 115 per cent of the label claim.

Category. Pharmaceutical aid.

Description. A white to cream-coloured granular powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *polyvinyl alcohol IPRS* or with reference spectrum of polyvinyl alcohol.

B. Dissolve 0.5 g of Polyvinyl Alcohol in 10 ml of *water*, with the aid of heat if necessary, and let the solution cool to room temperature. Transfer 5 ml of the solution so obtained to a test tube, add 1 drop of *iodine TS*, mix, and allow to stand; a dark red to blue colour is produced. Add 10 ml of *ethanol* to the remaining 5 ml of the polyvinyl alcohol solution, and mix; a white, turbid, or flocculent precipitate is formed.

Tests

pH (2.4.24). 5.0 to 8.0, determined in a 4.0 per cent w/v solution.

Viscosity (2.4.28). 85 per cent to 115 per cent of the label claim.

After determining the loss on drying, weigh quantity of undried Polyvinyl Alcohol, equivalent to 6.0 g on the dried basis. Over a period of seconds, transfer the test specimen with continuous slow stirring to about 140 ml of *water* contained in a suitable tared flask. When the specimen is well wetted, increase the rate of stirring, avoiding mixing in excess air. Heat the mixture to 90°, and maintain the temperature at 90° for about 5 minutes. Discontinue heating, and continue stirring for 1 hour. Add water to make the mixture weigh 150 g. Resume stirring to obtain a homogenous solution. Filter the solution through a tared 100-mesh screen into a 250-ml conical flask; cool the filtrate to about 15°, and mix. Determine its viscosity at 20°.

Acid value (2.3.23). Not more than 3.0.

Add 200 ml of *water* to a borosilicate round-bottomed flask attached to a reflux condenser. Heat the water on a water-bath with constant stirring. Add 10.0 g of Polyvinyl Alcohol to the water, and continue heating for 30 minutes with continuous stirring. Remove the flask from the water bath, and continue stirring until room temperature is reached. Transfer this solution to a 250-ml volumetric flask, dilute with *water* to volume and mix. Add 1 ml of *phenolphthalein solution* to 50 ml of the solution and titrate with *0.05 M potassium hydroxide* until the pink colour persists for 15 seconds.

Calculate the acid value from the expression:

$$2.805V/2$$

V is the number of millilitres of *0.05 M potassium hydroxide* used.

Ester value (2.3.26). 90.0 per cent to 110.0 per cent of the value stated on the label.

Saponify 1.0 g in a mixture of 25.0 ml of *0.5 M alcoholic potassium hydroxide* and 25.0 ml of *water*.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 1.0 per cent.

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g by drying in an oven at 110°.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states (1) viscosity in terms of mPas (2) ester value.

Potassium Chloride and Dextrose Injection

Potassium Chloride in Dextrose Injection; Potassium Chloride and Dextrose Intravenous Infusion; Potassium Chloride and Glucose Intravenous Infusion

Potassium Chloride and Dextrose Injection is a sterile solution of Potassium Chloride and Dextrose in Water for Injections.

Potassium Chloride and Dextrose Injection contains not less than 95.0 per cent and not more than 110.0 per cent of the stated amount of potassium chloride, KCl, and not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of dextrose, $C_6H_{12}O_6$. It contains no antimicrobial agent.

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Usual strengths: Injections containing the following amounts of potassium chloride, KCl, and dextrose, $C_6H_{12}O_6$.

per cent w/v of Potassium Chloride (KCl)	per cent w/v of Dextrose ($C_6H_{12}O_6$)
0.075	5
0.15	5
0.225	5
0.30	5

Description. A clear, colourless or faintly straw-coloured solution.

Identification

A. To 1 ml add 0.05 ml of *potassium cupri-tartrate solution*; the solution remains blue and clear. Heat to boiling, a copious red precipitate is formed.

B. It gives reaction (B) of potassium salts and reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 3.5 to 6.5.

5-Hydroxymethylfurfural and Related substances. Dilute a volume containing 1.0 g of Dextrose to 500.0 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 284 nm, absorbance at about 284 nm (2.4.7); not more than 0.25.

Heavy metals (2.3.13). Evaporate a volume containing 4.0 g of Dextrose to 10 ml and add 2 ml of *dilute acetic acid* and sufficient *water* to produce 25 ml. The solution complies with the limit test for heavy metals, Method A (5 ppm).

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *For potassium chloride* — Titrate a measured volume containing 0.1 g of Potassium Chloride with *0.1 M silver nitrate* using *potassium chromate solution* as indicator.

1 ml of *0.1 M silver nitrate* is equivalent to 0.007455 g of KCl.

For dextrose — To a measured volume containing 2 g to 5 g of Dextrose, add 0.2 ml of *5 M ammonia* and sufficient *water* to produce 100.0 ml. Mix well, allow to stand for 30 minutes and measure the optical rotation in a 2-dm tube (2.4.22). The observed rotation in degrees multiplied by 0.9477 represents the weight, in g, of dextrose, $C_6H_{12}O_6$, in the volume taken for assay.

Storage. Store in single dose containers.

Labelling. The label states (1) the strength as the percentages w/v of Potassium Chloride and Dextrose; (2) the total osmolar concentration in mOsmol per litre; (3) where the contents are less than 100 ml, or where the label states that the Injection is not for direct use but is to be diluted before use, the label alternatively may state the total osmolar concentration in mOsmol per ml; (4) the content of potassium in millimoles; (5) that the injection containing visible particles in the solution should not be used.

Potassium Chloride, Sodium Chloride and Dextrose Injection

Potassium Chloride in Dextrose and Sodium Chloride Injection; Potassium Chloride, Sodium Chloride and Dextrose Intravenous Infusion; Potassium Chloride, Sodium Chloride and Glucose Intravenous Infusion

Potassium Chloride, Sodium Chloride and Dextrose Injection is a sterile solution of Potassium Chloride, Sodium Chloride and Dextrose in Water for Injections.

Potassium Chloride, Sodium Chloride and Dextrose Injection contains not less than 95.0 per cent and not more than 110.0 per cent of the stated amounts of sodium, Na, potassium, K, and chloride, Cl, and not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of dextrose, $C_6H_{12}O_6$. It contains no antimicrobial agent.

Usual strengths: Injections containing the following amounts of potassium chloride, KCl, sodium chloride, NaCl, and dextrose, $C_6H_{12}O_6$.

per cent w/v of Potassium Chloride (KCl)	per cent w/v of Sodium Chloride (NaCl)	per cent w/v of Dextrose ($C_6H_{12}O_6$)
0.075	0.250	5
0.075	0.330	5
0.075	0.450	5
0.150	0.225	5
0.150	0.225	10
0.150	0.330	5
0.150	0.450	5
0.150	0.900	5
0.225	0.225	5
0.225	0.225	10
0.225	0.330	5
0.225	0.450	5
0.300	0.225	5
0.300	0.330	5
0.300	0.450	5
0.300	0.900	5

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Potassium Clavulanate Diluted contains not less than 91.2 per cent and not more than 107.1 per cent of the stated amount of potassium clavulanate, $C_8H_8KNO_5$.

Category. Beta-lactamase inhibitor.

Description. A white or almost white powder, hygroscopic.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

B. It gives reaction (A) of potassium salts (2.3.1).

C. Depending on the diluent used, carry out one of the relevant identification tests given below.

(i) On a watch-glass place a quantity of the substance under examination corresponding to 20 mg of cellulose and disperse in 4 ml of *iodinated zinc chloride solution*. The powder becomes violet-blue in colour.

(ii) It gives the reaction of silicates (2.3.1).

Tests

pH (2.4.24) 4.8 to 8.0, determined on a quantity of the substance under examination containing 0.2 g of potassium clavulanate dissolved in 20 ml of *carbon dioxide-free water*.

Polymeric impurities and other impurities absorbing at 278 nm. Disperse a quantity of the substance under examination containing 50 mg of potassium clavulanate in 10 ml of *0.1 M phosphate buffer solution pH 7.0* and dilute to 50.0 ml with the buffer solution, filter. The absorbance of the solution determined at 278 nm is not more than 0.40.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE —Prepare the solutions immediately before use.

Test solution. Dissolve a quantity of the substance under examination containing 0.25 g of potassium clavulanate in 5 ml of mobile phase A, dilute to 25.0 ml with mobile phase A and filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A.

Reference solution (b). Dissolve 10 mg of *amoxycillin trihydrate IPRS* in 1 ml of the test solution and dilute to 100.0 ml with mobile phase A.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. a 0.78 per cent w/v solution of *sodium dihydrogen phosphate*, adjusted to pH 4.0 with *dilute phosphoric acid*,

B. a mixture of equal volumes of mobile phase A and *methanol*,

- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
4	100	0
15	50	50
18	50	50
24	100	0

Inject reference solution (b). The resolution between the clavulanate (first peak) and amoxycillin (second peak) is not less than 13.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent) and the sum of areas of all the secondary peaks is not more than 2 times the area of the principal peak in the chromatogram obtained with the reference solution (a) (2.0 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.3.43). Not more than 2.5 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve a quantity of the substance under examination containing about 50.0 mg of potassium clavulanate in a 0.4 per cent w/v solution of *sodium acetate*, the adjusted to pH 6.0 with *glacial acetic acid*, dilute to 50.0 ml with the same solution and filter.

Reference solution (a). Dissolve 50.0 mg of *lithium clavulanate IPRS* in a 0.4 per cent w/v solution of *sodium acetate* with the pH previously adjusted to 6.0 with *glacial acetic acid* and dilute to 50.0 ml with the same solution.

Reference solution (b). Dissolve 10 mg of *amoxycillin trihydrate IPRS* in 10 ml of reference solution (a).

Chromatographic system

- a stainless steel column 30 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 5 volumes of *methanol* and 95 volumes of a 1.5 per cent w/v solution of *sodium dihydrogen phosphate*, adjusted to pH 4.0 with *dilute orthophosphoric acid*,
- flow rate: 1 ml per minute,

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– spectrophotometer set at 230 nm,
– injection volume: 10 µl.

Inject reference solution (b). The resolution between the clavulanate peak and amoxycillin peak is not less than 3.5.

Inject reference solution (a) and the test solution.

Calculate the content of $C_8H_8KNO_5$.

1 mg of clavulanate ($C_8H_9NO_5$) is equivalent to 1.191 mg of $C_8H_8KNO_5$.

Storage. Store protected from moisture.

Labelling. The label states the contents of potassium clavulanate and the diluent used to prepare the mixture.

Potassium Permanganate

$KMnO_4$ Mol. Wt. 158.0

Potassium Permanganate contains not less than 99.0 per cent and not more than 100.5 per cent of $KMnO_4$.

Category. Antiseptic.

Description. A dark purple or brownish black, granular powder or dark purple or almost black slender, prismatic crystals, having a metallic lustre. It decomposes on contact with certain organic substances.

Identification

A. A solution in *water* acidified with *sulphuric acid* and heated to 70° is decolorised by *hydrogen peroxide solution (20 vol)*.

B. Heated to redness, it decrepitates, evolves oxygen and leaves a black residue which with *water* forms *potassium hydroxide solution*; the resulting solution when neutralised with *dilute hydrochloric acid* gives the reactions of potassium salts (2.3.1).

Tests

Appearance of solution. Dissolve 1.5 g in 50 ml of distilled *water*, heat on a water-bath and add gradually 6 ml of *ethanol (95 per cent)*, cool, dilute to 60 ml with distilled *water* and filter. The filtrate (solution A) is colourless (2.4.1).

Chlorides (2.3.12). 40 ml of solution A complies with the limit test for chlorides (250 ppm).

Sulphates (2.3.17). 10 ml of solution A complies with the limit test for sulphates (600 ppm).

Water-insoluble matter. Dissolve 0.5 g in 50 ml of *water*, heat to boiling, filter through a tared, sintered-glass filter (porosity No. 4) and wash with *water* until the filtrate is colourless. The weight of the residue, dried at 105° to constant weight, is not more than 5 mg (1.0 per cent).

Assay. Dissolve 0.3 g in sufficient *water* to produce 100.0 ml. To 20.0 ml add 20 ml of *water*, 1 g of *potassium iodide* and 10 ml of *2 M hydrochloric acid* and titrate the liberated iodine with *0.1 M sodium thiosulphate* using *starch solution*, added towards the end of the titration, as indicator.

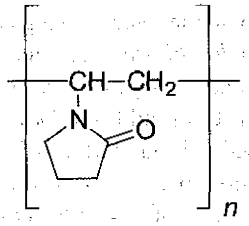
1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.003160 g of $KMnO_4$.

Storage. Store protected from moisture.

CAUTION — Great care should be taken in handling potassium permanganate as dangerous explosions are liable to occur if it is brought into contact with organic or other readily oxidisable substances, either in solution or in the dry condition.

Povidone

Polyvinylpyrrolidone; Polyvidone



$(C_6H_9NO)_n$ Mol. Wt. $(111.2)_n$

Povidone is poly(2-oxopyrrolidin-1-yl-ethylene) and consists of linear polymers of 1-vinylpyrrolidin-2-one. The different types of Povidone are characterised by their viscosity in solution, expressed as K-value, in the range 10 to 95.

Povidone with a nominal K-value of 15 or less has a K-value of not less than 85.0 per cent and not more than 115.0 per cent of the declared value. The K-value of povidone with a nominal K-value of more than 15, or a nominal K-value range with an average of more than 15, is not less than 90.0 per cent and not more than 107.0 per cent of the declared value or of the average of the declared range. It contains not less than 11.5 per cent and not more than 12.8 per cent of nitrogen, N, calculated on the anhydrous basis.

Category. Pharmaceutical aid (tablet binder, coating agent, dispersing and suspending agent).

Description. A white or yellowish white powder or flakes; hygroscopic.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *povidone IPRS*.

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B. Add 2.5 g in small portions to a suitable volume of *carbon dioxide-free water*, stirring with a magnetic stirrer, and dilute to 25 ml with the same solvent (solution A). To 0.4 ml of solution A add 10 ml of *water*, 5 ml of *2 M hydrochloric acid* and 2 ml of *potassium dichromate solution*; an orange-yellow precipitate is produced.

C. To 1 ml of solution A add 0.2 ml of *dimethylaminobenzaldehyde reagent* and 0.1 ml of *sulphuric acid*; a pink colour is produced.

D. To 0.1 ml of solution A add 5 ml of *water* and 0.2 ml of *0.05 M iodine*; a red colour is produced.

Tests

Appearance of solution. Solution A is clear (2.4.1), and not more intensely coloured than reference solution BS6 or BYS6 (2.4.1).

Heavy metals. Mix 2.0 g with 0.5 g of *magnesium oxide* in a silica crucible. Ignite to dull red heat until a homogeneous white or greyish white mass is produced. Heat at 800° for about 1 hour, dissolve the residue using two quantities, each of 5 ml, of *5 M hydrochloric acid*, add 0.1 ml of *phenolphthalein solution* and *strong ammonia solution* until a pink colour is produced. Cool, add *glacial acetic acid* until the solution is decolorised and add a further 0.5 ml. Filter if necessary and dilute the solution to 20 ml with *water*. To 12 ml of the resulting solution add 2 ml of *acetate buffer pH 3.5*, mix, add 1.2 ml of *thioacetamide reagent*, mix immediately and allow to stand for 2 minutes. Any brown colour produced is not more intense than that obtained by treating in the same manner a mixture of 2 ml of the test solution obtained above and 10 ml of the 20 ml of solution obtained by repeating the procedure using 2 ml of *lead standard solution (10 ppm Pb)* in place of the substance under examination, adding 0.5 g of *magnesium oxide* in a silica crucible and continuing as described above beginning at the words "Ignite to dull red heat...." (10 ppm).

Aldehydes. Boil 20.0 g in 180 ml of a 25 per cent v/v solution of *sulphuric acid* in a round-glass-stoppered flask under a reflux condenser for 45 minutes and allow to cool. Fit a distillation head, distil and collect 60 ml of the distillate in 20 ml of a 7.0 per cent w/v solution of *hydroxylamine hydrochloride*, previously adjusted to pH 3.1 using *1 M sodium hydroxide*, and cooled in ice. Titrate with *0.1 M sodium hydroxide* to pH 3.1. Carry out a blank titration. Not more than 9.1 ml of *0.1 M sodium hydroxide* is required (0.2 per cent, calculated as acetaldehyde, C_2H_4O).

1-vinylpyrrolidin-2-one. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 0.25 g of the substance under examination in 10.0 ml of the mobile phase.

Reference solution (a). A 0.05 per cent w/v solution of *1-vinylpyrrolidin-2-one* in *methanol*. Dilute 1.0 ml of the solution to 100.0 ml with *methanol*. Further dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of *1-vinylpyrrolidin-2-one* and 0.5 g of *vinyl acetate* in 100.0 ml of *methanol*. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.0 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 10 volumes of *acetonitrile* and 90 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 235 nm,
- injection volume: 50 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to 1-vinylpyrrolidin-2-one and vinyl acetate is not less than 2.0 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 2.0 in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of the peak due to 1-vinylpyrrolidin-2-one is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 5.0 per cent determined on 0.5 g.

K-value. For Povidone with a stated K-value of 18 or less, prepare a 5.0 per cent w/v solution. For Povidone with a declared K-value of more than 18, prepare a 1.0 per cent w/v solution. Allow the solution to stand for 1 hour and carry out Method B for the determination of viscosity (2.4.28), at 25° ± 0.2° using a size no. 1 viscometer with a minimum flow time of 100 seconds. Calculate the K-value (K_o) from the expression

$$K_o = \frac{1.5 \log z - 1}{0.15 + 0.003c} + \frac{\sqrt{300c \log z + (c + 1.5c \log z)^2}}{0.15c + 0.003c^2}$$

where c is the percentage concentration w/v of the substance under examination, calculated on the anhydrous basis, and z is the viscosity of the solution relative to that of *water*.

Nitrogen (2.3.30). Follow Method C, using 0.3 g, weighed and 11 ml of *nitrogen-free sulphuric acid*. For complete destruction of organic matter repeat the addition of *hydrogen peroxide (10 vol)* (usually 3 to 6 times) until a clear, light-green solution is obtained, then heat for a further 4 hours.

Storage. Store protected from moisture.

Labelling. The label states the viscosity in terms of a K-value or K-range.

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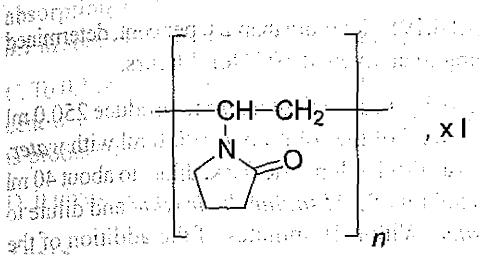
Povidone-Iodine



Povidone-Iodine is a complex produced by interaction between iodine and poly(2-oxopyrrolidin-1-ylethylene).

Povidone-Iodine contains not less than 9.0 per cent and not more than 12.0 per cent of available iodine, I, calculated on the dried basis.

Category. Topical anti-infective; antiseptic.

Description. A yellowish brown amorphous powder.

Identification

A. Add 0.05 ml of a 10 per cent w/v solution to a mixture of 1 ml of *starch solution* and 9 ml of *water*; a deep blue colour is produced.

B. Spread 1 ml of a 10 per cent w/v solution over an area of about 20 cm x 20 cm on a glass plate and allow it to dry in air at room temperature and in an atmosphere of low humidity overnight; a brown, dry, non-smearing film is formed which dissolves readily in *water*.

Tests

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Nitrogen (2.3.30). 9.5 to 11.5 per cent, calculated on the dried basis, determined on about 0.3 g, by Method A.

Iodide. Not more than 6.6 per cent, calculated on the dried basis, determined by the following method. Weigh 0.5 g and dissolve in 100 ml of *water*. Add sufficient *sodium bisulphite solution* until the colour of iodine is discharged. Add 25.0 ml of *0.1 M silver nitrate* and 10 ml of *nitric acid* and mix. Titrate with *0.1 M ammonium thiocyanate*, using *ferric ammonium sulphate solution* as indicator. Repeat the procedure without the substance under examination. The difference between the titrations represents the amount of silver nitrate required. 1 ml of *0.1 M silver nitrate* is equivalent to 0.01269 g of I. From the percentage of total iodine, calculated on the dried basis, subtract the percentage of available iodine determined in the Assay and obtain the percentage of iodide.

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 8.0 per cent, determined on 0.5 g by drying in an oven at 105°.

Assay. Weigh 3 g, transfer to a beaker and add 200 ml of *water*. Cover the beaker and stir with a mechanical stirrer at room temperature for not more than 1 hour to dissolve as completely as possible. Titrate immediately thereafter with *0.1 M sodium thiosulphate* using 3 ml of *starch solution*, added towards the end of the titration, as indicator.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.01269 g of I.

Storage. Store protected from light.

Povidone-Iodine Solution

Povidone-Iodine Solution is a solution of Povidone-Iodine in Purified Water. It may contain a small amount of Ethanol.

Povidone-Iodine Solution contains not less than 85.0 per cent and not more than 120.0 per cent of the stated amount of iodine, I.

Usual strengths. 5 per cent w/v; 7.5 per cent w/v; 10 per cent w/v.

Description. A deep brown liquid.

Identification

A. Dilute 1 ml to 20 ml with *water* and add 1 ml of the resulting solution to a mixture of 1 ml of *starch solution* and 9 ml of *water*; a deep blue colour is produced.

B. Transfer 10 ml to a small flask and cover the mouth of the flask with a filter paper soaked in *starch solution*; no blue colour is produced on the paper within 60 seconds.

C. Dilute 20 ml to 100 ml with *water*. To 10 ml add dropwise *0.1 M sodium thiosulphate* until the colour is just discharged. To 5 ml of the resulting solution add 5 ml of *2 M hydrochloric acid* and 2 ml of *potassium dichromate solution*; a red precipitate is produced.

Tests

pH (2.4.24). 3.0 to 6.5.

Ethanol (if present) (2.3.45). 90.0 to 110.0 per cent of the stated amount of C_2H_5OH, determined by Method A after decolorising the solution with just sufficient quantity of a 10 per cent w/v solution of *sodium thiosulphate* and treating with a few drops of *dilute sodium hydroxide solution*.

Assay. To a measured volume containing about 50 mg of iodine add sufficient *water* to produce not less than 30 ml and titrate immediately with *0.02 M sodium thiosulphate*, using 3 ml of

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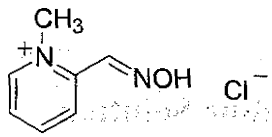
starch solution, added towards the end of the titration, as indicator. Carry out a blank titration.

1 ml of *0.02 M sodium thiosulphate* is equivalent to 0.002538 g of I.

Storage. Store protected from light.

Labelling. The label states the quantities of iodine and ethanol (if present) as percentages w/v.

Pralidoxime Chloride



$C_7H_9ClN_2O$ Mol. Wt. 172.6

Pralidoxime Chloride is 2-hydroxyiminomethyl-1-methylpyridinium chloride.

Pralidoxime Chloride contains not less than 97.0 per cent and not more than 103.0 per cent of $C_7H_9ClN_2O$, calculated on the dried basis.

Category. Antidote for cholinesterase inhibitors.

Description. A white to pale yellow, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pralidoxime chloride IPRS* or with the reference spectrum of pralidoxime chloride.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.0005 per cent w/v solution in *0.1 M hydrochloric acid* shows absorption maxima at about 242 nm and 292 nm and in *0.1 M sodium hydroxide*, a maximum at about 336 nm.

C. To 0.1 ml of a 20 per cent w/v solution add 1 ml of a 0.6 per cent w/v solution of *ferric chloride*; an amber-brown colour is produced.

D. It gives the reactions of chlorides (2.3.1).

Tests

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method A (20 ppm).

Chloride content. 20.2 to 20.8 per cent, calculated on the dried basis, determined by the following method. Dissolve 0.3 g in about 150 ml of *water*, add 20 ml of *glacial acetic acid* and 10 drops of *(4-tert-octylphenoxy) nonaethoxyethanol* and titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (2.4.25).

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of Cl.

Sulphated ash (2.3.18). Not more than 0.5 per cent.

Loss on drying (2.4.19). Not more than 2.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 0.5 g in sufficient *water* to produce 250.0 ml and mix. Dilute 5.0 ml of the solution to 100.0 ml with *water*. Transfer 5.0 ml to a 50-ml volumetric flask, dilute to about 40 ml with *water*, add 5.0 ml of *1 M sodium hydroxide* and dilute to volume with *water*. Within 10 minutes of the addition of the alkali, measure the absorbance of the solution at the maximum at about 332 nm (2.4.7), using a solution of 5.0 ml of *1 M sodium hydroxide* diluted with *water* to 50.0 ml as the blank.

Calculate the content of $C_7H_9ClN_2O$ from the absorbance obtained by carrying out the assay simultaneously using about 0.5 g, weighed, of *pralidoxime chloride IPRS*.

Storage. Store protected from moisture.

Pralidoxime Chloride Injection

Pralidoxime Chloride Injection is a sterile material consisting of Pralidoxime Chloride with or without buffering agents and other excipients. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Pralidoxime Chloride Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pralidoxime chloride, $C_7H_9ClN_2O$.

Usual strength. 1 g.

Description. A white to pale yellow powder.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pralidoxime chloride IPRS* or with the reference spectrum of pralidoxime chloride.

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B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.0005 per cent w/v solution in *0.1 M hydrochloric acid* shows absorption maxima at about 242 nm and 292 nm and in *0.1 M sodium hydroxide*, a maximum at about 336 nm.

C. To 0.1 ml of a 20 per cent w/v solution add 1 ml of a 0.6 per cent w/v solution of *ferric chloride*; an amber-brown colour is produced.

D. It gives the reactions of chlorides (2.3.1).

Tests

pH (2.4.24): 3.5 to 4.5, determined in a 5.0 per cent w/v solution.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method A (20 ppm).

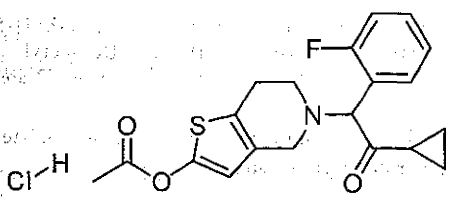
Bacterial endotoxins (2.2.3). Not more than 0.1 Endotoxin Unit per mg of pralidoxime chloride.

Assay. Dissolve 0.5 g in sufficient *water* to produce 250.0 ml and mix. Dilute 5.0 ml of the solution to 100.0 ml with *water*. Transfer 5.0 ml to a 50-ml volumetric flask, dilute to about 40 ml with *water*, add 5.0 ml of *1 M sodium hydroxide* and dilute to volume with *water*. Within 10 minutes of the addition of the alkali, measure the absorbance of the solution at the maximum at about 332 nm (2.4.7), using a solution of 5.0 ml of *1 M sodium hydroxide* diluted with *water* to 50.0 ml as the blank.

Calculate the content of $C_7H_9ClN_2O$ from the absorbance obtained by carrying out the assay simultaneously using about 0.5 g, weighed, of *pralidoxime chloride IPRS*.

Labelling. The label states the period within which the constituted injection should be used.

Prasugrel Hydrochloride



$C_{20}H_{20}FNO_3S, HCl$ Mol. Wt. 409.9

Prasugrel Hydrochloride is Ethanone, 2-[2-(acetyloxy)-6,7-dihydrothieno[3,2-c]pyridin-5(4*H*)-yl]-1-cyclopropyl-2-(2-fluorophenyl)-hydrochloride.

Prasugrel Hydrochloride contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{20}H_{20}FNO_3S, HCl$, calculated on the anhydrous basis.

Category. Anti thrombotic Agent.

Description. A white to almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prasugrel hydrochloride IPRS* or with the reference spectrum of prasugrel hydrochloride .

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

C. It gives reactions of chlorides (2.3.1).

Tests

Related substances. A. Determine by liquid chromatography (2.4.14).

Solvent mixture. 70 volumes of *acetonitrile* and 30 volumes of *water.*

Test solution. Dissolve 75 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). A 0.0015 per cent w/v *prasugrel hydrochloride IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 50.0 ml with the solvent mixture.

Reference solution (c). Transfer 5 mg of *prasugrel hydrochloride IPRS* to a 50-ml volumetric flask, add 20 ml of the solvent mixture and mix to dissolve. Add 1 ml of 0.0003 per cent v/v of piperidine in *acetonitrile* and dilute to volume with the solvent mixture. Heat the solution at 50° for one hour and cool to room temperature. Dilute 2.0 ml of the solution to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 40°,
- sample temperature: 10°,
- mobile phase: a mixture of 65 volumes of 0.01M *monobasic potassium phosphate*, 25 volumes of *tetrahydrofuran* and 10 volumes of *acetonitrile*,
- flow rate: 0.9 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 10 µl.

Name	Relative retention time
Desacetyl prasugrel diastereomer 1[1]	0.43
Desacetyl prasugrel diastereomer 2[1]	0.45
Desfluoro prasugrel[2]	0.9
Prasugrel	1.0
4-Fluoro prasugrel[3]	1.2
3-Fluoro prasugrel[4]	1.3

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[1]5-[2-Cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-5,6,7,7a-tetrahydrothieno[3,2-c]pyridine-2(4H)-one. Desacetyl prasugrel diastereomer 1and desacetyl prasugrel diastereomer 2 are a pair of diastereomers. They are used for resolution measurement only,

[2]5-(2-Cyclopropyl-2-oxo-1-phenylethyl)-4,5,6,7- tetrahydrothieno [3,2-c]pyridin-2-yl acetate,

[3]5-[2-Cyclopropyl-1-(4-fluorophenyl)-2-oxoethyl] -4,5,6,7- tetra-hydrothieno[3,2-c]pyridin-2-yl acetate,

[4]5-[2-Cyclopropyl-1-(3-fluorophenyl)-2-oxoethyl] -4,5,6,7- tetra-hydrothieno[3,2-c]pyridin-2-yl acetate.

Inject reference solution (a), (b) and (c). The test is not valid unless the resolution between the peaks due to desacetyl prasugrel diastereomer 1 and desacetyl prasugrel diastereomer 2 is not less than 1.5 in the chromatogram obtained with reference solution (c). The relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (a) and the signal to noise ratio is not less than 10 in the chromatogram obtained with reference solution (b).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any peak corresponding to desfluoro prasugrel is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any peak corresponding to 4-fluoro prasugrel is not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent), and the area of any peak corresponding to 3-fluoro prasugrel is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent).

B. Determine by liquid chromatography (2.4.14).

Solvent mixture. 70 volumes of *acetonitrile* and 30 volumes of *water.*

Test solution. Dissolve 250 mg of the substance under examination in the solvent mixture and dilute to 25.0 ml with the solvent mixture.

Reference solution (a). A 0.01 per cent w/v *prasugrel hydrochloride IPRS* in the solvent mixture.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Reference solution (c). Transfer 5 mg of *prasugrel hydrochloride IPRS* in to a 50-ml volumetric flask, add 20 ml of the solvent mixture and mix to dissolve. Add 1 ml of 0.0003 v/v of piperidine in *acetonitrile* and dilute to volume with the solvent mixture. Heat the solution at 50° for one hour and cool to room temperature.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with dodecylsilane bonded to porous silica (4 µm), (Such as Synergi Max RP)
- column temperature: 45°,
- sample temperature: 10°,
- mobile phase: A. a mixture of 90 volumes of *0.025M monobasic potassium phosphate* and 10 volumes of *acetonitrile,*

  B. a mixture of 90 volumes of *acetonitrile* and 10 volumes of *water,*
- flow rate: 1.5 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 210 nm,
- injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
2	100	0
30	0	100
37	0	100
38	100	0
45	100	0

Name	Relative retention time	Correction factor
Desacetyl hydroxyprasugrel[1]	0.74	—
Prasugrel diketone[2]	0.78	—
Desacetyl prasugrel diastereomer 1[3]	0.86	—
Desacetyl prasugrel diastereomer 2[3]	0.87	—
Prasugrel	1.0	—
Prasugrel chlorobutyryl analog[4]	1.06	1.37

[1]5-[2-Cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-7a-hydroxy5,6,7,7a-tetrahydrothieno[3,2-c]pyridine-2(4H)-one,

[2]1-Cyclopropyl-2-(2-fluorophenyl)ethane-1,2-dione,

[3]5-[2-Cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-5,6,7,7a-tetrahydrothieno[3,2-c]pyridin-2(4H)-one. Desacetyl prasugrel diastereomer 1 and desacetyl prasugrel diastereomer 2 are a pair of diastereomers,

[4]5-[5-Chloro-1-(2-fluorophenyl)-2-oxoethyl]-4,5,6,7-tetrahydro-thieno[3,2-c]pyridin-2-yl acetate.

Inject reference solution (a), (b) and (c). The test is not valid unless the resolution between the peaks due to desacetyl prasugrel diastereomer 1 and desacetyl prasugrel diastereomer 2 is not less than 0.9 in the chromatogram obtained with the reference solution (c), the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with the reference solution (a) and the signal to noise ratio is not less than 10 in the chromatogram obtained with the reference solution (b).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any

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peak corresponding to desacetyl hydroxyprasugrel is not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent), the area of any peak corresponding to prasugrel diketone is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any peak corresponding to desacetyl prasugrel diastereomer 1 is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any peak corresponding to desacetyl prasugrel diastereomer 2 is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent), the area of any peak corresponding to prasugrel chlorobutyryl analog is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent), the area of any other secondary peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with the reference solution (a) (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent, using platinum crucible.

Water (2.3.43). Not more than 0.5 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 70 volumes of *acetonitrile* and 30 volumes of *water*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v *prasugrel hydrochloride IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 65 volumes of *0.01 M monobasic potassium phosphate* adjusted to pH 2.8 with *orthophosphoric acid* and 35 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.0 per cent. Inject the reference solution and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak.

Calculate the content of $C_{20}H_{20}FNO_3S$, HCl,

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Prasugrel Tablets

Prasugrel Hydrochloride Tablets

Prasugrel Tablets contain prasugrel hydrochloride equivalent to not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of prasugrel, $C_{20}H_{20}FNO_3S$.

Usual strengths. 5 mg; 10 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.01M hydrochloric acid.*
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14),

Test solution. Use the filtrate, dilute if necessary, with *acetonitrile.*

Reference solution. A 0.024 per cent w/v solution of *prasugrel hydrochloride IPRS* in *acetonitrile.* Dilute 5.0 ml of the solution to 100.0 ml with the dissolution medium and further dilute 5.0 ml of the solution to 50.0 ml with the *acetonitrile.*

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- sample temperature: 15°,
- mobile phase. a mixture of 48 volumes of a buffer solution prepared by dissolving 1.36 gm of *potassium dihydrogen orthophosphate* in 1000 ml of *water,* adjusted to pH 2.7 with *dilute orthophosphoric acid* and 52 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the

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tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{20}FNO_3S$ in the medium.

Q: Not less than 75 per cent of the stated amount of $C_{20}H_{20}FNO_3S$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 90 volumes of *acetonitrile* and 10 volumes of *water*.

Test solution. Disperse a quantity of the powdered tablets containing 50 mg of Prasugrel in 10 ml of *water* with the aid of ultrasound and dilute to 100.0 ml with *acetonitrile*.

Reference solution (a). A 0.0055 per cent w/v solution of *prasugrel hydrochloride IPRS* in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution (b). To 25 mg of *prasugrel hydrochloride IPRS*, add 2 ml of *hydrogen peroxide (5 per cent)*, warm in water-bath at 80° for one minute. Cool and dilute to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- sample temperature: 15°,
- mobile phase A. *0.01M potassium dihydrogen orthophosphate* adjusted to pH 3.1 with *dilute orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
40	50	50
50	25	75
60	25	75
62	70	30
70	70	30

Name	Relative retention time	Correction factor
Desacetyl hydroxyl prasugrel[1]	0.9	0.83
Prasugrel hydrochloride	1.0	—
Desacetyl prasugrel diastereomer1[2]	1.4	0.76
Desacetyl prasugrel diastereomer 2[2]	1.5	0.76

[1](5-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-7a-hydroxy-5,6,7,7a-tetrahydrothieno [3, 2-c]pyridine-2(4 H)-one),

[2](5-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-5,6,7,7a-tetra hydrothieno [3,2-c] pyridin-2(4H)-one) Desacetyl prasugrel diastereomer1 and Desacetyl prasugrel diastereomers 2 are pair of diastereomers.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 5000 theoretical plates, the tailing factor not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject reference solution (b) for identification of desacetyl prasugrel diastereomer 1 and desacetyl prasugrel diastereomer 2 peaks.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area any peak corresponding to desacetyl prasugrel diastereomer 1 and desacetyl prasugrel diastereomer 2 is not more than 30 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent), the area any peak corresponding to desacetyl hydroxyl prasugrel is not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent). The area of any other secondary peak is not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent) and the sum of areas of all the secondary peaks excluding desacetyl prasugrel diastereomer 1 and desacetyl prasugrel diastereomer 2 is not more than 30 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Transfer one intact tablet to a suitable volumetric flask, add 10 ml of *water* and disperse with the aid of ultrasound. Add 70 ml of *acetonitrile* and further sonicate for 30 minutes with intermittent shaking, dilute with the solvent mixture to obtain a solution containing 0.005 per cent w/v of prasugrel.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{20}FNO_3S$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 90 volumes of *acetonitrile* and 10 volumes of *water*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 50 mg of Prasugrel in 10 ml of *water* with the aid of ultrasound. Add 70 ml of *acetonitrile*

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and further sonicate for 30 minutes with intermittent shaking, dilute to 100.0 ml with *acetonitrile*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture and filter.

Reference solution. A 0.0055 per cent w/v solution of *prasugrel hydrochloride IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- sample temperature: 15°,
- mobile phase. a mixture of 60 volumes of a buffer solution prepared by dissolving 1.36 gm of *potassium dihydrogen phosphate* in 1000 ml of *water,* adjusted to pH 2.7 with *diluted orthophosphoric acid* and 40 volumes of *acetonitrile,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{20}FNO_3S$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of prasugrel.

Prasugrel and Aspirin Gastro-resistant Capsules

Prasugrel Hydrochloride and Aspirin Gastro-resistant Capsules

Prasugrel and Aspirin Gastro-resistant Capsules contain prasugrel hydrochloride equivalent to not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of prasugrel, $C_{20}H_{20}FNO_3S$ and aspirin, $C_9H_8O_4$.

Usual strength. Prasugrel 10 mg and Aspirin 75 mg.

Identification

In the Assay of prasugrel, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution and in the Assay of aspirin, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

For Prasugrel —

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.01M hydrochloric acid,*
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14)

Test solution. Use the filtrate , dilute if necessary, ith *acetonitrile* to obtain a solution having a known concentration similar to the expected concentration of the reference solution.

Reference solution. A 0.024 per cent w/v solution of *prasugrel hydrochloride IPRS* in *acetonitrile*. Dilute 5.0 ml of the solution to 100.0 ml with the dissolution medium. Further dilute 5.0 ml of the solution to 50.0 ml with *acetonitrile.*

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as Oyster ODS 3),
- sample temperature: 15°,
- mobile phase. a mixture of 60 volumes of *0.01M potassium dihydrogen orthophosphate,* adjusted to pH 2.7 with *dilute orthophosphoric acid* and 40 volumes of *acetonitrile,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{20}FNO_3S$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{20}H_{20}FNO_3S$.

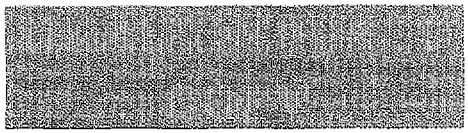
For Aspirin —

A. Apparatus No. 1 (Basket),
Medium. 1000 ml of *0.1 M hydrochloric acid,*
Speed and time. 100 rpm and 120 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14)

Test solution. Use the filtrate, if necessary, dilute suitably with the dissolution medium to obtain a solution with a known concentration similar to the expected concentration of the reference solution.



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Reference solution. Weight and transfer 30 mg of *aspirin IPRS* to 200- ml volumetric flak add 5 ml of *acetonitrile* and dissolve with the aid of ultrasound and dilute to volume with the dissolution medium. Dilute 5.0 ml of the solution to 100.0 ml with the dissolution medium.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS 2),
- sample temperature: 5°,
- mobile phase: A. a buffer solution prepared by dissolving 1.78 gm of *potassium dihydrogen ortophosphate* in 1000 ml of *water,* adjusted to pH 2.3 with *dilute orthophosphoric acid,*

B. a mixture of 70 volumes *acetonitrile* and 20 volumes *methanol.*

- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	55	45
10	55	45
11	20	80
15	20	80
16	55	45
20	55	45

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent. Inject the reference solution and the test solution.

Calculate the content of $C_9H_8O_4$ in the medium.

Complies with the acceptance criteria given under Acid stage.

B. Apparatus No. 1 (Basket),

Medium. 1000 ml of a buffer solution prepared by mixing of 75 volumes of *0.1 M hydrochloric acid* and 25 volumes of *0.2 M trisodium phosphate dodecahydrate,* adjusted to pH 6.8 with *2 M hydrochloric acid* or *2 M sodium hydroxide,*

Speed and time. 100 rpm and 90 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14)

Test solution. Dilute the filtrate, if necessary, with the dissolution medium.

Reference solution. Weight and transfer 30 mg of *aspirin IPRS* to 200-ml volumetric flak add 5 ml of *acetonitrile,* dissolve with the aid of ultrasound and dilute to volume with the dissolution medium. Dilute 5.0 ml of the solution to 10.0 ml with the dissolution medium.

Use chromatographic system as described under Acid stage.

Inject the reference solution and the test solution.

Calculate the content of $C_9H_8O_4$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_9H_8O_4$.

Related substances. Determine by liquid chromatography (2.4.14).

For Prasugrel —

Solvent mixture. 90 volumes of *acetonitrile* and 10 volumes of *water.*

Test solution. Disperse a quantity of the mixed powdered content of the capsules containing 100 mg of Prasugrel in 20 ml of *water,* with the aid of ultrasound. Add 140 ml *acetonitrile,* further sonicate for 30 minutes with intermittent shaking and dilute to 200.0 ml with *acetonitrile.*

Reference solution. A 0,0055 per cent w/v solution of *prasugrel hydrochloride IPRS* in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the the solvent mixture.

NOTE — Method is sensitive for buffer pH and column temperature.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as Inertsil ODS 3V),
- column temperature: 30°,
- sample temperature: 15°,
- mobile phase: A. *0.01M potassium dihydrogen orthophosphate,* adjusted to pH 3.1 with *dilute orthophosphoric acid,*

B. *acetonitrile,*

- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
40	50	50
50	25	75
60	25	75
62	70	30
70	70	30

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Name	Relative retention time	Correction factor
Prasugrel impurity HYTP [1]	0.9	0.83
Prasugrel	1.0	—
Prasugrel impurity A_1 [2]	1.4	0.76
Prasugrel impurity A_2 [2]	1.5	0.76

[1](5-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-7a-hydroxy-5,6,7,7a-tetrahydrothieno [3, 2-c]pyridine-2(4 H)-one),

[2](5-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4,5,6,7,tetra hydrothieno [3,2-c] pyridine-2(3H)-one).

Inject the reference solution. The test is not valid unless the column efficiency is not less than 5000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to prasugrel impurity A (Sum of the areas of prasugrel impurity A_1 and A_2) is not more than 30 times the area of the principal peak in the chromatogram obtained with the reference solution (3.0 per cent) and the area any peak corresponding to prasugrel impurity HYTP is not more than 7 times the area of the principal peak in the chromatogram obtained with the reference solution (0.7 per cent), the area of any other secondary peak is not more than 7 times the area of the principal peak in the chromatogram obtained with the reference solution (0.7 per cent) and the sum of the areas of all the secondary peaks, other than prasugrel impurity A_1 and A_2, is not more than 30 times the area of the principal peak in the chromatogram obtained with the reference solution (3.0 per cent). Ignore the peaks due to aspirin, salicylic acid and any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

For Aspirin —

Solvent mixture. 90 volumes of *acetonitrile* and 10 volumes of *water.*

Test solution. Disperse a quantity of the mixed powdered content of the capsules containing 375 mg of Aspirin in 40 ml of the solvent mixture, with the aid of ultrasound for 45 minutes with intermediate shaking and dilute to 50.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture and filter.

Reference solution. A 0.0075 per cent w/v solution of *aspirin IPRS* in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS 3V),
- sample temperature: 15°,
- mobile phase: A. *0.01M potassium dihydrogen orthophosphate,* adjusted to pH 2.5 with *dilute orthophosphoric acid,*

  B. *acetonitrile,*
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	60	40
15	60	40
25	20	80
26	60	40
35	60	40

Name	Relative retention time	Correction factor
Aspirin	1.0	—
Salicylic Acid	1.48	1.16

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to salicylic acid is not more than 30 times the area of the principal peak in the chromatogram obtained with the reference solution (3.0 per cent), the area of any other secondary peak is not more than 5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks, other than salicylic acid is not more than 20 times the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent). Ignore the peak due to prasugrel.

Uniformity of content. Complies with the test stated under Capsules.

Determine by liquid chromatography (2.4.14), as described under Assay of prasugrel with the following modification.

Test solution. Disperse one intact capsule in 20 ml *water*, with the aid of ultrasound. Add 140 ml *acetonitrile*, sonicate for 30 minutes with intermediate shaking and dilute to 200.0 ml with *acetonitrile*, filter,

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{20}FNO_3S$ in the capsule.

Other tests. Comply with the tests stated under Capsules.

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Assay. Determine by liquid chromatography (2.4.14).

For Prasugrel —

Solvent mixture. 90 volumes of *acetonitrile* and 10 volumes of *water*.

Test solution. Disperse a quantity of the mixed powdered content of 20 capsules containing 100 mg of Prasugrel in 20 ml *water* and 140 ml *acetonitrile* with the aid of ultrasound for 30 minutes with intermediate shaking and dilute to 200.0 ml with the *acetonitrile*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture and filter.

Reference solution. A 0.0055 per cent w/v solution of *prasugrel hydrochloride IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS 3V),
- sample temperature: 15°,
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 2.7 with *diluted orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{20}FNO_3S$ in the capsules.

For Aspirin —

Solvent mixture. 90 volumes of *acetonitrile* and 10 volumes of *water*.

Test solution. Disperse a quantity of the mixed powdered content of 20 capsules containing 375 mg of Aspirin in the solvent mixture, with the aid of ultrasound for 45 minutes with intermittent shaking and dilute to 50.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture and filter.

Reference solution. A 0.075 per cent w/v solution of *aspirin IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS 3V),
- sample temperature: 15°,
- mobile phase: a mixture of 60 volumes *0.01M potassium dihydrogen phosphate*, adjusted to pH 2.5 with *dilute orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

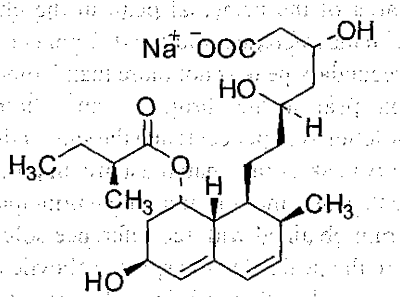
Inject the reference solution and the test solution.

Calculate the content of $C_9H_8O_4$ in the capsules.

Storage. Store protected from moisture, at a temperature not exceeding 25°.

Labelling. The label states the strength in terms of the equivalent amount of prasugrel and aspirin.

Pravastatin Sodium



$C_{23}H_{35}NaO_7$ Mol. Wt. 446.5

Pravastatin Sodium is sodium (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*S*,8*S*,8a*R*)-6-hydroxy-2-methyl-8-[[(2*S*)-2-methylbutanoyl]oxy]-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]heptanoate

Pravastatin Sodium contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{23}H_{35}NaO_7$, calculated on the anhydrous and ethanol free basis.

Category. Antihyperlipidaemic.

Description. A white to yellowish white powder or crystalline powder, hygroscopic.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pravastatin sodium IPRS* or with the reference spectrum of pravastatin sodium.

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B. 1 ml of a 5 per cent w/v solution in *carbon dioxide-free water* (Solution A) gives reaction (A) of sodium salts (2.3.1).

Tests

Appearance of solution. Dilute 2.0 ml of solution A to 10 ml with *water*. The solution is clear (2.4.1) and not more intensely coloured than the reference solution BYS6 (2.4.1).

pH (2.4.24): 7.2 to 9.0, determined in solution A.

Specific optical rotation (2.4.22). +153° to +159°, determined in a 0.5 per cent w/v solution in *water*.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 9 volumes of *methanol* and 11 volumes of *water*.

Test solution (a). Dissolve 100 mg of the substance under examination in 100 ml of the solvent mixture.

Test solution (b). Dilute 10.0 ml of test solution (a) to 100.0 ml with the solvent mixture.

Reference solution (a). Dissolve the contents of a vial of *pravastatin impurity A IPRS* in 1 ml of test solution (b).

Reference solution (b). Dilute 2.0 ml of test solution (a) to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution (c). Dissolve 12.4 mg of *pravastatin 1,1,3,3- tetramethylbutylamine IPRS* in 100.0 ml of the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 1 volume of *glacial acetic acid*, 1 volume of *triethylamine*, 450 volumes of *methanol* and 550 volumes of *water*,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to pravastatin impurity A and pravastatin is not less than 7.0.

The relative retention times with reference to pravastatin for (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*S*,8*S*,8a*R*)-6-hydroxy-8-[[(2*S*,3*R*)-3-hydroxy-2-methylbutanoyl]oxy]-2-methyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl] heptanoic acid (3″-hydroxypravastatin) (pravastatin impurity B), (3*R*,5*R*)-3,5-dihydroxy-7-[(1S,2S,6S,8S,8a*R*)-6-hydroxy-8-[[(2S,3S)-3-hydroxy-2-methylbutanoyl]oxy]-2-methyl-1,2,6,7,8,8a-hexahydronapthalen-1-yl] heptanoic acid (3″-(*S*)-hydroxypravastatin) (pravastatin impurity E), (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*R*,8*S*,8a*R*)-6-hydroxy-2-methyl-8-[[(2*S*)-2-methylbutanoyl]oxy]-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]heptanoic acid (6'-epipravastatin) (pravastatin impurity A), (1*S*,3*S*,7*S*,8*S*,8a*R*)-3-hydroxy-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-7-methyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl (2*S*)-2-methylbutanoate (pravastatin lactone) (pravastatin impurity D) and (3*R*,5*R*) -3,5-dihydroxy-7-[(1*S*,2*S*,6*S*,8*S*,8a*R*)-6-hydroxy-2-methyl-8-[[(2*S*)-2-methyl-pentanoyl]oxy]-1,2,6,7,8,8a-hexahydro-naphthalen-1-yl] heptanoic acid) (pravastatin impurity C) and (3*R*, 5*R*)-7-[(1*S*,2*S*,6*S*,8*S*,8a*R*)-6,8-dihydroxy-2-methyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]-3,5-dihydroxyheptanoic acid (pravastatin impurity F) are about 0.2, 0.3, 0.6, 1.9 and 2.1 respectively.

Inject reference solution (b) and test solution (a). Run the chromatogram 2.5 times the retention time of the principal peak of pravastatin. In the chromatogram obtained with test solution (a), the area of peak due to pravastatin impurity A is not more than 1.5 times the area of the principal peak obtained with reference solution (b) (0.3 per cent), the area of peaks due to pravastatin impurities B, C, D and E is not more than the area of the principal peak obtained with reference solution (b) (0.2 per cent), the area of peak due to pravastatin impurity F is not more than 0.75 times the area of the principal peak obtained with reference solution (b) (0.15 per cent) the area of other secondary peak is not more than 0.5 times the area of the principal peak obtained with reference solution (b) (0.1 per cent) and sum of the areas of all the secondary peaks is not more than 3 times the area of the principal peak obtained with reference solution (b) (0.6 per cent). Ignore any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). Dissolve 2.0 g in a mixture of 15 volumes of *water* and 85 volumes of *methanol* and dilute to 20 ml with the same solvent mixture and use 12 ml of the solution. The resulting solution complies with the limit test for heavy metals, Method D (20 ppm), using 10 ml of *lead standard solution (2 ppm Pb)* in the same solvent misture..

Ethanol (2.3.45). Not more than 3.0 per cent (determined by method I).

Water (2.3.43). Not more than 4.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14), as described under test for Related substances with the following modification.

Inject reference solution (c) and test solution (b).

Calculate the content of $C_{23}H_{35}NaO_7$ from the declared content of pravastatin in *pravastatin 1,1,3,3-tetramethylbutylamine IPRS*.

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1 mg of pravastatin is equivalent to 1.052 mg of pravastatin sodium.

Storage. Store protected from light and moisture.

Pravastatin Tablets

Pravastatin Sodium Tablets

Pravastatin Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of pravastatin sodium, $C_{23}H_{35}NaO_7$.

Usual strengths. 10 mg; 20 mg; 40 mg.

Identification

A. Shake a quantity of the powdered tablets containing about 10 mg of Pravastatin Sodium with 80 ml of *water* for 10 minutes, dilute to 100 ml with *water*, filter. Dilute 1 ml to 10 ml with *water*. When examined in the range 220 to 350 nm (2.4.7), exhibits a maximum only at 238 nm.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the medium, if necessary, at the maxima at about 238 nm (2.4.7). Calculate the content of $C_{23}H_{35}NaO_7$ in the medium from the absorbance obtained from a solution of known concentration of *pravastatin 1,1,3,3-tetramethylbutylamine IPRS* in the dissolution medium.

NOTE — To Calculate the content of $C_{23}H_{35}NaO_7$, use the conversion factor of (446.51/553.78), in which 446.51 and 553.78 are the molecular weights of pravastatin sodium and pravastatin 1,1,3,3-tetramethylbutylamine respectively.

Q. Not less than 80 per cent of the stated amount of $C_{23}H_{35}NaO_7$.

Related substances. Determine by liquid chromatography (2.4.14).

Solution A. Dissolve 4.1 g of *anhydrous sodium acetate* in 400 ml of *water*, adjusted to pH 5.6 with *glacial acetic acid* and add sufficient *water* to produce 500.0 ml.

Solution B. Mix 20 volumes of *methanol* with 80 volumes of solution A.

Test solution. Add 20 ml of solution A to a quantity of the powdered tablets containing about 10 mg of Pravastatin Sodium, mix using a vortex mixer and then with the aid of ultrasound for 15 minutes, centrifuge. Dilute 1 ml of the clear supernatant liquid to 5 ml with solution B.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with solution B.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 5.0 ml with solution B.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 4.0 ml with solution B.

Reference solution (d). Dissolve 2 mg of *(3R,5R)-3,5-dihydroxy-7-[(1S,2S,6R,8S,8aR)-6-hydroxy-2-methyl-8-[[(2S)-2-methylbutanoyl]oxy]-1,2,6,7,8,8a- hexahydronaphthalen-1-yl]heptanoic acid IPRS (pravastatin impurity A IPRS)* in the minimum volume of *methanol* and dilute to 20.0 ml with the test solution.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Ultrasphere ODS),
- mobile phase: a mixture of 1 volume of *glacial acetic acid*, 1 volume of *triethylamine*, 450 volumes of *methanol* and 550 volumes of *water*,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 20 µl.

Inject reference solution (d). The test is not valid unless the resolution between the peaks due to pravastatin impurity A and pravastatin is not less than 7.0.

Inject reference solution (a), (b), (c) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2 per cent), the area of one such peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent), not more than a further one such peak has an area more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than three times the area of the principal peak in the chromatogram obtained with reference solution (a) (3 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Add 20 ml of solution A to a quantity of the powdered tablets containing about 10 mg of Pravastatin

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Sodium, mix using a vortex mixer and then with the aid of ultrasound for 15 minutes, centrifuge. Dilute 1.0 ml of the clear supernatant liquid to 5.0 ml with solution B.

Reference solution (a). Dissolve 12.4 mg of *pravastatin 1,1,3,3,-tetramethylbutylamine IPRS* in 20 ml of solution A. Dilute 1 ml of the solution to 5 ml with solution B.

Reference solution (b). Dissolve 2 mg of *pravastatin impurity A IPRS* in the minimum volume of *methanol* and dilute to 20.0 ml with the test solution.

Use the chromatographic system, solution A and B as described under Related substances.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to pravastatin impurity A and pravastatin is not less than 7.0.

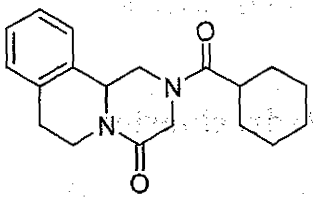
Inject reference solution (a) and the test solution.

Calculate the content of $C_{23}H_{35}NaO_7$ in the tablets using the declared content of pravastatin in *pravastatin 1,1,3,3-tetramethylbutylamine IPRS*.

1 mg of $C_{23}H_{36}O_7$ is equivalent to 1.052 mg of $C_{23}H_{35}NaO_7$.

Storage. Store protected from light and moisture.

Praziquantel



$C_{19}H_{24}N_2O_2$ Mol.Wt. 312.4

Praziquantel is (11b*RS*)-2-(cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4*H*-pyrazino[2,1-*a*]isoquinolin-4-one.

Praziquantel contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{19}H_{24}N_2O_2$, calculated on the dried basis.

Category. Antihelminthic.

Description. A white or almost white, crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *praziquantel IPRS* or with the reference spectrum of praziquantel.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution (a). Dissolve 40 mg of the substance under examination in 10 ml of the mobile phase.

Test solution (b). Dilute 1.0 ml of test solution (a) to 20.0 ml with the mobile phase.

Reference solution (a). Dissolve 40 mg of *praziquantel IPRS* in 10.0 ml of the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *(11bRS)-2-benzoyl-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]isoquinolin-4-one IPRS (praziquantel impurity A IPRS)* in 25 ml of reference solution (a). Dilute 2.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of test solution (a) to 20.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 45 volumes of *acetonitrile* and 55 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks corresponding to praziquantel impurity A and praziquantel is not less than 3.0.

Inject reference solution (b), (c) and test solution (a). Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with test solution (a), the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent), the area of one such peak is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent) and the sum of the areas of all other secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent). Ignore any peak with an area 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 50° over *diphosphorus pentoxide* at a pressure not exceeding 0.7 kPa for 2 hours.

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Assay. Determine by liquid chromatography (2.4.14) as described under Related substances.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject reference solution (a) and test solution (b).

Calculate the content of $C_{19}H_{24}N_2O_2$.

Storage. Store protected from light.

Praziquantel Tablets

Praziquantel Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of Praziquantel, $C_{19}H_{24}N_2O_2$.

Usual strength. 600 mg.

Identificaton

Extract a quantity of the powdered tablets containing 50 mg of praziquantel with 20 ml of *dichloromethane*, filter and evaporate the filtrate to dryness. The residue complies with the following test.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *praziquantel IPRS* or with the reference spectrum of praziquantel.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. Ethyl acetate.

Test solution. Shake a quantity of the powdered tablets containing 30 mg of praziquantel in 5 ml of *methanol* for 5 minutes, centrifuge and use the clear supernatant liquid.

Reference solution. A 0.6 per cent w/v solution of *praziquantel IPRS* in *methanol*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1 M hydrochloric acid* containing 2.0 mg per ml of *sodium lauryl sulphate*,

Speed and time. 50 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate at the maximum at about 263 nm (2.4.7). Calculate the content of $C_{19}H_{24}N_2O_2$ in the medium from the absorbance obtained from a solution of known concentration of *praziquantel IPRS* in the same medium.

Q. Not less than 75 per cent of the stated amount of $C_{19}H_{24}N_2O_2$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powder containing 150 mg of Praziquantel, add 70 ml of mobile phase, sonicate for 5 minutes, dilute to 100.0 ml with mobile phase, mix and filter. Dilute 3.0 ml of the solution to 25.0 ml with the mobile phase.

Reference solution. A 0.018 per cent w/v solution of *praziquentel IPRS* in the mobile phase.

Chromatographic System

- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase : a mixture of 60 volumes of *acetonitrile* and 40 volumes of *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

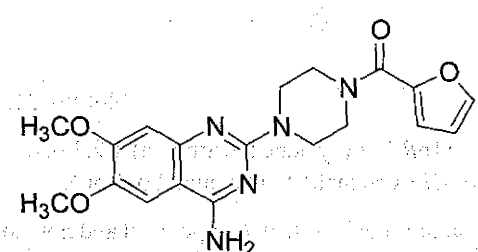
Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{24}N_2O_2$ in the tablets.

Storage. Store protected from light.

Prazosin Hydrochloride



$C_{19}H_{21}N_5O_4,HCl$ Mol. Wt. 419.9

Prazosin Hydrochloride is 2-[4-(2-furoyl)piperazin-1-yl]-6,7-dimethoxyquinazolin-4-ylamine hydrochloride.

Prazosin Hydrochloride contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{19}H_{21}N_5O_4,HCl$, calculated on the anhydrous basis.

Category. Antihypertensive.

Description. A white or almost white powder.

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Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prazosin hydrochloride IPRS* or with the reference spectrum of prazosin hydrochloride.

B. Prepare a 0.05 per cent w/v solution of the substance under examination in a 0.1 per cent v/v solution of *hydrochloric acid* in *methanol*. Dilute separately 1.0 ml and 5.0 ml of the solution to 100 .0ml with the same acid solution (solution A and B respectively). When examined in the range 220 nm to 280 nm (2.4.7), solution A shows an absorption maximum at about 247 nm; absorbance at the maximum, 0.66 to 0.70. When examined in the range 280 nm to 400 nm, solution B exhibits two maxima at about 330 nm and 343 nm; absorbances at the two maxima, 0.65 to 0.70 and 0.60 to 0.66 respectively.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Solvent mixture. 1 volume of *diethylamine*, 10 volumes of *methanol* and 10 volumes of *methylene chloride*.

Mobile phase. A mixture of 5 volumes of *diethylamine* and 95 volumes of *ethyl acetate*.

Test solution. Dissolve 10 mg of the substance under examination in 10 ml of solvent mixture.

Reference solution. A 0.1 per cent w/v solution of *prazosin hydrochloride IPRS* in solvent mixture.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 8 cm. Dry the plate in warm air and examine under ultra-violet light at 254 nm. The principal spot in the chroma togram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

D. A 0.1 per cent w/v solution gives the reactions of chlorides (2.3.1).

Tests

Iron. To 1.0 g add dropwise about 1.5 ml of *nitric acid*, heat cautiously on a water-bath until fumes are no longer evolved. Ignite by slowly raising the temperature from 150° to 1000°, maintaining the final temperature for 1 hour. Cool, dissolve the residue in 20 ml of *2 M hydrochloric acid*, evaporate to about 5 ml, dilute to 25 ml with *2 M hydrochloric acid* and examine the resulting solution by atomic absorption spectrophotometry (2.4.2), measuring at 248 nm using an iron hollow-cathode light as a source of radiation, an air-acetylene flame and *iron standard solution (8 ppm Fe)*, diluted as necessary with *water* to prepare the standard solutions (100 ppm).

Reserve about 10 ml of the solution for the Nickel test.

Nickel. Examine the final solution prepared in the test for Iron by atomic absorption spectrophotometry (2.4.2), measuring at 232 nm using a nickel hollow-cathode light as a source of radiation, an air-acetylene flame and using *nickel standard solution (10 ppm Ni)*, diluted as necessary with *water* to prepare the standard solution (50 ppm).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50.0 mg of the substance under examination in the mobile phase and dilute to 50 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 8 mg of *metoclopramide hydrochloride IPRS* in 1 ml of the test solution and dilute to 25 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

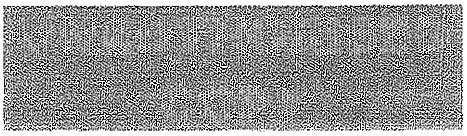
Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of *methanol* and 50 volumes of a solution containing 3.484 g per litre of *sodium pentanesulphonate* and 3.64 g per litre of *tetramethylammonium hydroxide* adjusted to pH 5.0 with *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (b). The retention times are: prazocine about 9 minutes and metoclopramide about 5 minutes.The test is not valid unless the resolution between the peaks corresponding to prazocine and metoclopramide is at least 8.

Inject reference solution (a) and the test solution. Continue the chromatography of the test solution for 6 times the retention time of the principal peak. Any secondary peak obtained with the test solution is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of all the impurities is not more than five times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). Determine on the residue obtained in the test for Sulphated ash. Dissolve in sufficient *2 M nitric acid* to produce 10 ml. The resulting solution complies with the limit test for heavy metals, Method B (20 ppm).



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Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). Not more than 0.5 per cent w/w, using 2.0 g dissolved in a mixture of equal volumes of *dichloromethane* and *methanol*.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 96 volumes of *methanol*, 2 volumes of *glacial acetic acid* and 2 volumes of *water*

Test solution. Dissolve 20 mg of substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.002 per cent w/v solution of *prazosin hydrochloride IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 20 cm x 4 mm, packed with porous silica particles (5 µm),
- mobile phase: 0.01 per cent w/v solution of *diethylamine* in the solvent mixture,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{21}N_5O_4,HCl$.

Storage. Store protected from light.

Prazosin Tablets

Prazosin Hydrochloride Tablets

Prazosin Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of prazosin, $C_{19}H_{21}N_5O_4$.

Usual strengths. The equivalent of 500 µg; 1 mg; 2 mg; 5 mg of prazosin.

Identification

Shake a quantity of the powdered tablets containing about 10 mg of prazosin with a mixture of 10 ml of *dichloromethane* and 10 ml of *0.05 M potassium hydroxide*, filter the organic layer through cotton, evaporate to dryness and dry the residue at 60° at a pressure not exceeding 2 kPa at 60° for 2 hours.

On the residue determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prazosin hydrochloride IPRS* treated in the same manner or with the reference spectrum of prazosin.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Solvent mixture. 10 volumes of *dichloromethane*, 10 volumes of *methanol* and 1 volume of *diethylamine*.

Mobile phase. A mixture of 95 volumes of *ethyl acetate* and 5 volumes of *diethylamine*.

Test solution. Shake a quantity of the powdered tablets containing 5 mg of prazosin with 2 ml of the solvent mixture, centrifuge and pass the supernatant liquid through a 0.5 µm PTFE (Polytetrafluoroethylene) filter.

Reference solution (a). Dilute 1 volume of test solution to 200 volumes with the solvent mixture

Reference solution (b). Dilute 2 volumes of reference solution (a) to 5 volumes with the solvent mixture.

Apply to the plate 20 µl of each solution. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than two such spots are more intense than the spot in the chromatogram obtained with reference solution (b).

Uniformity of content. Complies with the tests stated under Tablets.

Determine by liquid chromatography (2.4.14).

Test solution. Shake one tablet for 1 hour in a suitable volume of a mixture of 96 volumes of *methanol*, 2 volumes of *glacial acetic acid* and 2 volumes of *water* to produce a solution containing 0.002 per cent w/v solution of prazosin, centrifuge and use the supernatant liquid.

Reference solution. A 0.0022 per cent w/v solution of *prazosin hydrochloride IPRS* in the same solvent mixture.

Chromatographic system

- a stainless steel column 20 cm x 4 mm, packed with porous silica particles, (5 µm),
- mobile phase: 0.01 per cent w/v solution of *diethylamine* in a mixture of 96 volumes of *methanol*, 2 volumes of *glacial acetic acid* and 2 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Calculate the content of $C_{19}H_{21}N_5O_4$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Shake a quantity of the powdered tablets containing about 2 mg of prazosin with 100 ml in a mixture of 96 volumes of *methanol*, 2 volumes

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of *glacial acetic acid* and 2 volumes of *water* for 30 minutes, centrifuge and use the supernatant liquid.

Reference solution. A 0.0022 per cent w/v solution of *prazosin hydrochloride IPRS* in a mixture of 96 volumes of *methanol*, 2 volumes of *glacial acetic acid* and 2 volumes of *water*.

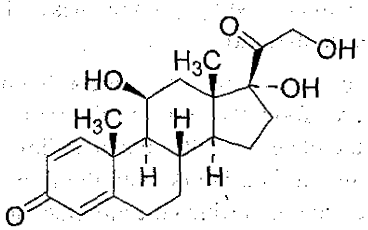
Use chromatographic conditions as described under Uniformity of content.

Calculate the content of $C_{19}H_{21}N_5O_4$ in the tablets.

Storage. Store protected from light.

Labelling. The label states the strength in terms of the equivalent amount of prazosin.

Prednisolone



$C_{21}H_{28}O_5$ Mol. Wt. 360.4

Prednisolone is 11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione.

Prednisolone contains not less than 96.0 per cent and not more than 104.0 per cent of $C_{21}H_{28}O_5$, calculated on the dried basis.

Category. Adrenocortical steroid.

Description. A white or almost white, crystalline powder; hygroscopic.

Identification

Test A may be omitted if tests B and C are carried out. Test C may be omitted if tests A and B are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prednisolone IPRS* or with the reference spectrum of prednisolone.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. A mixture of 90 volumes of *acetone* and 10 volumes of *formamide*.

Mobile phase. *Chloroform*.

Test solution. Dissolve 25 mg of the substance under examination in 10 ml of the solvent mixture.

Reference solution (a). Dissolve 25 mg of *prednisolone IPRS* in 10 ml of the solvent mixture.

Reference solution (b). Mix equal volumes of the test solution and reference solution (a).

Place the dry plate in a tank containing a shallow layer of the solvent mixture, allow the solvent mixture to ascend to the top, remove the plate from the tank and allow the solvent to evaporate. Use within 2 hours, with the flow of the mobile phase in the direction in which the aforementioned treatment was done.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of warm air, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulphuric acid (20 per cent v/v)*. Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

C. Dissolve 2 mg in 2 ml of *sulphuric acid* by shaking and allow to stand for 5 minutes; an intense red colour is produced with a reddish brown fluorescence when examined under ultraviolet light at 365 nm. Pour the solution into 10 ml of *water* and mix; the colour fades and there is a yellow fluorescence under ultra-violet light (365 nm).

Tests

Specific optical rotation (2.4.22). + 96.0° to + 102°, determined in a 1.0 per cent w/v solution in *dioxan*.

Light absorption (2.4.7). Absorbance of a 0.001 per cent w/v solution in *ethanol (95 per cent)* at the maximum at about 240 nm, 0.40 to 0.43.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in 2 ml of *tetrahydrofuran* and dilute to 10ml with *water*.

Reference solution (a). Dissolve 2 mg of *prednisolone IPRS* and 2 mg of *hydrocortisone IPRS* in the mobile phase and dilute to 100 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with base deactivated end-capped octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,

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– mobile phase: a mixture of 22 volumes of *tetrahydrofuran* and 78 volumes ml of *water*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 254 nm,
– injection volume: 20 µl.

Equilibrate the column with the mobile phase for about 30 minutes.

The retention times are: prednisolone, about 14 minutes and hydrocortisone about 15.5 minutes.

Inject reference solution (a). The test is not valid unless the resolution between the peaks corresponding to prednisolone and hydrocortisone is at least 2.2. If necessary, adjust the concentration of *tetrahydrofuran* in the mobile phase.

Inject reference solution (ab) and the test solution. Run the chromatogram 4.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and not more than one such peak has an area more than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than 2.0 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent). Ignore any peak obtained with the blank run and any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (b).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Weigh 0.1 g and dissolve in sufficient *ethanol* to produce 100.0 ml. Dilute 2.0 ml of the solution to 100.0 ml with *ethanol*. Measure the absorbance of the resulting solution at the maximum at about 243.5 nm. Calculate the content of $C_{21}H_{28}O_5$ taking 415 as the specific absorbance at 243.5 nm.

Storage. Store protected from light and moisture.

Prednisolone Tablets

Prednisolone Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of prednisolone, $C_{21}H_{28}O_5$.

Usual strengths. 5 mg; 10 mg; 20 mg.

Identification

Extract a quantity of the powdered tablets containing 30 mg of Prednisolone with 10 ml of *chloroform*, filter and evaporate the filtrate to dryness. The residue complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prednisolone IPRS* or with the reference spectrum of prednisolone.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. A mixture of 90 volumes of *acetone* and 10 volumes of *formamide*.

Mobile phase. Chloroform.

Test solution. Disperse a quantity of powdered tablets containing 25 mg of Prednisolone in 10 ml of the solvent mixture.

Reference solution (a). Dissolve 25 mg of *prednisolone IPRS* in 10 ml of the solvent mixture.

Reference solution (b). Mix equal volumes of the test solution and reference solution (a).

Place the dry plate in a tank containing a shallow layer of the solvent mixture, allow the solvent mixture to ascend to the top, remove the plate from the tank and allow the solvent to evaporate. Use within 2 hours, with the flow of the mobile phase in the direction in which the aforementioned treatment was done.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of warm air, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulphuric acid (20 per cent v/v)*. Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium: 900 ml of *water*,

Speed and time: 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium, filter and dilute a suitable volume of the filtrate with the same solvent. Measure the absorbance of the filtrate at the maximum at about 240 nm (2.4.7). Calculate the content of $C_{21}H_{28}O_5$ in the medium from the absorbance obtained from a solution of known concentration of *prednisolone IPRS* prepared by dissolving in *methanol* and diluting with the dissolution medium.

Q: Not less than 70 per cent of the stated amount of $C_{21}H_{28}O_5$.

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Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets containing 10 mg of Prednisolone with 25 ml of *methanol* for 10 minutes and mix with the aid of ultrasound for 2 minutes; filter the extract (Whatman GF/F is suitable), wash the filter with two 10-ml quantities of *methanol*, combine the filtrate and washings and evaporate to dryness using a rotary evaporator and a warm water-bath, dissolve the residue in 10 ml of *tetrahydrofuran* and dilute to 20 ml with *water*.

Reference solution (a). Dissolve 2 mg of *prednisolone IPRS* and 2 mg of *hydrocortisone IPRS* in the mobile phase and dilute to 100 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a 50 per cent v/v solution of *tetrahydrofuran*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: a mixture of 22 volumes of *tetrahydrofuran* and 78 volumes ml of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Equilibrate the column with the mobile phase for about 30 minutes.

The retention times are: prednisolone, about 14 minutes and hydrocortisone about 15.5 minutes.

Inject reference solution (a). The test is not valid unless the resolution between the peaks corresponding to prednisolone and hydrocortisone is not less than 2.2. If necessary, adjust the concentration of *tetrahydrofuran* in the mobile phase.

Inject reference solution (ab) and the test solution. Run the chromatogram 4.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and the sum of the areas of all the secondary peaks is not greater than three times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent). Ignore any peak obtained with the blank run and any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent) and any peak with a retention time of 3 minutes or less.

Uniformity of content. Complies with the test stated under Tablets.

Powder one tablet, add 50 ml of *ethanol (95 per cent)*, shake for 30 minutes, add sufficient *ethanol (95 per cent)* to produce 100.0 ml. Centrifuge and pipette a suitable volume of the supernatant liquid containing 0.5 mg of Prednisolone and dilute to 50.0 ml with *ethanol (95 per cent)*. Measure the absorbance of the resulting solution at the maximum at about 240 nm (2.4.7). Calculate the content of $C_{21}H_{28}O_5$ taking 415 specific absorbance at 240 nm.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 5 mg of Prednisolone, add 58 ml of *methanol*, shake for 10 minutes and add sufficient *water* to produce 100.0 ml. Mix well and filter.

Reference solution (a). A solution containing 0.005 per cent w/v of *prednisolone IPRS* and 0.0075 per cent w/v of *dexamethasone* (internal standard) in a mixture of 58 volumes of *methanol* and 42 volumes of *water*.

Reference solution (b). A 0.005 per cent w/v solution of *prednisolone IPRS* in a mixture of 58 volumes of *methanol* and 42 volumes of *water*.

Chromatographic system
- a stainless steel column 20 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 42 volumes of *water* and 58 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

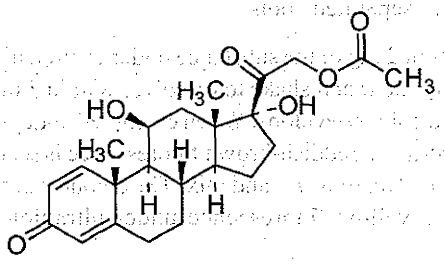
Inject reference solution (a). The test is not valid unless the resolution between the peaks due to prednisolone and dexamethasone is not less than 2.5 and the column efficiency, determined using the peak due to prednisolone in the chromatogram obtained with the test solution is not less than 15,000 theoretical plates per metre.

Inject reference solution (b) and the test solution.

Calculate the content of $C_{21}H_{28}O_5$ in the tablets.

Storage. Store protected from light.

Prednisolone Acetate



$C_{23}H_{30}O_6$ Mol.Wt. 402.5

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Prednisolone Acetate is 11β,17α-dihydroxy-3,20-dioxopregna-1,4-dien-21-yl acetate.

Prednisolone Acetate contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{23}H_{30}O_6$, calculated on the dried basis.

Category. Antiinflammtory; immunosuppressant.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prednisolone acetate IPRS* or with the reference spectrum of prednisolone acetate.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel F254*.

Solvent mixture. 10 volumes of *methanol* and 90 volumes of *dichloromethane*.

Mobile phase. Add a mixture of 1.2 volumes of *water* and 8 volumes of *methanol* to a mixture of 15 volumes of *ether* and 77 volumes of *dichloromethane*.

Test solution. Dissolve 10 mg of the substance under examination in 10.0 ml of the solvent mixture.

Reference solution (a). A 0.1 per cent w/v solution of *prednisolone acetate IPRS* in the solvent mixture.

Reference solution (b). Dissolve 10 mg of *prednisolone pivalate IPRS* in 10.0 ml of reference solution (a).

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. Spray with *alcoholic solution of sulphuric acid*. Heat at 105° for 10 minutes or until the spots appear. Allow to cool and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

C. Add about 2 mg of the substance under examination in 2 ml of *sulphuric acid* and shake to dissolve. Within 5 minutes, an intense red colour develops. When examined under ultraviolet light at 365 nm, a reddish-brown fluorescence is seen. Add the solution to 10 ml of *water* and mix. The colour fades and there is greenish-yellow fluorescence under ultraviolet light at 365 nm.

D. It gives the reaction of acetyl (2.3.1).

Tests

Specific optical rotation (2.4.22). +128° to +137°, determined in a 0.35 per cent w/v solution in *methanol*.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Prepare the solutions immediately before use.

Solvent mixture. Equal volumes of *acetonitrile* and buffer solution pH 4 prepared by mixing 1 volume of *dilute hydrochloric acid*, 5 volumes of a 6.81 per cent w/v solution of *sodium acetate*, 15 volumes of a 3.73 per cent w/v solution of *potassium chloride* and 79 volumes of *water*.

Test solution. Dissolve 25 mg of the substance under examination in 10 ml of *methanol*.

Reference solution (a). Dissolve 2 mg each of *prednisolone acetate IPRS* and *hydrocortisone acetate IPRS (prednisolone impurity A IPRS)* in 100 ml of the solvent mixture.

Reference solution (b). Dilute 1.0 ml of the test solution to 100 ml with the solvent mixture. Dilute 2.0 ml of the solution to 10 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 35 volumes of *acetonitrile* and 65 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention time with reference to prednisolone acetate for prednisolone acetate impurity B is about 0.4, for prednisolone acetate impurity A is about 1.1, for prednisolone acetate impurity C is about 2.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to prednisolone acetate and prednisolone acetate impurity A is not less than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of secondary peak corresponding to prednisolone acetate impurity A and B, each of, is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), the area of secondary peak corresponding to prednisolone acetate impurity C is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any other secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with

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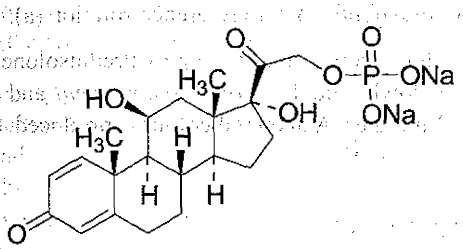
reference solution (b) (0.1 per cent) and the sum of the areas of all secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent). Ignore any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.1 g in 100.0 ml of *ethanol (95 per cent)*. Dilute 2.0 ml of the solution to 100.0 ml with the same solvent. Measure the absorbance at the maximum at about 243 nm (2.4.7). Calculate the content of $C_{23}H_{30}O_6$ taking 370 as the specific absorbance at 243 nm.

Storage. Store protected from light.

Prednisolone Sodium Phosphate



$C_{21}H_{27}Na_2O_8P$ Mol. Wt. 484.4

Predisolone Sodium Phosphate is 11β,17α, 21-trihydroxy-pregna-1,4-diene-3,20-dione-21-(dihydrogenphosphate) disodium salt.

Prednisolone Sodium Phosphate contains not less than 96.0 per cent and not more than 102.0 per cent of $C_{21}H_{27}Na_2O_8P$, calculated on the anhydrous basis.

Category. Antiinflammtory; immunosuppressant.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prednisolone sodium phosphate IPRS* or with the reference spectrum of prednisolone sodium phosphate. (If the spectra obtained in the solid state show differences, dissolve the substance under examination and the reference substance separately in the minimum volume of *ethanol (95 per cent)* evaporate to dryness on a water-bath and record the spectra again using the residues).

B. To about 40 mg add 2 ml of *sulphuric acid* and heat gently until white fumes are evolved. Add *nitric acid* dropwise, continue the heating until the solution is almost colourless and cool. Add 2 ml of *water*, heat until white fumes are again evolved, cool, add 10 ml of *water* and neutralise to *red litmus paper* with *dilute ammonia solution*. The solution complies with reaction (A) of sodium salts and reaction (B) of phosphates (2.3.1).

Tests

pH (2.4.24). 7.5 to 10.5 determined in 1.0 per cent w/v solution.

Specific optical rotation (2.4.22). +95.0° to +102.0°, determined in a 1.0 per cent w/v solution in a mixture of 9 volumes of *phosphate buffer pH 7.0* and 1 volume of *carbon dioxide-free water*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 62.5 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 25 mg of *prednisolone sodium phosphate IPRS* and 25 mg of *prednisolone IPRS* in the mobile phase and dilute to 25.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 25.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica or ceramic microparticles (5 µm),
- mobile phase: mix 1.36 g of *potassium dihydrogen phosphate* and 0.6 g of *hexylamine* and allow to stand for 10 minutes and dissolve in 185 ml of *water*, add 65 ml of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Equilibrate the column with the mobile phase for about 30 minutes.

The retention times are: prednisolone sodium phosphate, about 6.5 minutes and prednisolone, about 8.5 minutes.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to prednisolone sodium phosphate and prednisolone is at least 4.5; if this resolution is not achieved, increase the concentration of *acetonitrile* or increase the concentration of *water* in the mobile phase.

Inject reference solution (b) and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak other than that from the principal peak is not

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greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent) and not more than one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent); the sum of the areas of all the peaks other than the principal peak is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent). Ignore any peak due to the solvent and any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Inorganic phosphate. Not more than 1.0 per cent of phosphate, PO_4.

Dissolve 50 mg in sufficient *water* to produce 100 ml. To 10 ml of the resulting solution add 5 ml of *molybdovanadic reagent*, mix and allow to stand for 5 minutes. Any yellow colour in the solution is not more intense than that produced in a standard prepared at the same time in the same manner using 10 ml of *phosphate standard solution (5 ppm PO_4)*.

Water (2.3.43). Not more than 6.5 per cent, determined on 1.0 g.

Assay. Dissolve 0.1 g in sufficient *water* to produce 100.0 ml and mix. Dilute 5.0 ml of the resulting solution to 250.0 ml with *water*. Measure the absorbance at the maximum at about 247 nm (2.4.7). Calculate the content of $C_{21}H_{27}Na_2O_8P$ taking 312 as the specific absorbance at 247 nm.

Storage. Store protected from light.

Prednisolone Sodium Phosphate Eye Drops

Prednisolone Sodium Phosphate Eye Drops is a sterile solution of Prednisolone Sodium Phosphate in Purified Water.

Prednisolone Sodium Phosphate Eye Drops contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of prednisolone sodium phosphate, $C_{21}H_{27}Na_2O_8P$.

Identification

For eye drops containing less than 0.1 per cent w/v of Prednisolone Sodium Phosphate carry out test B only.

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

NOTE— Prepare the solutions immediately before use.

Mobile phase. A mixture of 33 volumes of *water*, 47 volumes of *glacial acetic acid* and 120 volumes of *butan-1-ol*.

Test solution. Dilute the eye drops to obtain a solution containing 0.1 per cent w/v solution of Prednisolone Sodium Phosphate in *water*.

Reference solution (a). A 0.1 per cent w/v solution of *prednisolone sodium phosphate IPRS* with *water*.

Reference solution (b). A mixture of equal volumes of test solution and reference solution (a).

Reference solution (c). A mixture of equal volumes of reference solution (a) and 0.1 per cent w/v solution of *betamethasone sodium phosphate IPRS* in *water*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air, heat at 110° for 10 minutes and examine under ultraviolet light at 254 nm. The chromatogram obtained with the test solution, reference solution (a) and reference solution (b) show single principal spots with similar R_f values. The chromatogram obtained with reference solution (c) shows two principal spots with almost identical Rf values.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

C. To a volume containing 0.2 mg of Prednisolone Sodium Phosphate slowly add 1 ml of *sulphuric acid* and allow to stand for 2 minutes. A deep red colour is produced.

Tests

pH (2.4.24). 7.0 to 8.5.

Free prednisolone. Determine by liquid chromatography (2.4.14).

For eye drops containing 0.01 per cent w/v or more of Prednisolone Sodium Phosphate. Use 50 µl of the following solutions —

Test solution. Dilute the volume of eye drops with the mobile phase to produce a solution containing 0.01 per cent w/v of Prednisolone Sodium Phosphate.

Reference solution. A 0.0004 per cent w/v solution of *prednisolone IPRS* in the mobile phase.

For eye drops containing less than 0.01 per cent w/v of Prednisolone Sodium Phosphate. Use 0.2 ml of the following solutions —

Test solution. Dilute the volume of eye drops with the mobile phase to produce a solution containing 0.001 per cent w/v solution of Prednisolone Sodium Phosphate.

Reference solution. A 0.00004 per cent w/v solution of *prednisolone IPRS* in the mobile phase.

Chromatographic system

— a stainless steel column 20 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (10 µm),

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- mobile phase: a mixture of 45 volumes of *methanol* and 55 volumes of *citro-phosphate buffer pH 5.0*;
- flow rate: 2 ml per minute,
- spectrophotometer set at 247 nm.

Inject the reference solution and the test solution. In the chromatogram obtained with test solution, the area of any peak corresponding to prednisolone is not more than the area of the principal peak in the chromatogram obtained with the reference solution (4.0 per cent).

Other tests. Comply with the tests stated under Eye Drops.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a quantity of the eye drops, if necessary, to obtain a solution containing 0.001 per cent w/v of Prednisolone Sodium Phosphate in *water*.

Reference solution (a). A 0.001 per w/v solution of *prednisolone sodium phosphate IPRS* in *water*.

Reference solution (b). A solution containing 0.01 per cent w/v of *betamethasone sodium phosphate IPRS* and 0.001 per cent w/v of *prednisolone sodium phosphate IPRS* in *water*. Dilute 10.0 ml of the solution to 100.0 ml with *water*.

Chromatographic system
- a stainless steel column 20 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 45 volumes of *methanol* and 55 volumes of *citro-phosphate buffer pH 5.0*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 247 nm,
- injection volume: 50 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to betamethasone sodium phosphate and prednisolone sodium phosphate is not less than 2.5.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{21}H_{27}Na_2O_8P$ in the eye drop.

Storage. Store protected from light.

Prednisolone Sodium Phosphate Injection

Prednisolone Sodium Phosphate Injection is a sterile solution of Prednisolone Sodium Phosphate in Water for Injections.

Prednisolone Sodium Phosphate Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of prednisolone phosphate, $C_{21}H_{29}O_8P$.

Usual strength. 20 mg per ml.

Description. A clear, colourless liquid.

Identification

A. In the Assay, the chromatogram obtained with the test solution corresponds to the peak due to prednisolone sodium phosphate in the chromatogram obtained with reference solution (a).

B. To a volume containing 0.2 mg of Prednisolone Sodium Phosphate slowly add 1 ml of *sulphuric acid* and allow to stand for 2 minutes. A deep red colour is produced.

Tests

pH (2.4.24). 7.0 to 8.0.

Bacterial endotoxins (2.2.3). Not more than 5.0 Endotoxin Units per mg of prednisolone phosphate.

Other tests. Complies with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a measured volume of the injection to obtain a solution containing 0.001 per cent w/v of prednisolone phosphate.

Reference solution (a). Weigh 10 mg of *prednisolone sodium phosphate IPRS*, dissolve in sufficient *water* to produce 100.0 ml (solution A) and dilute 10.0 ml of the solution to 100.0 ml with *water*.

Reference solution (b). Add 10 ml of a 0.01 per cent w/v solution of *betamethasone sodium phosphate IPRS* in *water* to 10 ml of solution A and dilute to 100.0 ml with *water*.

Chromatographic system
- a stainless steel column 20 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica or ceramic microparticles (10 µm) (such as Spherisorb ODS 1),
- mobile phase: a mixture of 45 volumes of *methanol* and 55 volumes of *citro-phosphate buffer pH 5.0*,
- flow rate: 2.0 ml per minute,
- spectrophotometer set at 247 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to betamethasone sodium phosphate and prednisolone sodium phosphate is at least 2.5.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{21}H_{29}O_8P$ in the injection.

Storage. Store protected from light, in a single-dose or in multi-dose containers.

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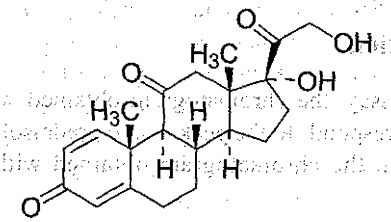
Prednisone



$C_{21}H_{26}O_5$ Mol. Wt. 358.4

Prednisone is 17α,21-dihydroxypregna-1,4-diene-3,11,20-trione.

Prednisone contains not less than 96.0 per cent and not more than 104.0 per cent of $C_{21}H_{26}O_5$, calculated on the dried basis.

Category. Adrenocortical steroid.

Description. A white or almost white, crystalline powder.

Identification

Tests A and B may be omitted if tests C and D are carried out. Tests C and D may be omitted if tests A and B are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prednisone IPRS* or with the reference spectrum of prednisone.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. 90 volumes of *acetone* and 10 volumes of *formamide*.

Mobile phase. *Chloroform*.

Test solution. Dissolve 25 mg of the substance under examination in 10 ml of the solvent mixture.

Reference solution (a). Dissolve 25 mg of *prednisone IPRS* in 10 ml of the solvent mixture.

Reference solution (b). Mix equal volumes of the test solution and reference solution (a).

Place the dry plate in a tank containing a shallow layer of the solvent mixture, allow the solvent mixture to ascend to the top, remove the plate from the tank and allow the solvent to evaporate. Use within 2 hours, with the flow of the mobile phase in the direction in which the aforementioned treatment was done.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of warm air, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulphuric acid (20 per cent v/v)*. Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

C. Dissolve 2 mg in 2 ml of *sulphuric acid* and allow to stand for 5 minutes; an orange colour is produced within 5 minutes, which exhibits a blue fluorescence under ultraviolet light at 365 nm. Pour the solution into 10 ml of *water*; the colour changes first to yellow and then fades gradually but the blue fluorescence under ultraviolet light remains.

D. Dissolve 1 mg in 1 ml of *ethanol (95 per cent)*, evaporate to dryness at a pressure not exceeding 0.7 kPa, add 5 ml of *1 M sodium hydroxide* and heat at 70° for 30 minutes; not more than a slight yellow colour is produced (distinction from cortisone acetate).

Tests

Specific optical rotation (2.4.22). + 167° to + 175°, determined in a 1.0 per cent w/v solution in *dioxan*.

Light absorption (2.4.7). Absorbance of a 0.001 per cent w/v solution in *methanol* at the maximum at about 240 nm, 0.40 to 0.43; the ratio of the absorbance at the maximum at about 240 nm to that at about 263 nm, 1.85 to 2.05.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in *methanol* and dilute to 10.0 ml with *methanol*.

Reference solution (a). Dissolve 2.0 mg of *prednisolone IPRS* and 2.0 mg of *prednisone IPRS* in methanol and dilute to 100.0 ml with *methanol*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilyl silica gel (5 µm),
- column temperature: 45°,
- mobile phase: A. a mixture of 100 ml of *acetonitrile*, 200 ml of *methanol* and 650 ml of *water*, allowed to equilibrate, diluted to 1000 ml with *water* and mixed again,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 2.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

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Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
25	100	0
40	40	60
41	0	100
46	0	100
47	100	0
52	100	0

Equilibrate the column with the mobile phase B for at least 30 minutes and then with mobile phase A for 5 minutes. For subsequent chromatograms, use the conditions described from 40.0 to 52.0 minutes.

The retention times are: prednisone, about 19 minutes and prednisolone about 23 minutes.

Inject reference solution (a). The test is not valid unless the resolution between the peaks corresponding to prednisone and prednisolone is at least 2.7. If necessary, adjust the concentration of acetonitrile in mobile phase A.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent) and the sum of the areas of all the secondary peaks is not more than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.75 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 0.1 g in sufficient *ethanol* and dilute to 100.0 ml with *ethanol*. Dilute 2.0 ml of the solution to 100.0 ml with *ethanol*. Measure the absorbance of the resulting solution at the maximum at about 238 nm. Calculate the content of $C_{21}H_{26}O_5$ taking 425 as the specific absorbance at 238 nm.

Storage. Store protected from light.

Prednisone Tablets

Prednisone Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of prednisone, $C_{21}H_{26}O_5$.

Usual strengths. 5 mg; 10 mg.

Identification

Shake a quantity of the powdered tablets containing 30 mg of Prednisone with 10 ml of *chloroform*, filter and evaporate the filtrate to dryness. The residue complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prednisone IPRS* or with the reference spectrum of prednisone.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. A mixture of 90 volumes of *acetone* and 10 volumes of *formamide*.

Mobile phase. Chloroform.

Test solution. Dissolve 25 mg of the residue in 10 ml of the solvent mixture.

Reference solution (a). Dissolve 25 mg of *prednisone IPRS* in 10 ml of the solvent mixture.

Reference solution (b). Mix equal volumes of the test solution and reference solution (a).

Place the dry plate in a tank containing a shallow layer of the solvent mixture, allow the solvent mixture to ascend to the top, remove the plate from the tank and allow the solvent to evaporate. Use within 2 hours, with the flow of the mobile phase in the direction in which the aforementioned treatment was done.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of warm air, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulphuric acid (20 per cent v/v)*. Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate at the maximum at about 240 nm (2.4.7). Calculate the content of $C_{21}H_{26}O_5$ in the medium from

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the absorbance obtained from a solution of known concentration of *prednisone IPRS*.

Q. Not less than 70 per cent of the stated amount of $C_{21}H_{26}O_5$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of substance under examination in *methanol* and dilute to *methanol*.

Reference solution (a). A 0.002 per cent w/v, each of solution, of *prednisolone IPRS* and *prednisone IPRS* in *methanol*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: A. a mixture of 100 ml of *acetonitrile*, 200 ml of *methanol* and 650 ml of *water*, allowed to equilibrate, diluted to 1000 ml with *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 2.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
25	100	0
40	40	60
41	0	100
46	0	100
47	100	0
52	100	0

Equilibrate the column with the mobile phase B for at least 30 minutes and then with mobile phase A for 5 minutes. For subsequent chromatograms use the conditions described from 40.0 to 52.0 minutes.

The relative retention time with reference to prednisone to prednisolone is about 1.21.

Inject reference solution (a). The test is not valid unless the resolution between the peaks corresponding to prednisone and prednisolone is at least 2.7. If necessary, adjust the concentration of acetonitrile in mobile phase A.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak other than the principal peak is not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent) and the sum of the areas of all the peaks other secondary peaks is not more than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.75 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Powder one tablet, add 50 ml of *ethanol (95 per cent)*, shake for 30 minutes, add sufficient *ethanol (95 per cent)* to produce 100.0 ml. Centrifuge and pipette a suitable volume of the supernatant liquid equivalent to 0.5 mg of Prednisone and dilute to 50.0 ml with *ethanol (95 per cent)*. Measure the absorbance of the resulting solution at the maximum at about 240 nm (2.4.7). Calculate the content of $C_{21}H_{26}O_5$ taking 415 as specific absorbance at 240 nm.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Internal standard solution. Dissolve a weighed quantity of *acetanilide* in a 50 per cent v/v solution of *methanol* to obtain a solution having a known concentration of 0.11 mg per ml.

Test solution. Weigh and powder 20 tablets. To a weighed quantity of the powder containing about 20 mg of Prednisone, add 5 ml of *water*, mix with the aid of ultrasound for one minute, add 50 ml of *methanol* and mix with the aid of ultrasound and dilute with *water* to 100.0 ml and mix. To 5.0 ml of the solution add 5.0 ml of internal standard solution and dilute to 50.0 ml with a 50 per cent v/v solution of *methanol* and mix.

Reference solution. Weigh a suitable quantity of *prednisone IPRS* and dissolve in a 50 per cent v/v solution of *methanol* to obtain a solution having a concentration of about 0.2 mg per ml. Dilute to 5.0 ml of the solution add 5.0 ml of internal standard solution and dilute to 50.0 ml with a 50 per cent v/v solution of *methanol*.

Chromatographic system
- a stainless steel column 25 cm x 4 mm, packed with octadecylsilane bonded to porous silica (3 to 10 µm),
- mobile phase: a suitable filtered mixture of 688 volumes of *water*, 250 volumes of *peroxide-free tetrahydrofuran* and 62 volumes of *methanol* ,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

The relative retention time with reference to prednisone for acetonitrile is about 0.75.

Inject the reference solution. The test is not valid unless the resolution between prednisone and the internal standard is not less than 3.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

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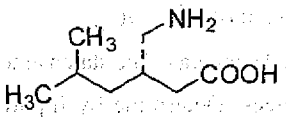
Inject the reference solution and the test solution. Calculate the content of $C_{21}H_{26}O_5$ in the tablets.

Storage. Store protected from light.

Pregabalin



$C_8H_{17}NO_2$ Mol. Wt. 159.2

Pregabalin is (*S*)-4-amino-3-(2-methylpropyl)butyric acid.

Pregabalin contains not less than 98.0 per cent and not more than 102.0 per cent of $C_8H_{17}NO_2$, calculated on the dried basis.

Category. Anticonvulsant.

Description. A white to off-white powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pregabalin IPRS* or with the reference spectrum of pregabalin.

Tests

Specific optical rotation (2.4.22). + 10° to + 12.0°, determined on 1.0 per cent w/v solution.

Enantiomeric purity. Determine by liquid chromatography (2.4.14).

Marfey's reagent (1, Fluoro-2, 4-dinitrophenyl-5-L-alaninamide). A 0.3 per cent w/v solution of *marfey 's reagent* in *acetone.*

NOTE — Store the reagent at 2° to 8°.

Test solution (a). Dissolve about 100 mg of the substance under examination in 100.0 ml of *water.*

Test solution (b). To 2.0 ml of test solution (a), add 0.5 ml of merfey's reagent and 0.5 ml of *1 M sodium bicarbonate* in the test tube. Heat at 40° for 60 minutes. Allow to equilibrate to room temperature.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil BDS C18),
- mobile phase: a mixture of 62 volumes of a buffer solution prepared by diluting about 7.18 ml of *triethylamine* to 1000 ml *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 38 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 340 nm,
- injection volume: 20 µl.

The retention time for S- Pregabalin is about 9 minutes and R- Pregabalin is about 12 minutes.

Inject test solution (b). The area of peak due to R-pregabalin is not more than 0.5 per cent the area of the S-pregabalin.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 1.2 g of *monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 6.9 with *5 M potassium hydroxide.*

Test solution. Dissolve 125 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as Kromasil 100),
- mobile phase: A. dissolve 1.2 g of *monobasic potassium phosphate* in 940 ml of *water*, adjusted to pH 6.9 with *5 M potassium hydroxide* and 60 ml of *acetonitrile*,
  B. dissolve 1.2 g of *monobasic potassium phosphate* in 700 ml of *water*, adjusted to pH 6.7 with *5 M potassium hydroxide* and 300 ml of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 40 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
4	100	0
45	0	100
46	100	0
60	100	0

The relative retention time with reference to pregabalin for lactum impurity (4-isobutylpyrrolidine-2-one) is 6.11.

Inject the test solution. The area of any peak corresponding to lactum impurity (4-isobutylpyrrolidin-2-one) is not more than 0.5 per cent. The area of any other secondary peak is not more than 0.5 per cent and the sum of the areas of all the secondary peaks is not more than 1.0 per cent, calculated by area normalisation.

Heavy Metals (2.3.13). 1 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent determined on 1.0 g by drying in an oven at 105° for 3 hours.

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Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 200 mg of the substance under examination in 100.0 ml of the mobile phase.

Reference solution. A 0.2 per cent w/v solution of *pregabalin IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS-3),
- mobile phase: a mixture of 95 volumes of *water* and 5 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation of replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{17}NO_2$.

Storage. Store protected from light and moisture.

Pregabalin Capsules

Pregabalin Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pregabalin, $C_8H_{17}NO_2$.

Usual strengths. 25 mg; 50 mg; 75 mg; 100 mg; 150 mg; 225 mg; 300 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.05 M hydrochloric acid*,

Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.
Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *pregabalin IPRS* in the dissolution medium and dilute with the dissolution medium to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Use chromatographic system as described under Assay using injection volume 100 µl.

Inject the reference solution. The test is not valid unless the theoretical plates of the principal peak is not less than 2000, tailing factor is not more than 2.0 and the relative standard deviation of replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{17}NO_2$.

Q. Not less than 70 per cent of the stated amount of $C_8H_{17}NO_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 1.2 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.9 with *dilute potassium hydroxide*.

Test solution. Weigh and disperse a quantity of the contents of capsules containing about 750 mg of Pregabalin, in about 25 ml of the solvent mixture, with the aid of ultrasound for 30 minutes and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). A 0.015 per cent w/v solution of *pregabalin IPRS* in the solvent mixture.

Reference solution (b). Dissolve 4.5 mg of *lactam IPRS* in 10.0 ml of the solvent mixture.

Reference solution (c). Dissolve 750 mg of *pregabalin IPRS* in 30 ml of solvent mixture. Add about 5 ml of reference solution (b) and dilute to 50.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS-3V),
- mobile phase: A. a mixture of 90 volumes of the solvent mixture and 10 volumes of *acetonitrile*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	100	0
25	35	65
30	100	0
40	100	0

The relative retention time with reference to lactam peak for lactose conjugate impurity is about 0.78.

Inject reference solution (a). The test is not valid unless the theoretical plates is not less than 3000, the tailing factor is not

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more than 2.0 and the relative standard deviation of replicate injections is not more than 2.0 per cent.

Inject reference solution (a), (c) and the test solution. In the chromatogram obtained with the test solution, the area of the peak due to lactam multiplied with relative response factor, 0.07 is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent), the area of lactose conjugate impurity peak multiplied with relative response factor, 0.05 is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent) and the sum of areas of all the secondary peaks is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent). Ignore any peak with an area is not more than 0.05 times of the area of principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Mix the contents of 20 capsules. Disperse a quantity of powder containing about 200 mg of Pregabalin with 35 ml of the mobile phase, sonicate for 10 minutes and dilute to 50 ml with the mobile phase.

Reference solution. A 0.4 per cent w/v solution of *pregabalin IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS-3),
- mobile phase: a mixture of 92 volumes of solution containing 2.72 g of *potassium dihydrogen orthophosphate* in 900 ml of *water*, add 2 ml of *triethylamine* and adjusted to pH 6.0 with *orthophosphoric acid*, 5 volumes of *methanol* and 3 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the theoretical plates are not less than 2000, tailing factor is not more than 2.0 and the relative standard deviation of replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{17}NO_2$ in the capsules.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of Pregabalin.

Pregabalin and Methylcobalamin Capsules

Pregabalin and Methylcobalamin Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pregabalin, $C_8H_{17}NO_2$ and not less than 90.0 per cent of methylcobalamin, $C_{63}H_{91}CoN_{13}O_{14}P$.

Usual strengths. Pregabalin, 75 mg and Methylcobalamin, 500 mcg; Pregabalin, 75 mg and Methylcobalamin, 750 mcg; Pregabalin, 150 mg and Methylcobalamin, 500 mcg.

Identification

A. In the Assay of pregabalin, the principal peak in the chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution.

B. In the Assay of methylcobalamin, the principal peak in the chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

For Pregabalin —

Apparatus No. 2 (Paddle),
Medium. 500 ml of *water;*
Speed and time: 100 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14) using the chromatographic system as described under Assay of pregabalin.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Weigh accurately about 15 mg of *pregabalin IPRS* dissolve and dilute to 20.0 ml with mobile phase. Dilute 5.0 ml of the solution to 25.0 ml with dissolution medium.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{17}NO_2$.

Q. Not less than 70 per cent of the stated amount of $C_8H_{17}NO_2$.

Uniformity of content. Complies with the test stated under Capsules.

Determine by liquid chromatography (2.4.14), using the chromatographic system prescribed under Assay.

Test solution. Transfer the whole contents of a capsule in 25-ml volumetric flask, add 20 ml of mobile phase and disperse with the aid of ultrasound for about 5 minutes, cool and dilute to volume with mobile phase, and filter. Dilute further with mobile phase, if necessary.

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Reference solution. A 0.002 per cent w/v solution of *methylcobalamin IPRS* in mobile phase. Dilute further with mobile phase, if necessary.

Inject the reference solution and test solution.

Calculate the content of $C_{63}H_{91}CON_{13}O_{14}$ in each capsule.

Other tests. Comply with the tests stated under Capsules.

Assay

For Pregabalin — Determine by liquid chromatography (2.4.14).

Test solution. Weigh accurately a quantity of the mixed contents of 20 capsules containing about 75 mg of Pregabalin in 100-ml volumetric flask, add 50 ml of the mobile phase and disperse with the aid of ultrasound for 10 minutes and dilute to volume with mobile phase and filter.

Reference solution. A 0.075 per cent w/v solution of *pregabalin IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 90 volumes of a buffer solution prepared by dissolving 2.72 g of *potassium dihydrogen orthophosphate* in 900 ml of *water*, add 2 ml of *triethylamine* and adjusted to pH 6.0 with *orthophosphoric acid*, 6 volumes of *methanol* and 4 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{17}NO_2$ in the capsules.

For Methylcobalamin — Determine by liquid chromatography (2.4.14).

NOTE — All the test for methylcobalamin shall be carried out in dark environment.

Test solution. Weigh accurately a quantity of the mixed contents of 20 capsules containing about 0.75 mg of Methylcobalamin in 25-ml volumetric flask, add 20 ml of mobile phase and disperse with the aid of ultrasound for about 5 minutes, cool and dilute to volume with mobile phase and filter.

Reference solution. 0.003 per cent w/v solution of *methylcobalamin IPRS* in mobile phase

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- sample temperature: 15°,
- mobile phase: a mixture of a solution containing 5.80 g of *disodium hydrogen phosphate* in 810 ml of *water*, adjusted to pH 3.5 with *citric acid*, and add 3.76 g of *sodium 1-hexane sulphonate sodium salt* and add 190 ml *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 266 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{63}H_{91}CON_{13}O_{14}P$ in the capsules.

Storage. Protected from light and moisture at a temperature below 30°.

Pregelatinised Starch

Pregelatinised Starch is prepared from maize starch, potato starch or rice starch by mechanical processing in the presence of *water*, with or without heat, to rupture all or part of the starch granules and subsequent drying. It contains no added substances but it may be modified to render it compressible and to improve its flow characteristics.

Description. A white or yellowish-white powder.

Identification

A. *Microscopic* — In a mixture of equal volumes of *glycerol* and *water*, it presents irregular, translucent, white or yellowish-white flakes or pieces with an uneven surface. Under polarised light (between crossed nicol prisms), starch granules with a distinct black cross intersecting at the hilum may be seen.

B. Disperse 0.5 g in 2 ml of *water* without heating, add 0.05 ml of *iodine solution*; a reddish-violet to blue colour is produced.

Tests

pH (2.4.24). 4.5 to 7.0, determined in 3.0 per cent w/v solution in *carbon dioxide-free water*.

Oxidising substances. Transfer 4.0 g to a glass-stoppered, 125 ml conical flask and add 50.0 ml of a mixture of equal volumes of *water* and *methanol*. Insert the stopper and swirl for 5 minutes. Transfer to a glass-stoppered 50 ml centrifuge tube and centrifuge. Transfer 30.0 ml of the clear supernatant liquid to a glass-stoppered 125-ml conical flask. Add 1 ml of *glacial acetic acid* and 0.5 g to 1.0 g of *potassium iodide*

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Insert the stopper, swirl, and allow to stand for 30 minutes in the dark. Add 1 ml of *starch solution* and titrate with *0.002 M sodium thiosulphate* until the starch-iodine colour disappears. Carry out a blank titration. Not more than 1.4 ml of *0.002 M sodium thiosulphate* is required (0.002 per cent, calculated as H_2O_2).

1 ml of 0.002 M *sodium thiosulphate* is equivalent to 0.034 mg of oxidising substances, calculated as H_2O_2.

Sulphur dioxide (2.3.40). Not more than 50 ppm.

Iron (2.3.14). Dissolve the residue obtained in the test for Sulphated ash, in 10 ml of *dilute hydrochloric acid*, filter. This solution complies with the limit test for iron (20 ppm).

Foreign matter. Examined under a microscope using a mixture of equal volumes of *glycerol* and *water*, not more than traces of matter other than starch granules are present.

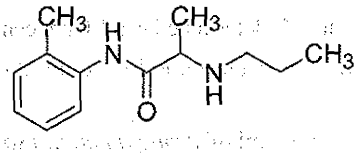
Sulphated ash (2.3.18). Not more than 0.6 per cent, determined on 2 g.

Loss on drying (2.4.19). Not more than 15.0 per cent, determined on 1.0 g by drying in an oven at 130° for 90 minutes.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 10^3 CFU per g and total fungal count is not more than 10^2 CFU per g determined by plate count. 1 g is free from *Escherichia coli*.

Labelling. The label states the type of starch used as starting material.

Prilocaine



$C_{13}H_{20}N_2O$ Mol. Wt. 220.3

Prilocaine is propanamide, *N*-(2-methylphenyl)-2-(propylamino); 2-(Propylamino)-*o*-propionotoluidide; (*RS*)-*N*-(2-methylphenyl)-2-(propylamino) propanamide.

Prilocaine contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{13}H_{20}N_2O$, calculated on the anhydrous basis.

Category. Local anaesthetic

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prilocaine IPRS* or with the reference spectrum of prilocaine.

B. In the Assay, the principal peak in the chromatogram obtained with test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. A 0.25 per cent w/v solution of the substance under examination in the mobile phase.

Use chromatographic system as described under Assay.

Inject reference solution (b) and the test solution. Run the chromatogram 1.5 times the retention time of prilocaine peak. The test is not valid unless the resolution between the peaks due to prilocaine and prilocaine related compound B is not less than 3.0, the signal to noise ratio is not less than 10 for prilocaine peak, and the relative retention times of prilocaine and prilocaine related compound B are 1.0 and 1.2, respectively. The area of any individual peak is not more than 0.2 per cent and sum of the areas of all the secondary peak is not more than 0.5 per cent, calculated by area normalization.

Limit of Prilocaine related compound A. Determine by liquid chromatography (2.4.14).

Use chromatographic system as described under Assay.

Test solution. A 1.0 per cent w/v solution of the substance under examinationin the mobile phase.

Reference solution. A 0.00013 per cent w/v solution of *prilocaine related compound A IPRS* in the mobile phase.

Inject the reference solution. The test is not valid unless the signal to noise ratio is not less than 10 for prilocaine.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to prilocaine related compound A is not more the area of principal peak corresponding to chromatogram obtained with reference solution (0.01 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.5 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of substance under examination in 100.0 ml of the mobile phase.

Reference solution (a). A 0.025 per cent w/v solution of *prilocaine IPRS* in the mobile phase.

Reference solution(b). A 0.00025 per cent w/v solution of *prilocaine IPRS* and 0.0003 per cent w/v solution of *prilocaine related compound B IPRS* in the mobile phase.

Chromatographic system

– a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),

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- mobile phase: a mixture of 73 volumes of a buffer solution prepared by dissolving 0.18 g of *monobasic sodium phosphate* and 2.89 g of *dibasic sodium phosphate dihydrate* in 1000 ml of *water* and 27 volume *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 20 µl.

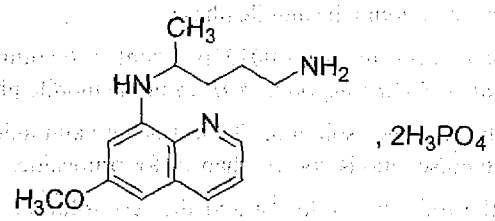
Inject reference solution (a), (b) and the test solution. Run the chromatogram 1.3 times the retention time of prilocaine peak. The test is not valid unless the resolution between the peaks due to prilocaine and prilocaine related compound B is not less than 3.0 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections in the chromatogram obtained with reference solution (a) is not more than 1.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{13}H_{20}N_2O$.

Storage. Store protected from the light and moisture, at a temperature below 25°.

Primaquine Phosphate



$C_{15}H_{21}N_3O,2H_3PO_4$ Mol. Wt. 455.3

Primaquine Phosphate is (*RS*)-8-(4-amino-1-methylbutylamino)-6-methoxyquinoline diphosphate.

Primaquine Phosphate contains not less than 98.5 per cent and not more than 101.5 per cent of $C_{15}H_{21}N_3O,2H_3PO_4$, calculated on the dried basis.

Category. Antimalarial.

Description. An orange, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *primaquine phosphate IPRS* or with the reference spectrum of primaquine phosphate.

B. When examined in the range 300 nm to 450 nm (2.4.7), a 0.015 per cent w/v solution in *0.01 M hydrochloric acid* shows absorption maxima at about 332 nm and 415 nm; absorbance at about 332 nm, about 0.68 to 0.78 and at about 415 nm, 0.41 to 0.53. Dilute 5 ml of the solution to 50 ml with *0.01 M hydrochloric acid*. When examined in the range 215 nm to 310 nm, the resulting solution shows absorption maxima at about 225 nm, 265 nm and 282 nm; absorbances at the maxima are 0.74 to 0.77, 0.50 to 0.53 and 0.50 to 0.52, respectively.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel F254*.

Mobile phase. A mixture of 60 volumes of *chloroform*, 40 volumes of *methanol* and 1 volume of *strong ammonia solution*.

Test solution. Dissolve 0.2 g in 5 ml of *water*, dilute to 10 ml with *methanol* and dilute 1 volume of the resulting solution to 10 volumes with *methanol (50 per cent)*.

Reference solution. Dissolve 20 mg of *primaquine phosphate IPRS* in 5 ml of *water*, dilute to 10 ml with *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

D. Dissolve 50 mg in 5 ml of *water*, add 2 ml of *2 M sodium hydroxide* and shake with two quantities, each of 5 ml, of *chloroform*. The aqueous layer, acidified by the addition of *nitric acid*, gives reaction (B) of phosphates (2.3.1).

Tests

pH (2.4.24). 2.5 to 3.5, determined in a 1.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Add 0.2 ml of *strong ammonia solution* to 1 ml of a 1.0 per cent w/v solution of the substance under examination, shake with 10 ml of the mobile phase and use the clear, lower layer.

Reference solution (a). Dilute 3.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase and dilute 1.0 ml of the resulting solution to 50.0 ml with the mobile phase.

Reference solution (c). Add 0.2 ml of *strong ammonia solution* to 1 ml of a 1.0 per cent w/v solution of the *primaquine phosphate IPRS*, shake with 10 ml of the mobile phase and use the clear, lower layer.

Chromatographic system

- a stainless steel column 20 cm x 4.6 mm, packed with porous silica particles (10 µm),

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– mobile phase: a mixture of 45 volumes of *chloroform*, 45 volumes of *hexane*, 10 volumes of *methanol* and 0.1 volume of *strong ammonia solution*,
– flow rate: 3 ml per minute,
– spectrophotometer set at 261 nm,
– injection volume: 20 µl.

The test is not valid unless in the chromatogram obtained with reference solution (c) there is a peak just before the principal peak with an area of about 6 per cent of that of the principal peak and the resolution between these peaks is not less than 2.0.

Inject each solution and record the chromatograms for at least twice the retention time of primaquine.

Inject reference solution (a) and the test solution. In the chromatogram the sum of the areas of all secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent). Ignore any peak the area of which is less than that of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent).

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.2 g in 40 ml of *anhydrous glacial acetic acid* with gentle heating. Titrate with *0.1 M perchloric acid*, determining the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02277 g of $C_{15}H_{21}N_3O,2H_3PO_4$.

Storage. Store protected from light and moisture.

Primaquine Tablets

Primaquine Phosphate Tablets

Primaquine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of primaquine, $C_{15}H_{21}N_3O$.

Usual strengths. 7.5 mg ; 15 mg.

(13 mg of Primaquine Phosphate is approximately equivalent to 7.5 mg of primaquine).

Identification

A. Dissolve a quantity of the powdered tablets containing 60 mg of primaquine in a mixture of 10 ml of *water* and 2 ml of *2 M sodium hydroxide* and extract with two quantities, each of 20 ml, of *chloroform*. Wash the chloroform extracts with *water*, dry over *anhydrous sodium sulphate*, evaporate to dryness and dissolve the residue in 2 ml of *chloroform*. On the solution, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *primaquine phosphate IPRS* treated in the same manner or with the reference spectrum of primaquine.

B. Extract a quantity of the powdered tablets containing 25 mg of primaquine with 10 ml of *water* and filter. To 2 ml of the filtrate, add 3 ml of *water* and 1 ml of a 5 per cent w/v solution of *ceric ammonium sulphate* in *2 M nitric acid*; a deep violet colour is produced immediately.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.01 M hydrochloric acid*,
Speed and time. 50 rpm and 60 minutes.
Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a weighed quantity of *primaquine phosphate IPRS* in the dissolution medium and dilute with dissolution medium to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Use chromatographic system as described under Assay.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Q. Not less than 75 per cent of the stated amount of $C_{15}H_{21}N_3O$.

Uniformity of content. Complies with the test stated under tablets.

Determine by liquid chromatography (2.4.14), as described under Assay, using the following solutions.

Test solution. Powder one tablet, dissolve in 20 ml of the mobile phase with the aid of ultrasound for 3 minutes and dilute to 50.0 ml with the mobile phase and filter.

Reference solution. Dissolve a weighed quantity of *primaquine phosphate IPRS* in the mobile phase and dilute with the mobile phase to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{21}N_3O$ in the tablet.

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Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 10 mg of primaquine, dissolve in 30 ml of the mobile phase and sonicate for 20 minutes. Make up the volume to 50.0 ml with mobile phase.

Reference solution. A 0.035 per cent w/v solution of *primaquine phosphate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 3.0 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 30°,
- mobile phase: a mixture of 40 volumes of *1- pentane sulphonate sodium* solution prepared by dissolving 960 mg of *sodium-1- pentane sulphonate* and 1 ml *glacial acetic acid* in 400 ml of *water* and 60 volumes of *methanol*,
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 264 nm,
- injection volume: 5 µl.

Inject the reference solution. The test is not valid unless the column efficiency in not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

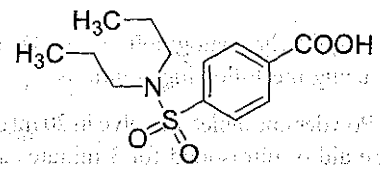
Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{21}N_3O$.

Storage. Store protected from moisture.

Labelling. The label states the strength in terms of the equivalent amount of primaquine.

Probenecid



$C_{13}H_{19}NO_4S$ Mol. Wt. 285.4

Probenecid is 4-(dipropylsulphamoyl)benzoic acid.

Probenecid contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{13}H_{19}NO_4S$, calculated on the dried basis.

Category. Uricosuric agent and for delaying renal excretion of penicillins and cephalosporins.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *probenecid IPRS* or with the reference spectrum of probenecid.

B. When examined in the range 210 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in a mixture of 9 volumes of *ethanol (95 per cent)* and 1 volume of *0.1 M hydrochloric acid* shows absorption maxima at about 223 nm and 248 nm; absorbance at about 248 nm is about 0.33.

C. Dissolve 0.2 g in about 0.6 ml of *2 M ammonia* and add 3 ml of 1.7 per cent w/v solution of *silver nitrate*; a white precipitate is produced which is soluble in an excess of *dilute ammonia solution*.

Tests

Appearance of solution. A 10.0 per cent w/v solution in *2 M sodium hydroxide* is clear (2.4.1), and not more intensely coloured than reference solution YS6 (2.4.1).

Acidity. Add 2.0 g to 100 ml of *water*, heat on a water-bath for 30 minutes, cool, filter and dilute with *water* to 100.0 ml. Titrate 50.0 ml of the solution with *0.1 M sodium hydroxide* using *phenolphthalein solution* as indicator. Not more than 0.5 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 55 volumes of *toluene*, 20 volumes of *di-isopropyl ether*, 15 volumes of *chloroform* and 10 volumes of *glacial acetic acid*.

Test solution. Dissolve 0.1 g of the substance under examination in 10 ml of *acetone*.

Reference solution. A 0.005 per cent w/v solution of the substance under examination in *acetone*.

Apply to the plate 20 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

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Assay. Weigh 0.5 g and dissolve in 100 ml of *ethanol (95 per cent)*, shaking well and heating gently if necessary. Cool and titrate with *0.1 M sodium hydroxide*, using *bromothymol blue solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.02854 g of $C_{13}H_{19}NO_4S$.

Storage. Store protected from moisture.

Probenecid Tablets

Probenecid Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of probenecid, $C_{13}H_{19}NO_4S$.

Usual strength. 500 mg.

Identification

A. Triturate a quantity of the powdered tablets containing 0.5 g of Probenecid with *ethanol (95 per cent)*, filter and concentrate the filtrate by evaporation on a water-bath. Cool, filter and recrystallise the residue from *ethanol (50 per cent)*. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *probenecid IPRS* or with the reference spectrum of probenecid.

B. When examined in the range 210 nm to 360 nm (2.4.7), the final solution obtained in the Assay shows absorption maxima at about 225 nm and 248 nm.

C. The residue obtained in test A melts at about 199° (2.4.21).

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 55 volumes of *toluene*, 20 volumes of *di-isopropyl ether*, 15 volumes of *chloroform* and 10 volumes of *glacial acetic acid*.

Test solution. Extract a quantity of the powdered tablets containing 0.2 g of Probenecid with 20 ml of *acetone*, filter and use the filtrate.

Reference solution. Dilute 1.0 ml of the test solution to 200.0 ml with *acetone*.

Apply to the plate 20 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Dissolution (2.5.2).

Apparatus. No. 2 (Paddle),
Medium. 900 ml of *phosphate buffer pH 7.6*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Dilute 4.0 ml of the filtrate to 100.0 ml with *0.1 M sodium hydroxide*. Measure the absorbance of the resulting solution at the maximum at about 244 nm (2.4.7). Similarly measure the absorbance of a solution of a known concentration of *probenecid IPRS*. Calculate the content of $C_{13}H_{19}O_4S$.

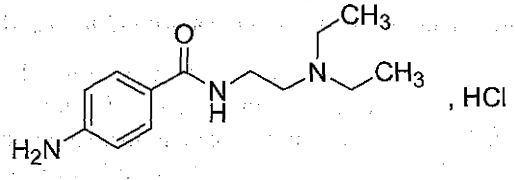
Q. Not less than 80 per cent of the stated amount of $C_{13}H_{19}NO_3S$.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.2 g of Probenecid in 200 ml of *ethanol (95 per cent)* and 5 ml of *1 M hydrochloric acid*, heat on a water-bath at 70° for 30 minutes, shaking occasionally. Cool, add sufficient *ethanol (95 per cent)* to produce 250.0 ml and filter. To 5.0 ml of the filtrate add 5 ml of *0.1 M hydrochloric acid*, dilute to 250.0 ml with *ethanol (95 per cent)* and measure the absorbance of the resulting solution at the maximum at about 248 nm (2.4.7). Calculate the content of $C_{13}H_{19}NO_4S$ taking 332 as specific absorbance at 248 nm.

Storage. Store protected from moisture.

Procainamide Hydrochloride



$C_{13}H_{21}N_3O,HCl$ Mol. Wt. 271.8

Procainamide Hydrochloride is 4-amino-*N*-[2-(diethylamino) ethyl)benzamide hydrochloride.

Procainamide Hydrochloride contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{13}H_{21}N_3O,HCl$, calculated on the dried basis.

Category. Antiarrhythmic.

Description. A white or very slightly yellow, crystalline powder; hygroscopic.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

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A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *procainamide hydrochloride IPRS* or with the reference spectrum of procainamide hydrochloride.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *0.1 M sodium hydroxide* shows an absorption maximum at about 273 nm; absorbance at about 273 nm, 0.58 to 0.61.

C. Dilute 1 ml of a solution, prepared by dissolving 2.5 g in sufficient *carbon dioxide-free water* to produce 25 ml, to 2 ml with *water*. 1 ml of the solution gives the reaction of primary aromatic amines (2.3.1).

D. A 2 per cent w/v solution gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution BS6 (2.4.1).

pH (2.4.24). 5.6 to 6.3, determined in a 10.0 per cent w/v solution.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *1-butanol*, 30 volumes of *water* and 15 volumes of *glacial acetic acid*.

Test solution. Dissolve 0.1 g of the substance under examination in 10 ml of *ethanol (95 per cent)*.

Reference solution. A 0.005 per cent w/v solution of the substance under examination in *ethanol (95 per cent)*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.25 g in 75 ml of *water* and 10 ml of *hydrochloric acid*, add 3 g of *potassium bromide*, cool in ice and carry out the nitrite titration (2.3.31).

1 ml of *0.1 M sodium nitrite* is equivalent to 0.02718 g of $C_{13}H_{21}N_3O$, HCl.

Storage. Store protected from light.

Procainamide Injection

Procainamide Hydrochloride Injection

Procainamide Injection is a sterile solution of Procainamide Hydrochloride in Water for Injections. It may contain Sodium Metabisulphite as a stabilising agent.

Procainamide Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of procainamide hydrochloride, $C_{13}H_{21}N_3O$, HCl.

Usual strength. 100 mg per ml.

Identification

A. Dilute a volume containing 0.25 g of Procainamide Hydrochloride to 25 ml with *water*, make alkaline with *5 M sodium hydroxide* and extract with two quantities, each of 5 ml, of *chloroform*. Filter the combined extracts through *anhydrous sodium sulphate*, evaporate the filtrate to dryness using a rotatory evaporator and dissolve the residue in 5 ml of *chloroform*.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *procainamide hydrochloride IPRS* treated in the same manner or with the reference spectrum of procainamide.

B. Dilute a suitable volume of the injection with *water* to produce a solution containing 0.0005 per cent w/v of Procainamide Hydrochloride. Absorbance of the resulting solution at about 280 nm, about 0.30 (2.4.7).

C. It gives the reactions of chlorides (2.3.1).

Tests

pH (2.4.24). 4.0 to 5.5.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *1-butanol*, 30 volumes of *water* and 15 volumes of *glacial acetic acid*.

Test solution. Dilute a volume of the injection containing 0.1 g of Procainamide Hydrochloride to 5 ml with *methanol*.

Reference solution. Dilute 1 volume of the test solution to 100 volumes with *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Bacterial endotoxins (2.2.3). Not more than 0.35 Endotoxin Unit per mg of procainamide hydrochloride.

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Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. To a volume containing about 0.25 g of Procainamide Hydrochloride add 45 ml of *6 M hydrochloric acid* and boil for 1 minute, add 3 g of *potassium bromide*, cool in ice. Carry out the nitrite titration (2.3.31).

1 ml of *0.1 M sodium nitrite* is equivalent to 0.02718 g of $C_{13}H_{21}N_3O,HCl$.

Storage. Store protected from light.

Procainamide Tablets

Procainamide Hydrochloride Tablets

Procainamide Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of procainamide hydrochloride, $C_{13}H_{21}N_3O,HCl$.

Usual strength. 250 mg.

Identification

A. Shake a quantity of the powdered tablets containing 0.25 g of Procainamide Hydrochloride with 25 ml of *water*, make alkaline with *5 M sodium hydroxide* and extract with two quantities, each of 5 ml, of *chloroform*. Filter the combined extracts through *anhydrous sodium sulphate*, evaporate the filtrate to dryness using a rotatory evaporator and dissolve the residue in 5 ml of *chloroform*.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *procainamide hydrochloride IPRS* treated in the same manner or with the reference spectrum of procainamide.

B. Triturate a quantity of the powdered tablets containing 1 g of Procainamide Hydrochloride with 10 ml of *water* and filter. The filtrate gives the reactions of chlorides (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1M hydrochloric acid*,
Speed and time. 50 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate suitably diluted with *0.01M sodium hydroxide* (not less than twice the volume of the portion of test solution), at the maximum at about 275 nm (2.4.7). Calculate the content of $C_{13}H_{21}N_3O.HCl$ in the medium from the absorbance obtained from a solution of known concentration of *procainamide hydrochloride IPRS* in the same medium.

Q. Not less than 80 per cent of the stated amount of $C_{13}H_{21}N_3O,HCl$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *1-butanol*, 30 volumes of *water* and 15 volumes of *glacial acetic acid*.

Test solution. Shake a quantity of the powdered tablets containing 0.4 g of Procainamide Hydrochloride with 20 ml of *methanol (90 per cent)* for 15 minutes and filter.

Reference solution. Dilute 1 volume of the test solution to 100 volumes with *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 0.5 g of procainamide in 350 ml of *methanol* and sonicate for 10 minutes at 40° in water-bath and dilute to 500.0 ml with *methanol*, cool to room temperature and centrifuge. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (a). A 0.005 per cent w/v solution of *procainamide IPRS* in the mobile phase.

Reference solution (b). A 0.01 per cent w/v solution of *p-amino benzoic acid* in the mobile phase. Dilute 10.0 ml of the solution and 10.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 140 volumes of *water*, 60 volumes of *methanol* and 1 volume of *triethylamine*, adjusted to pH 7.5 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to p-amino benzoic acid and procainamide is not less than 5.0 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{13}H_{21}N_3O,HCl$.

Storage. Store protected from light and moisture.

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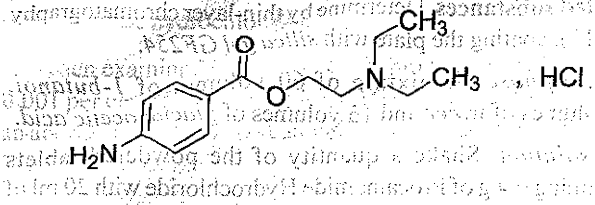
Procaine Hydrochloride



$C_{13}H_{20}N_2O_2,HCl$ Mol. Wt. 272.8

Procaine Hydrochloride is 2-(diethylamino)ethyl 4-aminobenzoate hydrochloride.

Procaine Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{13}H_{20}N_2O_2,HCl$, calculated on the dried basis.

Category. Local anaesthetic.

Description. Colourless crystals or a white, crystalline powder.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests B, C and D may be omitted if tests A and E are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *procaine hydrochloride IPRS* or with the reference spectrum of procaine hydrochloride.

B. To 0.2 ml of a 5 per cent w/v solution add 2 ml of *water* and 0.5 ml of *1 M sulphuric acid*, shake and add 1 ml of a 0.1 per cent w/v solution of *potassium permanganate*; the colour is immediately discharged.

C. To about 5 mg add 0.5 ml of *fuming nitric acid*, evaporate to dryness on a water-bath, cool, dissolve the residue in 5 ml of *acetone* and add 1 ml of *0.1 M ethanolic potassium hydroxide*; only a brownish red colour develops.

D. Dilute 1 ml of a 5 per cent w/v solution to 100 ml with *water*; 2 ml of the solution gives the reaction of primary aromatic amines (2.3.1).

E. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and colourless (2.4.1).

pH (2.4.24). 5.0 to 6.5, determined in a 2.0 per cent w/v solution.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 80 volumes of *dibutyl ether*, 16 volumes of *n-hexane* and 4 volumes of *glacial acetic acid*.

Test solution. A 10 per cent w/v solution of the substance under examination in *water*.

Reference solution. A 0.005 per cent w/v solution of *4-aminobenzoic acid* in *water*.

Apply to the plate 5 µl of each solution. After development, dry the plate at 105° for 10 minutes and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution. The principal spot remains on the line of application.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method A (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.3 g in 50 ml of *2 M hydrochloric acid*, add 3 g of *potassium bromide*, cool in ice and titrate slowly with *0.1 M sodium nitrite*, stirring constantly. Determine the end point potentiometrically (2.4.25).

1 ml of *0.1 M sodium nitrite* is equivalent to 0.02728 g of $C_{13}H_{20}N_2O_2,HCl$.

Procaine Hydrochloride intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.6 Endotoxin Unit per mg of procaine hydrochloride.

Storage. Store protected from light.

Procaine and Adrenaline Injection

Procaine Hydrochloride and Adrenaline Bitartrate Injection; Procaine Hydrochloride and Epinephrine Bitartrate Injection; Adrenaline Bitartrate and Procaine Hydrochloride Injection

Procaine and Adrenaline Injection is a sterile solution of Procaine Hydrochloride and Adrenaline Bitartrate in Water for Injections.

Procaine and Adrenaline Injection contains not less than 1.9 per cent and not more than 2.1 per cent w/v of procaine hydrochloride, $C_{13}H_{20}N_2O_2$, HCl and the equivalent of not less than 0.00175 per cent and not more than 0.00225 per cent w/v of adrenaline, $C_9H_{13}NO_3$.

Description. A clear, colourless solution.

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Identification

A. To 5 ml add 5 ml of *water* and 10 ml of *picric acid solution*, shake gently and set aside for 1 hour; the crystalline precipitate, after washing with *water* and drying at 100°, melts at about 134° (2.4.21).

B. To 5 ml add 1 ml of *hydrochloric acid*, cool to 0°, add 5 ml of *sodium nitrite solution* and pour the mixture into 2 ml of *2-naphthol solution* containing 1 g of *sodium acetate*; an orange-red colour is produced.

C. To 10 ml add 4 ml of *disodium hydrogen phosphate solution* and sufficient *0.1 M iodine* to produce a distinct brown colour. Add *0.1 M sodium thiosulphate* to remove the excess of iodine; a pink colour is produced.

Tests

pH (2.4.24). 3.0 to 5.5.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *For procaine hydrochloride* — To 10.0 ml of the injection add 0.5 g of *sodium carbonate* and extract with three quantities, each of 20 ml, of a mixture of 1 volume of *2-propanol* and 3 volumes of *chloroform* until complete extraction of procaine is effected. Shake the combined extracts with 5 ml of *water*, wash the water with the solvent mixture and add the washing to the combined extracts. Shake the combined extracts and washings with 10.0 ml of *0.1 M hydrochloric acid*, separate the acid layer, wash the combined extracts and washings with 5 ml of *water*, add the aqueous extract to the separated acid layer and titrate with *0.1 M sodium hydroxide*, using *methyl red-methylene blue solution* as indicator.

1 ml of *0.1 M hydrochloric acid* is equivalent to 0.02728 g of $C_{13}H_{20}N_2O_2,HCl$.

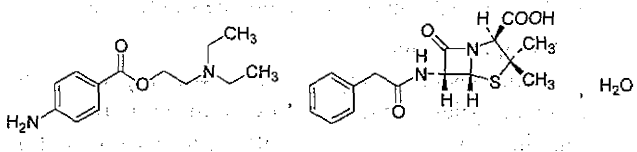
For adrenaline — To 10.0 ml of the injection add 20 mg of *sodium metabisulphite*, 0.1 ml of *ferrous sulphate-citrate solution*, 1 ml of *glycine buffer solution* and mix. Allow to stand for 10 minutes, extract with 10 ml of *ether*, allow to separate, reject the *ether* and measure the absorbance of a 4-cm layer of the solution at about 540 nm (2.4.7). Calculate the content of adrenaline, $C_9H_{13}NO_3$, from a reference curve prepared by treating suitable aliquots of a solution of *adrenaline bitartrate IPRS* in the same manner.

1 mg of adrenaline bitartrate is equivalent to 0.5497 mg of $C_9H_{13}NO_3$.

Storage. Store protected from light.

Labelling. The label states the strength as "Procaine Hydrochloride, 2 per cent w/v; Adrenaline, 0.002 per cent w/v".

Procaine Penicillin



$C_{13}H_{20}N_2O_2,C_{16}H_{18}N_2O_4S,H_2O$ Mol. Wt. 588.7

Procaine Penicillin is 2-diethylaminoethyl 4-aminobenzoate (6*R*)-6-(2-phenylacetamido)penicillanate monohydrate.

Procaine Penicillin contains not less than 51 per cent and not more than 59.6 per cent of penicillin G, calculated as $C_{16}H_{18}N_2O_4S$, and not less than 37.5 per cent and not more than 43.0 per cent of procaine, $C_{13}H_{20}N_2O_2$, both calculated on the anhydrous basis.

Category. Antibacterial.

Description. A white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *procaine penicillin IPRS* or with the reference spectrum of procaine penicillin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution (a).

C. A turbid solution of 0.1 g in 2 ml of *2 M hydrochloric acid* gives the reaction of primary aromatic amines (2.3.1).

Tests

pH (2.4.24). 5.0 to 7.5, determined in a solution prepared by shaking 50 mg in sufficient *carbon dioxide-free water* to produce 15 ml until dissolution is complete.

Specific optical rotation (2.4.22). + 165° to + 180°, determined in a solution prepared by dissolving 0.25 g in sufficient of a mixture of 3 volumes of *acetone* and 2 volumes of *water* to produce 25 ml.

Water (2.3.43). 2.8 to 4.2 per cent, determined on 0.5 g.

Assay. Determine the contents of benzyl penicillin and procaine by liquid chromatography (2.4.14).

Test solution. Weigh 70 mg of the substance under examination and dissolve in 30 ml of the mobile phase with the aid of ultrasound and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 42 mg of *benzylpenicillin potassium IPRS* and 32 mg of *procaine hydrochloride IPRS* in 25 ml of the mobile phase with the aid of ultrasound and dilute to 50.0 ml with the mobile phase.

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Reference solution (b). Mix 1 volume of a 0.24 per cent w/v solution of *phenoxymethylpenicillin potassium IPRS* in the mobile phase with 3 volumes of reference solution (a).

Chromatographic system

- a stainless steel column 30 cm x 4 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 50 volumes of a solution prepared by mixing 14 g of *potassium dihydrogen phosphate* in 6.5 g of *tetrabutylammonium hydroxide (40 per cent)* and adjusted to pH 7.0 with *1 M potassium hydroxide* or *dilute phosphoric acid*, 25 volumes of *acetonitrile* and 25 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 235 nm,
- injection volume: 20 µl.

Inject reference solution (b). The resolution between benzyl penicillin potassium and phenoxymethylpenicillin potassium is not less than 2. The relative retention time of procaine with reference to benzyl penicillin is about 2.2.

Inject reference solution (a) and the test solution. Record the peak responses of the main peaks of benzyl penicillin and procaine. Calculate the content of benzyl penicillin, $C_{16}H_{18}N_2O_4S$ and the content of procaine, $C_{13}H_{20}N_2O_2$ using the factor of 0.866 for converting the content of procaine hydrochloride to that of procaine.

Procaine Penicillin intended for use in the manufacture of Parenteral Preparations without a further procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.10 Endotoxin Unit per mg.

Procaine Penicillin intended for use in the manufacture of Parenteral Preparations without a further sterilisation procedure complies with the following additional requirement.

Sterility. Complies with the test for sterility (2.2.11).

Storage. Store protected from moisture. If the material is intended for use in the manufacture of parenteral preparations, the container should be sterile and sealed so as to exclude micro-organisms.

Labelling. The label states (1) where applicable, that the contents are free from bacterial endotoxins; (2) where applicable, that the contents are sterile.

Fortified Procaine Penicillin Injection

Procaine Penicillin with Benzylpenicillin Injection

Fortified Procaine Penicillin Injection is a sterile mixture of five parts of Procaine Penicillin and one part of Benzylpenicillin Potassium or Benzylpenicillin Sodium, together with suitable dispersing and buffering agents. It is filled in a sealed container.

The injection is constituted by suspending the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

Storage. The constituted injection should be used within 24 hours (4 days if a buffering agent is present) of preparation when stored at a temperature not exceeding 20° or within 7 days (14 days if a buffering agent is present) when stored at a temperature between 2° and 8°.

Fortified Procaine Penicillin Injection contains a quantity of total penicillins calculated as $C_{16}H_{17}N_2NaO_4S$ and equivalent to not less than 60.0 per cent and not more than 74.0 per cent of the stated amounts of procaine penicillin and benzylpenicillin potassium or benzylpenicillin sodium and a quantity of procaine, $C_{13}H_{20}N_2O_2$, equivalent to not less than 36.0 per cent and not more than 44.0 per cent of the stated amount of procaine penicillin.

Usual strengths. Procaine Penicillin, 300 mg (300,000 Units) and Benzylpenicillin Potassium or Benzylpenicillin Sodium 60 mg (100,000 Units), Procaine Penicillin 3 g (3,000,000 Units) and Benzylpenicillin Potassium or Benzylpenicillin Sodium, 600 mg (1,000,000 Units).

Description. A white or almost white powder.

The contents of the sealed container comply with the tests stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Identification

A. Dissolve 10 mg in 10 ml of *water* and add 0.5 ml of *neutral red solution*. Add sufficient *0.01 M sodium hydroxide* to give a permanent orange colour and then add 1 ml of *penicillinase solution*; a red colour is produced rapidly.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

C. It give the reaction of primary aromatic amines (2.3.1), producing a bright, orange-red precipitate.

Tests

Water (2.3.43). Not more than 3.5 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the mixed contents of 10 containers equivalent to about 70 mg of procaine penicillin and dissolve in 30 ml of the mobile phase with the aid of ultrasound and dilute to 50.0 ml with the mobile phase.

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Reference solution (a). Weigh 56 mg of *benzylpenicillin potassium IPRS* and 32 mg of *procaine hydrochloride IPRS* and dissolve in 25 ml of the mobile phase with the aid of ultrasound and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Mix 1 volume of a 0.24 per cent w/v solution of *phenoxymethylpenicillin potassium IPRS* in the mobile phase with 3 volumes of reference solution (a).

Chromatographic system

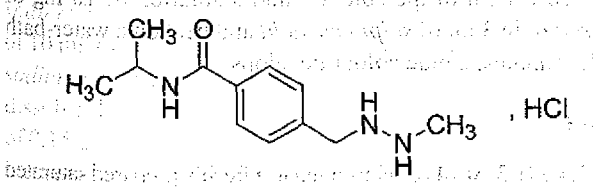
- a stainless steel column 30 cm x 4 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 50 volumes of a solution containing 1.4 per cent w/v of *potassium dihydrogen orthophosphate* and 0.65 per cent w/v *tetrabutyl ammonium hydroxide* adjusted to pH 7.0 with *1 M potassium hydroxide* or *orthophosphoric acid*, 25 volumes of *acetonitrile* and 25 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 235 nm,
- injection volume: 20 µl.

Inject reference solution (b). The resolution between benzylpenicillin potassium and phenoxymethylpenicillin potassium is not less than 2. The relative retention time of procaine with reference to benzylpenicillin is about 2.2.

Inject reference solution (a) and the test solution. Record the peak responses of the main peaks of benzyl penicillin and procaine. Calculate the total penicillin content as benzylpenicillin sodium, $C_{16}H_{17}N_2NaO_4S$ and the content of procaine, $C_{13}H_{20}N_2O_2$ using the factor of 0.866 for converting the content of procaine hydrochloride to that of procaine.

Labelling. The label states (1) the quantities of Procaine Penicillin and Benzylpenicillin Potassium or Benzylpenicillin Sodium contained in it; (2) the names of any added dispersing and buffering agents; (3) "for intramuscular injection only".

Procarbazine Hydrochloride



$C_{12}H_{19}N_3O,HCl$ Mol. Wt. 257.8

Procarbazine Hydrochloride is *N-iso*.Propyl-4-[(2-methylhydrazino)methyl]benzamide hydrochloride.

Procarbazine Hydrochloride contains not less than 98.5 per cent and not more than 100.5 per cent of $C_{12}H_{19}N_3O,HCl$.

CAUTION — Handle with exceptional care, since it is a highly potent agent.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *procarbazine hydrochloride IPRS* or with the reference spectrum of procarbazine hydrochloride.

B. When examined in the range 200 nm to 400 nm (2.4.7), a 0.001 per cent w/v solution in *0.1 M hydrochloric acid* shows absorption maximum at 232 nm. (*NOTE — Use low actinic glassware).*

Tests

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method A (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Weigh 0.75 g in 100 ml of *water*. Titrate with *0.1 M sodium hydroxide*. Determine the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.02578 g of $C_{12}H_{19}N_3O,HCl$.

Storage. Store protected from light and moisture.

Procarbazine Hydrochloride Capsules

Procarbazine Hydrochloride Capsules contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of procarbazine $C_{12}H_{19}N_3O$.

Usual strength. 50 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *water*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Dilute the filtrate if necessary with dissolution medium, measure the absorbance at the maximum at about 233 nm (2.4.7). Calculate the content of $C_{12}H_{19}N_3O$ in the medium from the absorbance obtained by repeating the determination using a solution of known concentration of *procarbazine hydrochloride IPRS* in *water*.

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Q. Not less than 75 per cent of the stated amount of $C_{12}H_{19}N_3O$

Other tests. Comply with the test stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of mixed content of 20 capsules containing 50 mg of Procarbazine and disperse in 200.0 ml of *water* with the aid of ultrasound for 5 minutes, cool and dilute to 250.0 ml with same solvent and filter. Dilute 5.0 ml of the solution to 10.0 ml with mobile phase.

Reference solution. A solution containing 0.012 per cent w/v solution of *procarbazine hydrochloride IPRS* in mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 14 volumes of *acetonitrile*, 14 volumes of *methanol* and 72 volumes of buffer solution prepared by dissolving 2.0 g of *sodium hexanesulphonate* and 4 ml of *triethylamine* in 1000 ml of *water*, adjusted to pH 3.5 with *orthophosphoric acid*,
- flow: 1 ml per minute,
- spectrophotometer set at 232 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

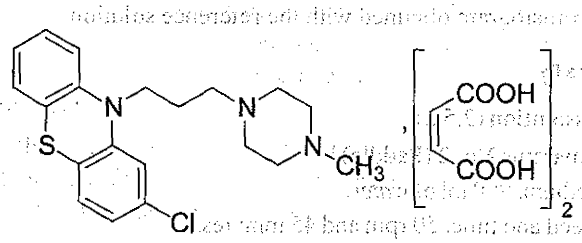
Inject the reference solution and the test solution.

Calculate the content of $C_{12}H_{19}N_3O$ in the capsules.

Storage. Store in airtight containers, at a temperature not exceeding 25°.

Labelling. The label states the strength in terms of the equivalent amount of procarbazine.

Prochlorperazine Maleate



$C_{20}H_{24}ClN_3S,2C_4H_4O_4$ Mol. Wt. 606.1

Prochlorperazine Maleate is 2-chloro-10-[3-(4-methylpiperazin-1-yl)propyl]phenothiazine di(hydrogen maleate).

Prochlorperazine Maleate contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{20}H_{24}ClN_3S,2C_4H_4O_4$, calculated on the dried basis.

Category. Antipsychotic; antiemetic.

Description. A white or pale yellow, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. To 20 mg add 5 ml of *water* and 1 ml of *1 M sodium hydroxide*, shake and extract with 10 ml of *ether*. Wash the ether extract with 5 ml of *water*, dry with *anhydrous sodium sulphate*, evaporate to dryness and dissolve the residue in 0.2 ml of *chloroform*.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prochlorperazine maleate IPRS*, treated in the same manner or with the reference spectrum of prochlorperazine.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *ethanol (95 per cent)* containing 0.01 per cent v/v of *strong ammonia solution* shows an absorption maximum at about 258 nm and a less well-defined maximum at about 313 nm; absorbance at about 258 nm, about 0.63.

C. Complies with the test for identification of phenothiazines (2.3.3).

Test solution. A solution containing 0.1 per cent w/v of the substance under examination in a mixture of equal volumes of *methanol* and *chloroform*.

Reference solution. A 0.1 per cent w/v solution of *prochlorperazine maleate IPRS* in the same solvent mixture.

Apply 4 µl of each solution.

D. Triturate 0.2 g with a mixture of 3 ml of *water* and 1 ml of *10 M sodium hydroxide* and shake with three quantities, each of 5 ml, of *ether*. To 0.1 ml of the aqueous layer add a solution of 10 mg of *resorcinol* in 3 ml of *sulphuric acid* and heat on a water-bath for 15 minutes; no colour develops. To the remainder of the aqueous layer add 2 ml of *bromine solution*, heat on a water-bath for 15 minutes, then heat to boiling and cool. To 0.1 ml of the solution add a solution of 10 mg of *resorcinol* in 3 ml of *sulphuric acid* and heat on a water-bath for 15 minutes; a blue colour develops.

Tests

pH (2.4.24). 3.0 to 4.0, determined in a freshly prepared saturated solution.

Related substances. Complies with the test for Related substances in Phenothiazines (2.3.5), using mobile phase A.

NOTE— Prepare the test solution immediately before use.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

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Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh 0.2 g, in powder, dissolve in 50 ml of *anhydrous glacial acetic acid*, heating gently on a water-bath, allow to cool. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03031 g of $C_{20}H_{24}ClN_3S,2C_4H_4O_4$.

Storage. Store protected from light and moisture.

Prochlorperazine Tablets

Prochlorperazine Maleate Tablets

Prochlorperazine Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of prochlorperazine maleate, $C_{20}H_{24}ClN_3S,2C_4H_4O_4$.

Usual strengths. 5 mg; 25 mg.

Identification

A. To a quantity of the powdered tablets containing 40 mg of Prochlorperazine Maleate add 10 ml of *water* and 2 ml of *1 M sodium hydroxide*, shake and extract with 15 ml of *ether*. Wash the ether with 5 ml of *water*, dry with *anhydrous sodium sulphate*, evaporate to dryness and dissolve the residue in 0.4 ml of *chloroform*.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prochlorperazine maleate IPRS*, treated in the same manner or with the reference spectrum of prochlorperazine.

B. To a quantity of the powdered tablets containing 5 mg of Prochlorperazine Maleate add 5 ml of *sulphuric acid* and allow to stand for 5 minutes; a red colour is produced.

C. Shake a quantity of the powdered tablets containing 0.2 g of Prochlorperazine Maleate with 2 ml of *water* and 1 ml of *5 M sodium hydroxide*, mix and extract with three quantities, each of 10 ml, of *ether*. Dry the combined extracts with *anhydrous sodium sulphate*, filter, evaporate the filtrate to dryness and dissolve the residue in 10 ml of *methanol* and add a solution of 0.15 g of *picric acid* in 10 ml of *methanol*. The precipitate, after washing with a small quantity of *methanol*, melts at about 255°, with decomposition (2.4.21).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium: 500 ml of *0.1 M hydrochloric acid*,

Speed and time. 75 rpm for 60 minutes.

Withdraw a suitable volume of the medium and filter, rejecting the first few ml of filtrate. Dilute a suitable volume of the filtrate with the medium, if necessary. Measure the absorbance of the resulting solution at the maximum at about 254 nm (2.4.7).

Calculate the content of prochlorperazine maleate, $C_{20}H_{24}ClN_3S,2C_4H_4O_4$ in the medium from the absorbance obtained from a solution of known concentration of *prochlorperazine maleate IPRS* in the dissolution medium.

Q. Not less than 75 per cent of the stated amount of $C_{20}H_{24}ClN_3S,2C_4H_4O_4$.

Related substances. Comply with the test for Related substances in Phenothiazines (2.3.5), using mobile phase A.

NOTE— Prepare the following solutions freshly.

Test solution. Extract a quantity of the powdered tablets containing 0.1 g of Prochlorperazine Maleate with 10 ml of *methanol* containing 0.5 per cent v/v of *strong ammonia solution* and filter.

Reference solution. Dilute 1.0 ml of the test solution to 200.0 ml with the same solvent.

Apply to the plate 20 µl of each solution.

Uniformity of content. Complies with the test stated under Tablets.

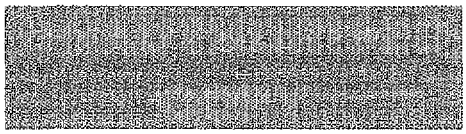
NOTE— Protect the solutions from light throughout the Assay.

Crush one tablet and extract with three quantities, each of 10 ml, of *ethanol* containing 1 per cent v/v of *strong ammonia solution*. Filter the extracts and to the combined filtrates add sufficient *ethanol* to produce 50.0 ml. Dilute 10.0 ml of the solution to 100.0 ml with *ethanol*. Dilute further with *ethanol*, if necessary, to give a final solution containing 10 µg of Prochlorperazine Maleate per ml and measure the absorbance of the resulting solution at the maximum at about 258 nm (2.4.7). Calculate the content of $C_{20}H_{24}ClN_3S,2C_4H_4O_4$ taking 620 as the specific absorbance at 258 nm.

Other tests. Comply with the tests stated under Tablets.

Assay. *Protect the solutions from light throughout the Assay.*

Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 25 mg of Prochlorperazine Maleate and extract with three quantities, each of 10 ml, of *ethanol* containing 1 per cent v/v of *strong ammonia solution*. Filter the extracts and to the combined filtrates add sufficient *ethanol* to produce 100.0 ml. Dilute 10.0 ml to 50.0 ml with *ethanol*, dilute 10.0 ml of the solution to 50.0 ml with *ethanol* and measure the absorbance of the resulting solution at the maximum at about 258 nm (2.4.7). Calculate the content of $C_{20}H_{24}ClN_3S,2C_4H_4O_4$ taking 620 as the specific absorbance at 258 nm.



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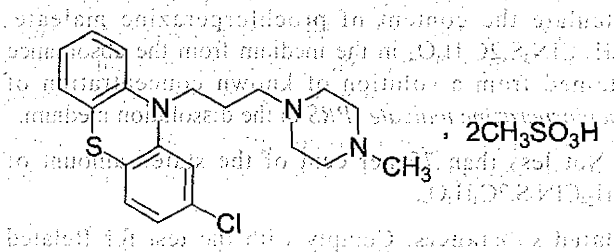
Storage. Store protected from light and moisture.

Prochlorperazine Mesylate



$C_{20}H_{24}ClN_3S,2CH_4SO_3$ Mol. Wt. 566.2

Prochlorperazine Mesylate is 2-chloro-10-[3-(4-methylpiperazin-1-yl)propyl]phenothiazine di(methanesulphonate).

Prochlorperazine Mesylate contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{20}H_{24}ClN_3S,2CH_4SO_3$, calculated on the dried basis.

Category. Antipsychotic; antiemetic.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prochlorperazine mesylate IPRS* or with the reference spectrum of prochlorperazine mesylate.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *ethanol* containing 0.01 per cent v/v of *strong ammonia solution* shows an absorption maximum at about 258 nm and a less well-defined maximum at about 313 nm; absorbance at about 258 nm, about 0.63.

C. Dissolve 5 mg in 2 ml of *sulphuric acid* and allow to stand for 5 minutes; a red colour is produced.

D. Mix 50 mg with 0.2 g of powdered *sodium hydroxide*, heat to fusion and continue the heating for a few seconds longer. Cool, add 0.5 ml of *water* and a slight excess of *2 M hydrochloric acid* and warm; sulphur dioxide is evolved which turns moistened *starch iodate paper* blue.

Tests

pH (2.4.24). 2.0 to 3.0, determined in a 2.0 per cent w/v solution.

Related substances. Complies with the test for Related substances in Phenothiazines (2.3.5), using mobile phase A.

Test solution. Dissolve the substance under examination in *methanol* containing 0.5 per cent v/v of *strong ammonia solution*.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 100° at a pressure not exceeding 0.7 kPa.

Assay. Dissolve 0.5 g in 10 ml of *water*, add 5 ml of *1 M sodium hydroxide* and extract with successive quantities of 50, 25, 25 and 25 ml of *ether*. Wash the combined *ether* extracts with 5 ml of *water*, shake the washings with 5 ml of *ether*, add the *ether* to the combined ether extracts and evaporate to dryness. Add 2 ml of *ethanol*, evaporate to dryness. Dissolve the residue in 50 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, using *1-naphtholbenzein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02831 g of $C_{20}H_{24}ClN_3S,2CH_4SO_3$.

Storage. Store protected from light and moisture.

Prochlorperazine Injection

Prochlorperazine Mesylate Injection

Prochlorperazine Injection is a sterile solution of Prochlorperazine Mesylate in Water for Injections free from dissolved air.

Prochlorperazine Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of prochlorperazine mesylate, $C_{20}H_{24}ClN_3S,2CH_4SO_3$.

Usual strength. 12.5 mg per ml.

Identification

A. To a volume containing 0.1 g of Prochlorperazine Mesylate add 20 ml of *water* and 2 ml of *10 M sodium hydroxide*. Shake and extract with 25 ml of *ether*. Wash the ether layer with two quantities, each of 5 ml, of *water*, dry with *anhydrous sodium sulphate* and evaporate to dryness. Dissolve the residue in 1 ml of *chloroform*.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prochlorperazine mesylate IPRS* treated in the same manner or with the reference spectrum of prochlorperazine.

B. To a volume containing 5 mg of Prochlorperazine Mesylate add carefully 2 ml of *sulphuric acid* and allow to stand for 5 minutes; a red colour is produced.

Tests

pH (2.4.24). 5.5 to 6.5.

Related substances. Carry out the test for Related substances in Phenothiazines (2.3.5), using mobile phase A.

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Test solution. Use the injection under examination.

Reference solution (a). Dilute 1.0 ml of the test solution to 40 ml with *methanol* containing 0.5 per cent v/v of *strong ammonia solution* immediately before use.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with the *methanol-ammonia mixture* immediately before use.

Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b).

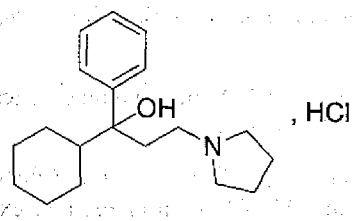
Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *Protect the solutions from light throughout the Assay.*

To a measured volume containing about 25 mg of Prochlorperazine Mesylate add sufficient *ethanol* containing 0.01 per cent v/v of *strong ammonia solution* to produce 200.0 ml. Dilute 5.0 ml of the solution to 100.0 ml with the *ammoniacal ethanol* and measure the absorbance of the resulting solution at the maximum at about 258 nm (2.4.7). Calculate the content of $C_{20}H_{24}ClN_3S, 2CH_4SO_3$ taking 635 as the specific absorbance at 258 nm.

Storage: Store protected from light.

Procyclidine Hydrochloride



$C_{19}H_{29}NO,HCl$ Mol. Wt. 323.9

Procyclidine Hydrochloride is (*RS*)-1-cyclohexyl-1-phenyl-3-pyrrolidin-1-ylpropan-1-ol hydrochloride.

Procyclidine Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{19}H_{29}NO,HCl$, calculated on the dried basis.

Category. Antiparkinson.

Description. A white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *procyclidine hydrochloride IPRS* or with the reference spectrum of procyclidine hydrochloride.

B. Dissolve 0.25 g in 10 ml of *water*, make alkaline with *5 M ammonia* and extract with three quantities, each of 10 ml, of *ether*. Dry the combined ethereal extracts over *anhydrous sodium sulphate*, filter, remove the ether and scratch the residue with a glass rod to induce solidification. The residue melts at about 85° (2.4.21).

C. It gives the reactions of chlorides (2.3.1).

Tests

pH (2.4.24), 4.5 to 6.5, determined in a 1.0 per cent w/v solution.

Related substances. A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 100 volumes of *ether* and 1 volume of *strong ammonia solution*.

Test solution. Dissolve 0.2 g of the substance under examination in 10 ml of *chloroform*.

Reference solution (a). A 0.004 per cent w/v solution of *1-phenyl-3-pyrrolidinopropan-1-one hydrochloride IPRS* in *chloroform*.

Reference solution (b). A 0.01 per cent w/v solution of the substance under examination in *chloroform*.

Apply to the plate 5 µl of each solution. After development, dry the plate at 105° for 15 minutes and examine under ultraviolet light at 254 nm. Any spot corresponding to 1-phenyl-3-pyrrolidinopropan-1-hydrochloride-1-one in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a). Spray the plate with *dilute potassium iodobismuthate solution*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (b).

B. Determine by gas chromatography (2.4.13).

Test solution. Add 5 ml of *1.25 M sodium hydroxide* to 20 ml of a 0.015 per cent w/v solution of the substance under examination and mix. Extract with two quantities, each of 20 ml, of *ether*, add to the combined extracts 5 ml of a 0.06 per cent w/v solution of *triphenylethylene* (internal standard) in *ether*, shake with *anhydrous sodium sulphate* and filter. Evaporate the filtrate and dissolve the residue in 1 ml of *ether*.

Reference solution (a). Prepare in the same manner as the test solution but using 20 ml of a 0.5 per cent w/v solution of the

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substance under examination and omitting the addition of the internal standard solution.

Reference solution (b). Prepare in the same manner as the test solution but using 20 ml of a 0.5 per cent w/v solution of the substance under examination.

Chromatographic system
- a glass column 1.5 m x 4 mm, packed with acid-washed, silanised diatomaceous support (Such as HP chromosorb W) coated with 10 per cent w/w of modified polyethylene glycol 20M (such as SP-1000) and 2 per cent w/w of *potassium hydroxide*,
- temperature :
  column. 240°,
  inlet port and detector at 240°,
- flow rate: 30 ml per minute of the carrier gas.

The ratio of the sum of the areas of any secondary peaks to the area of the peak due to the internal standard in the chromatogram obtained with reference solution (b) is not more than the ratio of the area of the principal peak to the area of the internal standard peak in the chromatogram obtained with the test solution.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.7 g in 75 ml of *anhydrous glacial acetic acid*, warm if necessary to effect solution and cool. Add 10 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03239 g of $C_{19}H_{29}NO,HCl$.

Storage. Store protected from moisture.

Procyclidine Tablets

Procyclidine Hydrochloride Tablets

Procyclidine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of procyclidine hydrochloride, $C_{19}H_{29}NO,HCl$.

Usual strengths. 2.5 mg; 5 mg.

Identification

A. Dissolve a quantity of the powdered tablets containing about 25 mg of Procyclidine Hydrochloride in 10 ml of *water*, shake with 20 ml of *ether* and discard the ether layer. Make the aqueous layer alkaline with *2 M sodium hydroxide* and extract with two quantities, each of 20 ml, of *ether*. Wash the combined ether extracts with two quantities, each of 10 ml, of *water*, dry by shaking with *anhydrous sodium sulphate*, filter and evaporate the filtrate to dryness. If necessary, induce crystallization by scratching with a glass rod.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *procyclidine hydrochloride IPRS* treated in the same manner or with the reference spectrum of procyclidine.

B. The powdered tablets give the reactions of chlorides (2.3.1).

Tests

Dissolution (2.5.2).
Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and pool the medium, filter. Follow the procedure described under Assay with necessary modifications and calculate the content of $C_{19}H_{29}NO,HCl$.

Q. Not less than 75 per cent of the stated amount of $C_{19}H_{29}NO,HCl$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 100 volumes of *ether* and 1 volume of *strong ammonia solution*.

Test solution. Shake a quantity of the powdered tablets containing 25 mg of Procyclidine Hydrochloride with 5 ml of *chloroform* and filter.

Reference solution (a). A 0.001 per cent w/v solution of *1-phenyl-3-pyrrolidinopropan-1-one hydrochloride IPRS* in *chloroform*.

Reference solution (b). Dilute 1 volume of test solution to 200 volumes with *chloroform*.

Apply to the plate 20 µl of each solution. After development, dry the plate at 105° for 15 minutes and examine under ultraviolet light at 254 nm. Any spot corresponding to 1-phenyl-3-pyrrolidinopropan-1-one in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent). Spray the plate with *dilute potassium iodobismuthate solution*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any spot due to excipients on the line of application.

Other tests. Comply with the tests stated under Tablets.

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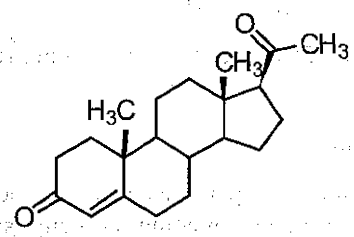
Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 2.5 mg of Procyclidine Hydrochloride, transfer to a 100-ml volumetric flask, add 10.0 ml of *water* and mix, and dilute to volume with a 0.025 per cent w/v solution of *bromocresol purple* in 2 per cent v/v solution of *glacial acetic acid*. Allow the undissolved particles to settle. Transfer 5.0 ml of the supernatant solution to a separating funnel, extract with 20.0 ml of *chloroform* and filter the extract, discarding the first 5 ml of the filtrate. Measure the absorbance of the filtrate at the maximum at about 405 nm (2.4.7), using as the blank a solution prepared by treating 0.5 ml of *water* and 4.5 ml of a 0.025 per cent w/v solution of *bromocresol purple* in the same manner beginning at the words "extract with 20 ml of *chloroform* ……".

Calculate the content of $C_{19}H_{29}NO,HCl$ from the absorbance obtained by repeating the operation using *procyclidine hydrochloride IPRS* in place of the powdered tablets.

Storage. Store protected from moisture.

Progesterone



$C_{21}H_{30}O_2$ Mol. Wt. 314.5

Progesterone is 4-Pregnene-3,20-dione.

Progesterone contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{21}H_{30}O_2$, calculated on the dried basis.

Category. Steroidal hormone.

Description. A white or almost white crystalline powder or colourless crystals. It shows polymorphism (2.5.11).

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *progesterone IPRS* or with the reference spectrum of progesterone.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel F254*.

Mobile phase. A mixture of 66 volumes of *dichloromethane* and 33 volumes of *ethyl acetate*.

Solvent mixture. 90 volumes of *dichloromethane* and 10 volumes of *methanol*.

Test solution. Dissolve 10 mg of the substance under examination in 10.0 ml of the solvent mixture.

Reference solution. A 0.1 per cent w/v solution of *progesterone IPRS* in the solvent mixture.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to the principal spot in the chromatogram obtained with the reference solution. Spray the plate with *ethanolic sulphuric acid (20 per cent)*, heat at 120° for 15 minutes, allow to cool and examine in day light. The principal spot in the chromatogram obtained with the test solution corresponds to the principal spot in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). + 186.0° to + 194.0°, determined in a 1.0 per cent w/v solution in *ethanol (95 per cent)*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in *methanol* and dilute to 50.0 ml with *methanol*.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with *methanol*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 241 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	50	50
20	50	50
27	20	80
45	50	50
50	50	50

Inject the reference solution. The test is not valid unless the column efficiency is not less than 4000 theoretical plates, tailing factor is not more than 2.0, and the relative standard deviation for replicate injections is not more than 5.0 per cent.

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Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of areas of all the secondary peaks is not more than 0.8 times the area of the principal peak in the chromatogram obtained with the reference solution (0.8 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 0.5 g by drying in an oven at 105° for 2 hours.

Assay. Weigh 10 mg and dissolve in 100.0 ml of *ethanol (95 per cent)*. Dilute 5.0 ml of the solution to 50.0 ml with *ethanol (95 per cent)*. Measure the absorbance of the resulting solution at the maximum at about 241 nm (2.4.7). Calculate the content of $C_{21}H_{30}O_2$ taking 535 as the specific absorbance at 241 nm.

Storage. Store protected from light.

Progesterone Injection

Progesterone Injection is a sterile solution of Progesterone in Ethyl Oleate or other suitable ester, in a suitable fixed oil or in any mixture of these. It may contain suitable alcohols.

Progesterone Injection contains not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of progesterone, $C_{21}H_{30}O_2$.

Usual strengths. 25 mg per ml; 50 mg per ml; 100 mg per ml; 250 mg per ml.

Identification

Dissolve a volume containing 50 mg of Progesterone in 8 ml of *light petroleum (40° to 60°)* and extract with three quantities, each of 8 ml, of a mixture of 7 volumes of *glacial acetic acid* and 3 volumes of *water*, wash the combined extract with 10 ml of *light petroleum (40° to 60°)*, dilute with *water*, until the solution becomes turbid, allow to stand in ice for 2 hours and filter. The precipitate, after washing with *water* and drying at 105°, on the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *progesterone IPRS* or with the reference spectrum of progesterone.

Tests

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. To a measured volume containing 50 mg of Progesterone add sufficient *chloroform* to produce 100.0 ml. Dilute 3.0 ml of the solution to 50.0 ml with *chloroform*. To 5.0 ml of the solution, add 10 ml of *isoniazid solution* and sufficient *methanol* to produce 20.0 ml. Allow to stand for 45 minutes and measure the absorbance of the resulting solution at the maximum at about 380 nm (2.4.7), using as the blank 5 ml of *chloroform* treated in the same manner. Calculate the content of $C_{21}H_{30}O_2$ from the absorbance obtained by repeating the procedure using a 0.003 per cent w/v solution of *progesterone IPRS* in *chloroform* and beginning at the words "To 5.0 ml of the solution....".

Storage. Store protected from light. If solid matter separates on standing, it should be redissolved by heating before use.

Labelling. The label states that the preparation is intended for intramuscular injection only.

Progesterone Injectable Suspension

Progesterone Injectable Suspension is a sterile suspension of Progesterone in Water for Injections.

Progesterone Injectable Suspension contains not less than 93.0 per cent and not more than 107.0 per cent of the stated amount of progesterone, $C_{21}H_{30}O_2$.

Usual Strengths. 25 mg per ml; 50 mg per ml; 100 mg per ml; 200 mg per ml.

Identification

A. Filter a volume of suspension containing about 100 mg of progesterone, through a medium-porosity, sintered-glass crucible, filter again if the fluid is not clear. Wash with several 5.0 ml portions of *water*, evaporate on a steam bath, dry the residue at 105°. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *progesterone IPRS* or with the reference spectrum of progesterone.

B. Melting point (2.4.21). 126° to 131°.

Tests

pH (2.4.24). 4.0 to 7.5.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 85 volumes of *ethanol (95 per cent)* and 15 volumes of *water*.

Internal standard solution. A 0.66 per cent w/v solution of *methyltestosterone IPRS* in the solvent mixture.

Test solution. Shake a volume of suspension containing about 25 mg of progesterone with 16 ml of the solvent mixture into a

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polytef-lined, screw-capped, 25 ml test tube until the solution is clear. Add 5.0 ml of the internal standard solution and dilute to 25.0 ml with the solvent mixture.

Reference solution. A 0.25 per cent w/v solution of *progesterone IPRS* in the solvent mixture. To 10.0 ml of the solution, add 5.0 ml of the internal standard solution and dilute to 25.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 100 cm x 2.1 mm, packed with octadecylsilane chemically bonded to porous silica (30-50 µm),
- mobile phase: a mixture of 72 volumes of *water* and 28 volumes of *isopropyl alcohol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 5 µl.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to progesterone and methyltestosterone is not less than 3.5 and the relative standard deviation for replicate injections is not more than 1.5 per cent.

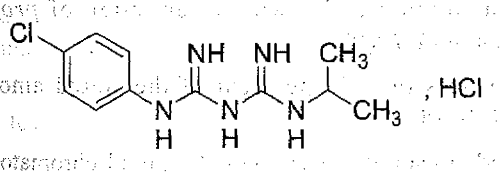
Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{30}O_2$ in the suspension.

Storage. Store in single dose or multiple dose containers, preferably of Type 1 glass.

Proguanil Hydrochloride

Chloroguanide Hydrochloride



$C_{11}H_{16}ClN_5, HCl$ Mol. Wt. 290.2

Proguanil Hydrochloride is 1-(4-chlorophenyl)-5-isopropylbiguanide hydrochloride.

Proguanil Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{11}H_{16}ClN_5, HCl$, calculated on the dried basis.

Category. Antimalarial.

Description. A white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *proguanil hydrochloride IPRS* or with the reference spectrum of proguanil hydrochloride.

B. To 10 ml of a saturated solution add 0.25 ml of *potassium ferrocyanide solution*; a white precipitate is produced which dissolves on addition of a few drops of *dilute nitric acid*.

C. Dissolve 5 mg in 5 ml of a warm 1.0 per cent w/v solution of *cetrimide* and add 1 ml of *5 M sodium hydroxide* and 1 ml of *bromine solution*; a deep red colour is produced.

D. It gives the reactions of chlorides (2.3.1).

Tests

Acidity or alkalinity. To 35 ml of *water* maintained at 60° to 65° add 0.2 ml of *methyl red solution*, neutralise with *0.01 M sodium hydroxide* or *0.01 M hydrochloric acid*, add 0.4 g of the substance under examination and stir until dissolved. The resulting solution is not acidic and requires for neutralisation not more than 0.2 ml of *0.01 M hydrochloric acid*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 10 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A 0.00005 per cent w/v solution of *proguanil impurity C IPRS (1,5-bis(4-chlorophenyl) biguanide IPRS)* in the mobile phase.

Reference solution (c). A 0.00005 per cent w/v solution of *proguanil impurity D IPRS (1,5-bis(1-methylethyl) biguanide IPRS)* in the mobile phase.

Reference solution (d). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase. Dilute to 1.0 ml of the solution, add 1.0 ml of reference solution (c) and mix.

Chromatographic system
- a stainless steel column 12.5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 3.78 g of *sodium hexanesulphonate* in a mixture of 1 volume of *glacial acetic acid*, 80 volumes of *water* and 120 volumes of *methanol*,
- flow rate: 1ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Inject reference solution (d). The test is not valid unless the resolution between the peaks due to proguanil impurity D and the principal peak is not less than 5.0.

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Inject reference solution (a) and the test solution. Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to proguanil impurity C is not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent), the area of the peak due to proguanil impurity D is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). The area of any other secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with refernce solution (a) (1.0 per cent). Ignore any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

4-Chloroaniline. Not more than 250 ppm.

Dissolve 0.1 g in 1 ml of *2 M hydrochloric acid*, add sufficient *water* to produce 20 ml, cool to 5°, add 1 ml of *0.05 M sodium nitrite*, allow to stand at 5° for 5 minutes, add 2 ml of a 5 per cent w/v solution of *ammonium sulphamate* and allow to stand for 10 minutes. Add 2 ml of a 0.1 per cent w/v solution of *N-(1-naphthyl)ethylenediamine dihydrochloride*, dilute to 50 ml with *water* and allow to stand for 30 minutes. Any magenta colour produced is not more intense than that obtained by treating in the same manner and at the same time 20 ml of a solution containing 1.25 µg per ml of *4-chloroaniline*.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.15 g in 25 ml of *anhydrous glacial acetic acid* and 10 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01451 g of $C_{11}H_{16}ClN_5,HCl$.

Storage. Store protected from light and moisture.

Proguanil Tablets

Proguanil Hydrochloride Tablets; Chloroguanide Hydrochloride Tablets

Proguanil Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of proguanil hydrochloride, $C_{11}H_{16}ClN_5,HCl$.

Usual strength. 100 mg.

Identification

A. Boil a quantity of the powdered tablets containing 0.5 g of Proguanil Hydrochloride with 5 ml of *dilute hydrochloric acid*, cool and filter. To the filtrate add a slight excess of *sodium hydroxide solution*, extract with 30 ml of *ether* and evaporate the ethereal extract; the residue, after drying at 105°, melts at about 131° (2.4.21).

Dissolve the residue obtained in test A in the minimum quantity of *dilute hydrochloric acid*; the solution diluted with *water* to about 40 ml and neutralised if necessary, with cautious addition of *dilute ammonia solution*, complies with the following tests.

B. To 10 ml add 0.25 ml of *potassium ferrocyanide solution*; a white precipitate is produced which dissolves on addition of a few drops of *dilute nitric acid*.

C. To 0.5 ml add 5 ml of a warm 1.0 per cent w/v solution of *cetrimide* and add 1 ml of *5 M sodium hydroxide* and 1 ml of *bromine solution*; a deep red colour is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.2 M hydrochloric acid* containing 0.2 per cent w/v solution of *sodium chloride*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Dilute a suitable volume of the filtrate, if necessary with dissolution medium. Measure the absorbance of the resulting solution at the maximum at about 242 nm (2.4.7), Calculate the content of $C_{11}H_{16}ClN_5,HCl$ in the medium from the absorbance obtained from a solution of known concentration of *proguanil hydrochloride IPRS*.

Q. Not less than 75 per cent of the stated amount of $C_{11}H_{16}ClN_5,HCl$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powder containing 100 mg of Proguanil Hydrochloride, dissolve in 50 ml of *methanol* and shake for 10 minutes, dilute to 100.0 ml with *methanol*. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of test solution to 100.0 ml with the mobile phase.

Reference solution (b). Mix 1.0 ml of 0.05 per cent w/v solution of *1-(2,5-dichlorophenyl)-5-isopropylbiguanide hydrochloride IPRS* in *methanol* and 9 ml of 0.01 per cent w/v solution of *proguanil hydrochloride IPRS* in the mobile phase.

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Chromatographic system
- a stainless steel column 10 cm x 5.0 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Nucleosil C18),
- column temperature: 40°,
- mobile phase: dissolve 1.89 g of *sodium hexanesulphonate* in a mixture of 500 ml of *methanol*, 500 ml of *water* and 5 ml of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution (b). The test is not valid unless the chromatogram shows two clearly separated peaks due to proguanil hydrochloride and 1-(2,5-dichlorophenyl)-5-isopropylbiguanide hydrochloride.

Inject reference solution (a) and the test solution. The sum of the areas of any secondary peaks in the chromatogram obtained with test solution is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

4-Chloroaniline. Not more than 250 ppm.

To a quantity of the powdered tablets containing about 0.1 g of Proguanil Hydrochloride add 5 ml of *ethanol (95 per cent)* and shake for 10 minutes. Add 2.5 ml of *2 M hydrochloric acid* and 15 ml of *water*, mix and filter through a wetted filter paper, washing the filter with 5 ml of *water*. Cool to 5°, add 1 ml of *0.05 M sodium nitrite*, allow to stand at 5° for 5 minutes, add 2 ml of a 5 per cent w/v solution of *ammonium sulphamate* and allow to stand for 10 minutes. Add 2 ml of a 0.1 per cent w/v solution of *N-(1-naphthyl)-ethylenediamine dihydrochloride*, dilute to 50 ml with *water* and allow to stand for 30 minutes. Any magenta colour produced is not more intense than that obtained by treating in the same manner and at the same time 20 ml of a solution containing 1.25 µg per ml of *4-chloroaniline*.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powder containing 100 mg of Proguanil Hydrochloride, dissolve in 50 ml of *methanol* and shake for 10 minutes, dilute to 100.0 ml with *methanol*. Dilute 5.0 ml to 50.0 ml with *methanol*. Dilute 10.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). A 0.002 per cent w/v solution of *proguanil hydrochloride IPRS* in the mobile phase.

Reference solution (b). Mix 1.0 ml of 0.05 per cent w/v solution of *1-(2,5-dichlorophenyl)-5-isopropylbiguanide hydrochloride IPRS* in *methanol* and 9 ml of 0.01per cent w/v solution of *proguanil hydrochloride IPRS* in the mobile phase.

Use chromatographic system as described under Related substances.

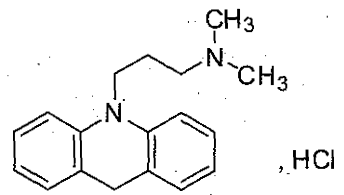
Inject reference solution (b). The test is not valid unless the chromatogram shows two clearly separated peaks due to proguanil hydrochloride and 1-(2,5-dichlorophenyl)-5-isopropylbiguanide hydrochloride.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{11}H_{16}ClN_5,HCl$.

Storage. Store protected from light and moisture.

Promazine Hydrochloride



$C_{17}H_{20}N_2S,HCl$ Mol. Wt. 320.9

Promazine Hydrochloride is 10-(3-dimethylaminopropyl) phenothiazine hydrochloride.

Promazine Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{17}H_{20}N_2S,HCl$, calculated on the dried basis.

Category. Neuroleptic.

Description. A white or almost white, crystalline powder, slightly hygroscopic in nature.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *promazine hydrochloride IPRS* or with the reference spectrum of promazine hydrochloride.

B. Complies with the test for identification of phenothiazine (2.3.3), using *promazine hydrochloride IPRS* to prepare reference solution.

C. Dissolve about 5 mg in 2 ml of *sulphuric acid* and allow to stand for 5 minutes. An orange colour is produced.

D. It gives reaction (B) of chlorides (2.3.1).

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Tests

pH (2.4.24). 4.2 to 5.2, determined on 5 per cent w/v solution in *carbon dioxide-free water*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

NOTE—Carry out the test protected from bright light. Prepare the solutions immediately before use.

Mobile phase. A mixture of 10 volumes of *acetone*, 10 volumes of *diethylamine* and 80 volumes of *cyclohexane*.

Solvent mixture. 5 volumes of *diethylamine* and 95 volumes of *methanol*.

Test solution. Dissolve 0.1 g of the substance under examination in 10 ml of the solvent mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with the solvent mixture.

Reference solution (b). Dissolve 10 mg of *chlorprothixene hydrochloride IPRS* in the solvent mixture, add 1.0 ml of the test solution and dilute to 10.0 ml with the same solvent.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.25 g in a mixture of 5.0 ml of *0.01 M hydrochloric acid* and 50 ml of *ethanol*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25). Read the volume added between the two points of inflexion.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.03209 g of $C_{17}H_{21}ClN_2S$.

Storage. Store protected from light.

Promazine Tablets

Promazine Hydrochloride Tablets

Promazine Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of promazine hydrochloride, $C_{17}H_{20}N_2S,HCl$.

Usual strengths. 25 mg; 50 mg; 100 mg.

Identification

A. To a quantity of the powdered tablets containing 40 mg of Promazine Hydrochloride, add 10 ml of *water* and 2 ml of *1 M sodium hydroxide*, shake and extract with 15 ml of *ether*. Wash the extract with 5 ml of *water*, dry with *anhydrous sodium sulphate*, evaporate to dryness and dissolve the residue in 0.4 ml of *chloroform*. On the resulting solution, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *promazine hydrochloride IPRS* treated in the same manner or with the reference spectrum of promazine.

B. To a quantity of the powdered tablets containing 5 mg of Promazine Hydrochloride, add 5 ml of *sulphuric acid* and allow to stand for 5 minutes. An orange colour is produced.

C. Triturate a quantity of the powdered tablets containing 0.2 g of Promazine Hydrochloride with 4 ml of *methanol*, centrifuge, filter the supernatant liquid, heat almost to boiling, immediately add 2 ml of a boiling 3.5 per cent w/v solution of *picric acid* in *methanol* and boil for 2 minutes. Cool in ice, filter, wash the crystals with three quantities of *methanol*, dissolve in 10 ml of hot *methanol* and repeat the crystallisation and washing. The rust red crystals so obtained, after drying at 105° for 1 hour, melts at about 144° (2.4.21).

Tests

Related substances. Comply with the test for Related substances in Phenothiazines (2.3.5), using mobile phase (a) and the following freshly prepared solutions.

Test solution. Extract a quantity of the powdered tablets containing 0.1 g of Promazine Hydrochloride with 10 ml of *methanol* and filter.

Reference solution. Dilute 1.0 ml of test solution to 200.0 ml with *methanol*.

Other tests. Comply with the tests stated under Tablets.

Assay.

NOTE—Carry out the test protected from light.

Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 50 mg of Promazine Hydrochloride and triturate with 10 ml of *2 M hydrochloric acid* and add 200 ml of *water*. Shake for 15 minutes, add sufficient *water* to produce 500 ml and centrifuge about 50 ml of the mixture. To 5 ml of the clear, supernatant liquid add 10 ml of *0.1 M hydrochloric acid* and sufficient *water* to produce 100 ml. Measure the absorbance (2.4.7) of the resulting solution at the maximum at about 251 nm.

Calculate the content of $C_{17}H_{20}N_2S,HCl$ taking 935 as the specific absorbance at 251 nm.

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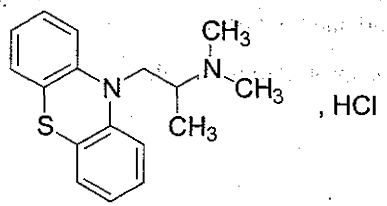
Promethazine Hydrochloride



$C_{17}H_{20}N_2S,HCl$ Mol. Wt. 320.9

Promethazine Hydrochloride is (*RS*)-dimethyl(2-phenothiazin-10-ylpropyl)amine hydrochloride.

Promethazine Hydrochloride contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{17}H_{20}N_2S,HCl$, calculated on the dried basis.

Category. Antiemetic.

Description. A white or faintly yellowish, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *promethazine hydrochloride IPRS* or with the reference spectrum of promethazine hydrochloride.

B. Complies with the test for Identification of Phenothiazines (2.3.3).

C. Dissolve 0.1 g in 3 ml of *water* and add 1 ml of *nitric acid* dropwise; a precipitate is produced which dissolves rapidly to give a red solution which becomes orange and then yellow. Heat the solution to boiling; it becomes orange and an orange-red precipitate is produced.

D. It gives reaction (B) of chlorides (2.3.1).

Tests

pH (2.4.24). 4.0 to 5.0, determined in a 10.0 per cent w/v solution prepared immediately before use.

Related substances. Carry out the test for Related substances in Phenothiazines (2.3.5), protected from bright light using mobile phase B.

NOTE—Prepare the following solutions immediately before use.

Test solution. Dissolve 0.2 g of the substance under examination in 10 ml of a mixture of 95 volumes of *methanol* and 5 volumes of *diethylamine*.

Reference solution (a). A 0.01 per cent w/v solution of the substance under examination in the same solvent mixture.

Reference solution (b). A 0.02 per cent w/v solution of *isopromethazine hydrochloride IPRS* in the same solvent mixture.

Apply to the plate 10 µl of each solution.

Any spot corresponding to isopromethazine in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (b) and any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (a).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.25 g in a mixture of 5 ml of *0.01 M hydrochloric acid* and 50 ml of *ethanol (95 per cent)* and titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25). Record the volume added between the two inflections.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.03209 g of $C_{17}H_{20}N_2S,HCl$.

Storage. Store protected from light and moisture.

Promethazine Injection

Promethazine Hydrochloride Injection

Promethazine Injection is a sterile solution of Promethazine Hydrochloride in Water for Injections free from dissolved air. It may contain suitable stabilising agents.

Promethazine Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of promethazine hydrochloride, $C_{17}H_{20}N_2S,HCl$.

Usual strengths. 25 mg in 1 ml; 50 mg in 2 ml.

Identification

A. To a volume containing 0.1 g of Promethazine Hydrochloride add 20 ml of *water* and 2 ml of *10 M sodium hydroxide*. Shake and extract the mixture with 25 ml of *ether*. Wash the ether layer with two quantities, each of 5 ml, of *water*, dry with *anhydrous sodium sulphate* and evaporate to dryness. Dissolve the residue in 1 ml of *chloroform*.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *promethazine hydrochloride IPRS* treated in the same manner or with the reference spectrum of promethazine.

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B. To a volume containing 0.2 g of Promethazine Hydrochloride add sufficient *potassium carbonate* to saturate the solution, extract with two quantities, each of 10 ml, of *ether* and evaporate the combined extracts to dryness. Dissolve the residue in 2 ml of *methanol* and pour into a solution of 0.4 g of *picric acid* in 10 ml of *methanol*, previously warmed to about 50°. Cool, scratch the sides of the tube to induce crystallisation, allow to stand for 3 to 4 hours and filter. The residue, after washing with *methanol* and drying, melts at about 160° (2.4.21).

C. To a volume containing 5 mg of Promethazine Hydrochloride add carefully 2 ml of *sulphuric acid* and allow to stand for 5 minutes; a red colour is produced.

Tests

pH (2.4.24). 5.0 to 6.0.

Related substances. Carry out the test for Related substances in Phenothiazines (2.3.5), using mobile phase B.

Test solution. Dilute a volume of the injection with a mixture of 95 volumes of *methanol* and 5 volumes of *diethylamine* to contain 1 per cent w/v of Promethazine Hydrochloride.

Reference solution (a). Dilute 1 volume of the test solution to 40 volumes with the same solvent mixture.

Reference solution (b). Dilute 1 volume of the test solution to 200 volumes with the same solvent mixture.

Reference solution (c). A 0.01 per cent w/v solution of *isopromethazine hydrochloride IPRS* in the same solvent mixture.

Apply separately to the plate 10 µl of each solution.

Any spot corresponding to isopromethazine in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (c). Any other secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b).

Bacterial endotoxins (2.2.3). Not more than 5.0 Endotoxin Units per mg of promethazine hydrochloride.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *Carry out the following procedure protected from light.*

To a measured volume containing about 25 mg of Promethazine Hydrochloride add sufficient *0.01 M hydrochloric acid* to produce 100.0 ml. Dilute 10.0 ml to 100.0 ml with *0.01 M hydrochloric acid*, dilute 10.0 ml of the solution to 50.0 ml with *0.01 M hydrochloric acid* and measure the absorbance of the resulting solution at the maximum at about 249 nm (2.4.7). Calculate the content of $C_{17}H_{20}N_2S$,HCl taking 910 as the specific absorbance at 249 nm.

Storage. Store protected from light.

Promethazine Syrup

Promethazine Hydrochloride Syrup; Promethazine Hydrochloride Oral Solution; Promethazine Oral Solution

Promethazine Syrup is a solution containing Promethazine Hydrochloride in a suitable flavoured vehicle.

Promethazine Syrup contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of promethazine hydrochloride, $C_{17}H_{20}N_2S$, HCl.

Usual strength. 5 mg per 5 ml.

Identification

Transfer an accurately measured volume of the sample containing about 25.0 mg of promethazine hydrochloride to a 250.0 ml of separator and 10.0 ml of *ammonia solution*. Extract the mixture with six quantities, each of 40.0 ml of *chloroform*, shaking vigorously. Wash the combined *chloroform* layer with 25.0 ml of 10 per cent *hydrochloric acid* and wash the acidic layer with 25.0 ml *chloroform* and add the washing to the main chloroform extract. Evaporate the combined extracts on a stream bath, with the aid of current of air, to a volume of 5.0 to 10.0ml, and finally evaporate, with the aid of current of air, to dryness and dissolve the residue in 2.5 ml of *carbon disulfide*. The resulting solution complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained by treating 25.0 mg of *promethazine hydrochloride IPRS* in the same manner.

Tests

Other tests. Comply with the tests stated under Oral Liquids.

Assay. *Carry out the following procedure protected from light.*

Transfer an accurately measured volume of the sample containing about 25 mg of Promethazine Hydrochloride to a 250.0 ml of separator and 10.0 ml of *ammonia solution*. Extract the mixture with six quantities, each of 40.0 ml of *chloroform*, shaking vigorously. Wash the combined *chloroform* layer with about 25.0 ml of 10 per cent *hydrochloric acid* and wash the acidic layer with 25.0 ml chloroform and add the washing to the main chloroform extract. Evaporate the combined extracts

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on a steam bath, with the aid of current of air, to a volume of 5.0 to 10.0 ml, and finally evaporate, using only a current of air, to dryness. Dissolve the residue, with slight warming in 1.0 per cent v/v *sulphuric acid*, and transfer to 500-ml volumetric flask and dilute to volume with the same medium. Mix well and filter, rejecting the first few ml of the filtrate. Measure the absorbance of the resulting solution at maximum at about 298 nm (2.4.7), using 1.0 per cent v/v *sulphuric acid* as blank. Calculate the content of $C_{17}H_{20}N_2S$,HCl in the sample from the absorbance obtained from a 0.005 per cent w/v solution of *promethazine hydrochloride IPRS* in 1.0 per cent v/v *sulphuric acid*.

Storage. Store protected from light and moisture.

Promethazine Tablets

Promethazine Hydrochloride Tablets

Promethazine Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of promethazine hydrochloride, $C_{17}H_{20}N_2S$, HCl. The tablets are coated.

Usual strengths. 10 mg; 25 mg; 50 mg.

Identification

A. To a quantity of the powdered tablets containing 40 mg of Promethazine Hydrochloride add 10 ml of *water* and 2 ml of *1 M sodium hydroxide*, shake and extract with 15 ml of *ether*. Wash the ether extract with 5 ml of *water*, dry with *anhydrous sodium sulphate*, evaporate to dryness and dissolve the residue in 0.4 ml of *chloroform*.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *promethazine hydrochloride IPRS* treated in the same manner or with the reference spectrum of promethazine.

B. Dissolve a quantity of the powdered tablets containing 0.2 g of Promethazine Hydrochloride in 2 ml of *water*, filter, add sufficient *potassium carbonate* to saturate the solution, extract with two quantities, each of 10 ml, of *ether* and evaporate the combined extracts to dryness. Dissolve the residue in 2 ml of *methanol* and pour into a solution of 0.4 g of *picric acid* in 10 ml of *methanol*, previously warmed to about 50°. Cool, scratch the sides of the tube to induce crystallisation, allow to stand for 3 to 4 hours and filter. The residue, after washing with *methanol* and drying, melts at about 160° (2.4.21).

C. To a quantity of the powdered tablets containing 5 mg of Promethazine Hydrochloride add 5 ml of *sulphuric acid* and allow to stand for 5 minutes; a red colour is produced.

Tests

Related substances. Carry out the test for Related substances in Phenothiazines (2.3.5), using mobile phase B.

Solvent mixture. 95 volumes of *methanol* and 5 volumes of *diethylamine.*

Test solution. A solution freshly prepared by extracting a quantity of the powdered tablets containing 0.1 g of Promethazine Hydrochloride with 10 ml of the solvent mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 40 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with the solvent mixture.

Reference solution (c). A 0.01 per cent w/v solution of *isopromethazine hydrochloride IPRS* in the solvent mixture.

Apply to the plate 10 µl of each solution. Any spot corresponding to isopromethazine in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (c). Any other secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b).

Uniformity of content. Complies with the test stated under Tablets.

Crush one tablet, add 1 ml of *dilute hydrochloric acid* and 30 ml of *water* and shake for 15 minutes. Add sufficient *water* to produce 50.0 ml and centrifuge. Complete the procedure described under Assay beginning at the words "To 5.0 ml of the clear supernatant liquid....".

Other tests. Comply with the tests stated under Tablets.

Assay. *Carry out the following procedure protected from light.*

Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 50 mg of Promethazine Hydrochloride with 10 ml of *2 M hydrochloric acid* and add 200 ml of *water*. Shake for 15 minutes, add sufficient *water* to produce 500.0 ml and centrifuge about 50 ml of the mixture. To 5.0 ml of the clear supernatant liquid add 10 ml of *0.1 M hydrochloric acid* and sufficient *water* to produce 100.0 ml. Measure the absorbance of the resulting solution at the maximum at about 249 nm (2.4.7). Calculate the content of $C_{17}H_{20}N_2S$, HCl taking 910 as the specific absorbance at 249 nm.

Storage. Store protected from light and moisture.

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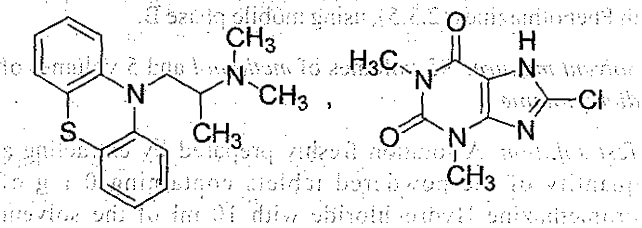
Promethazine Theoclate



$C_{17}H_{20}N_2S,C_7H_7ClN_4O_2$ Mol. Wt. 499.0

Promethazine Theoclate is the (*RS*)-dimethyl(2-phenothiazin-10-ylpropyl)amine salt of 8-chlorotheophylline.

Promethazine Theoclate contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{17}H_{20}N_2S,C_7H_7ClN_4O_2$, calculated on the dried basis.

Category. Antiemetic.

Description. A white or almost white powder.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests B, C and D may be omitted if tests A and E are carried out.

A. Shake 0.15 g with 2.5 ml of *water*, add 1 ml of *5 M ammonia* and extract with 30 ml of *ether*. Wash the ether extract with 10 ml of *water*, dry with *anhydrous sodium sulphate* and evaporate to dryness. Dissolve the residue in 1 ml of *chloroform*.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *promethazine theoclate IPRS* treated in the same manner or with the reference spectrum of promethazine.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.0007 per cent w/v solution in *ethanol* containing 0.01 per cent v/v of *strong ammonia solution* shows an absorption maximum at about 255 nm; absorbance is about 0.53.

C. Dissolve 5 mg in 2 ml of *sulphuric acid* and allow to stand for 5 minutes; a red colour is produced.

D. Shake 0.4 g with 10 ml of *water*, add 4 ml of *5 M ammonia*, shake with two quantities, each of 30 ml, of *ether* and add 4 ml of *hydrochloric acid* to the aqueous solution; a white precipitate is produced. Filter, wash with *water* and dry at 105°. Dissolve 10 mg of the residue in 1 ml of *hydrochloric acid*, add 0.1 g of *potassium chlorate* and evaporate to dryness; a reddish residue remains which becomes purple on exposure to the vapour of ammonia.

E. Fuse 50 mg of the residue obtained in test D with 0.5 g of *anhydrous sodium carbonate*, boil the residue with 5 ml of *water*, acidify to *litmus paper* with *nitric acid* and filter. The filtrate gives reaction (A) of chlorides (2.3.1).

Tests

Chlorides (2.3.12). Shake 1.5 g with 50 ml of *water* for 2 minutes and filter. 25 ml of the filtrate complies with the limit test for chlorides (330 ppm).

Related substances. Carry out the test for Related substances in Phenothiazines (2.3.5), protected from bright light, using mobile phase B.

NOTE-Prepare the following solutions immediately before use.

Test solution. Dissolve 0.2 g of the substance under examination in 10 ml of a mixture of 95 volumes of *methanol* and 5 volumes of *diethylamine*.

Reference solution (a). A 0.01 per cent w/v solution of the substance under examination in the same solvent mixture.

Reference solution (b). A 0.02 per cent w/v solution of *isopromethazine hydrochloride IPRS* in the same solvent mixture.

Apply to the plate 10 µl of each solution. Any spot corresponding to isopromethazine in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (b) and any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (a).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 1.0 g in 200 ml of *acetone*. Titrate with *0.1 M perchloric acid*, using 3 ml of a saturated solution of *methyl orange* in *acetone* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.04990 g of $C_{17}H_{20}N_2S,C_7H_7ClN_4O_2$.

Storage. Store protected from light and moisture.

Promethazine Theoclate Tablets

Promethazine Theoclate Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of promethazine theoclate, $C_{17}H_{20}N_2S,C_7H_7ClN_4O_2$.

Usual strengths. 25 mg; 50 mg.

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Identification

A. To a quantity of the powdered tablets containing 40 mg of Promethazine Theoclate add 10 ml of *water* and 2 ml of *1 M sodium hydroxide*, shake and extract with 15 ml of *ether*. Wash the ether extract with 5 ml of *water*, dry with *anhydrous sodium sulphate*, evaporate to dryness and dissolve the residue in 0.4 ml of *chloroform*.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *promethazine theoclate IPRS* treated in the same manner or with the reference spectrum of promethazine.

B. Dissolve a quantity of the powdered tablets containing 0.2 g of Promethazine Theoclate in 2 ml of *water*, filter, add sufficient *potassium carbonate* to saturate the solution, extract with two quantities, each of 10 ml, of *ether* and evaporate the combined extracts to dryness. Dissolve the residue in 2 ml of *methanol* and pour into a solution of 0.4 g of *picric acid* in 10 ml of *methanol*, previously warmed to about 50°. Cool, scratch the sides of the tube to induce crystallisation, allow to stand for 3 to 4 hours and filter. The residue, after washing with *methanol* and drying, melts at about 160° (2.4.21).

C. To a quantity of the powdered tablets containing 5 mg of Promethazine Theoclate add 5 ml of *sulphuric acid* and allow to stand for 5 minutes; a red colour is produced.

D. Extract a quantity of the powdered tablets containing 0.2 g of Promethazine Theoclate with *chloroform*, filter and evaporate the filtrate to dryness. Shake the residue with 10 ml of *water*, add 4 ml of *5 M ammonia* and extract with two quantities, each of 30 ml, of *ether*. Wash the combined extracts with 10 ml of *water*. Combine the aqueous layer and washings, add 4 ml of *hydrochloric acid*; a white precipitate is produced. Filter, wash the residue with *water* and dry at 105°. Dissolve 10 mg of the residue in 1 ml of *hydrochloric acid*, add 0.1 g of *potassium chlorate* and evaporate to dryness; a reddish residue remains which becomes purple on exposure to the vapour of ammonia.

Tests

Related substances. Carry out the test for related substances in phenothiazines (2.3.5), using mobile phase B.

Test solution. A solution freshly prepared by extracting a quantity of the powdered tablets containing 0.1 g of Promethazine Theoclate with 10 ml of a mixture of 95 volumes of *methanol* and 5 volumes of *diethylamine* and filtering.

Reference solution (a). Dilute 1 volume of the test solution to 40 volumes with the same solvent mixture.

Reference solution (b). Dilute 1 volume of the test solution to 200 volumes with the same solvent mixture.

Reference solution (c). A 0.01 per cent w/v solution of *isopromethazine hydrochloride IPRS* in the same solvent mixture.

Apply to the plate 10 µl of each solution. Any spot corresponding to isopromethazine in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (c). Any other secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b).

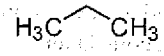
Other tests. Comply with the tests stated under Tablets.

Assay. *Carry out the following procedure protected from light.*

Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 50 mg of Promethazine Theoclate, add 5 ml of *water* and 1 ml of *strong ammonia solution* and allow to stand for 5 minutes. Add 50 ml of *ethanol*, shake for 5 minutes and filter, washing the residue with five quantities, each of 5 ml, of *ethanol*. Add sufficient *ethanol* to the filtrate to produce 100.0 ml. Dilute 10.0 ml to 100.0 ml with *ethanol* and dilute 10.0 ml of the solution to 100.0 ml with *ethanol*. Measure the absorbance of the resulting solution at the maximum at about 255 nm (2.4.7). Calculate the content of $C_{17}H_{20}N_2S,C_7H_7ClN_4O_2$ taking 755 as the specific absorbance at 255 nm.

Storage. Store protected from light and moisture.

Propane



C_3H_8 Mol. Wt. 44.1

Propane is dimethylmethane.

Propane contains not less than 98.0 per cent of C_3H_8.

CAUTION—Propane is highly flammable and explosive.

Identification

Determine by infrared absorption spectrophotometry (2.4.6), the solution give maxima at the wavelength of 3.4 µm, 6.8 µm and 7.2 µm.

Tests

Water (2.3.43). Not more than 0.001 per cent with the following modifications (a) provide the closed-system titrating vessel with an opening through which passes a coarse-porosity gas

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dispersion tube connected to a sample cylinder; (b) dilute the reagent with *anhydrous methanol* to give a water equivalence factor of between 0.2 and 1.0 mg per ml, age this diluted solution for not less than 16 hours before sanitation; (c) obtain a 100 g sample as directed under inhalation preparation, and introduce the sample into the titration vessel through the gas dispersion tube at a rate of about 100 ml of gas per minute.

High-boiling residues. Not more than 5 µg per ml, determined by the following method. Prepare a cooling coil from copper tubing (about 6 mm outside diameter × about 6.1 m long) to fit into a vacuum-jacketed flask. Immerse the cooling coil in a mixture of dry ice and *acetone* in a vacuum-jacketed flask, and connect one end of the tubing to the propellant sample cylinder. Carefully open the sample cylinder valve, flush the cooling coil with about 50 ml of the propellant, and discard this portion of liquefied propellant. Continue delivering liquefied propellant from the cooling coil, and collect it in a previously chilled 1000-ml sedimentation cone until the cone is filled to the 1000-ml mark. Allow the propellant to evaporate, using a warm water bath maintained at about 40° to reduce evaporating time. When all of the liquid has evaporated, rinse the sedimentation cone with two 50-ml portions of *pentane*, and combine the rinsings in a tared 150-ml evaporating dish. Transfer 100 ml of the *pentane* solvent to a second tared 150-ml evaporating dish, place both evaporating dishes on a water bath, evaporate to dryness, and heat the dishes in an oven at 100° for 60 minutes. Cool the dishes in a desiccator, and weigh. Repeat the heating for 15-minute periods until successive weighings are within 0.1 mg, and calculate the weight of the residue obtained from the propellant as the difference between the weights of the residues in the two evaporating dishes.

Acidity of residue. Add 10 ml of *water* to the residue obtained in the test for High boiling residues, mix by swirling for about 30 seconds, add 2 drops of *methyl orange solution*, insert the stopper in the tube, and shake vigorously; no pink or red color appears in the aqueous layer.

Sulphur. Carefully open the container valve to produce a moderate flow of gas. Do not direct the gas stream toward the face, but deflect a portion of the stream toward the nose, the odour free from the characteristic odour of sulphur compounds.

Assay. Determine by Gas chromatography (2.4.13).

Test solution. Connect 1 Propane cylinder to the chromatograph through a suitable sampling valve and a flow control valve downstream from the sampling valve. Flush the liquid sample through the sampling valve, taking care to avoid entrapment of gas or air in the sampling valve.

Chromatographic system

- an aluminum column 6 m x 3 mm, packed with 10 per cent of liquid phase G30 (Tetraethylene glycol dimethyle ether) on non-acid-washed support S1C (A support prepared from crushed firebrick and calcined or burned with a clay binder above 900° with subsequent acid-wash. It may be silanized),
- a thermal-conductivity detector,
- flow rate: 50 ml per minute using nitrogen as carrier gas.

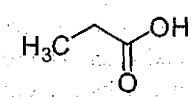
Inject the test solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 1.0.

Inject 2 µl of test solution.

Calculate the percentage purity by dividing 100 times the propane response by the sum of all of the responses in the chromatogram.

Storage. Store protected from moisture and prevent exposure to excessive heat.

Propionic Acid



$C_3H_6O_2$ Mol. Wt. 74.1

Propionic Acid contains not less than 99.5 per cent and not more than 100.5 per cent of $C_3H_6O_2$.

Tests

Weight per ml (2.4.29). 0.988 to 0.993.

Distilling range (2.4.8). 138.5° to 142.5°.

Heavy metals (2.3.13). To the residue obtained in the test for Nonvolatile residue, add 8 ml of *0.1 M hydrochloric acid*, warm gently until solution is complete, dilute with *water* to 100 ml. 12.0 ml of the solution complies with the limit test for heavy metals, Method D (10 ppm).

Limit of nonvolatile residue. Evaporate 20 g in a tared dish, and dry at 105° for 1 hour, the weight of the residue does not exceed 2 mg.

Readily oxidizable substances. Dissolve 15 g of *sodium hydroxide* in 50 ml of *water*, cool, add 6 ml of *bromine*, stirring to effect complete solution, and dilute with *water* to 2000 ml. Transfer 25.0 ml of the solution to a glass-stoppered, 250-ml conical flask containing 100 ml of *water*, and add 10 ml of 20.0 per cent w/v solution *sodium acetate* and 10.0 ml of *Propionic Acid*. Allow to stand for 15 minutes, add 5 ml of 25.0 per cent w/v solution of *potassium iodide* and 10 ml of *hydrochloric acid*, and titrate with *0.1 M sodium thiosulphate* just to the disappearance of the brown color. Carry out a blank titration. The difference between the volume of *0.1 M sodium thiosulphate* required for the blank and that required for the test solution is not more than 2.2 ml.

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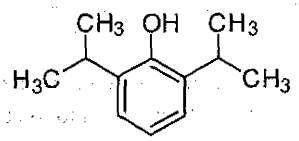
Aldehydes. Transfer 10.0 ml to a glass-stoppered, 250-ml conical flask containing 50 ml of *water* and 10.0 ml of 1.25 per cent w/v solution of *sodium bisulphite*, insert the stopper, and shake vigorously. Allow the mixture to stand for 30 minutes, then titrate with *0.1 M iodine* to the same brownish yellow endpoint obtained with a blank treated with the same quantities of the same reagents. The difference between the volume of *0.1 M iodine* required for the blank and that required for the test solution is not more than 1.75 ml.

Assay. Dissolve 1.5 g in 100 ml of recently boiled and cooled *water* in a 250-ml conical flask, and titrate with *0.5 M sodium hydroxide* using *dilute phenolphthalein solution* as indicator. 1 ml of *0.5 M sodium hydroxide* is equivalent to 0.03704 g of $C_3H_6O_2$.

Storage. Store protected from moisture.

Propofol



$C_{12}H_{18}O$ Mol. Wt. 178.3

Propofol is 2,6-diisopropylphenol.

Propofol contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{12}H_{18}O$.

Category. General anaesthetic.

Description. A colourless or very light yellow, clear liquid.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *propofol IPRS* or with the reference spectrum of propofol.

Tests

Refractive index (2.4.27). 1.5125 to 1.5145.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution (a). Dissolve 1.0 g of the substance under examination in 10 ml of *hexane*.

Test solution (b). Dissolve 0.24 g of the substance under examination in 100 ml of *hexane*.

Reference solution (a). A solution containing 5 mg of the substance under examination and 15 µl containing 15 mg of *2,6-bis(1-methylethyl)benzene-1,4-dione IPRS (propofol impurity J IPRS)* in 50 ml of *hexane*.

Reference solution (b). Dilute 0.1 ml of *propofol for peak identification IPRS* (containing 3,3',5,5'-tetrakis(1-methylethyl)biphenyl-4,4'-diol (propofol impurities E) and 2-(1-methylethoxy)-1,3-bis(1-methylethyl)benzene (propofol impurities G) to 1.0 ml with *hexane*.

Reference solution (c). Dilute 1.0 ml of test solution (a) to 100 ml with *hexane*. Dilute 1.0 ml of the solution to 10 ml with *hexane*.

Reference solution (d). A 0.24 per cent w/v solution of *propofol IPRS* in *hexane*.

Chromatographic system

- a stainless steel column 20 cm x 4.6 mm, packed with silica gel (5 µm),
- mobile phase: a mixture of 1 volume of *anhydrous ethanol*, 7.5 volumes of *acetonitrile* and 990 volumes of *hexane*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 275 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to propofol impurity J and propofol is not less than 4.0. The relative retention times with reference to propofol for propofol impurity G is about 0.5, for oxydibenzene (propofol impurity I) is about 0.6, for 2-(1-methylethenyl)-6-(1-methylethyl)phenol (propofol impurity B) is about 0.7, for 4-hydroxy-3,5-bis(1-methylethyl)benzoic acid (propofol impurity N) is about 2.3, for 2,5-bis(1-methylethyl) phenol (propofol impurity D) is about 2.5 , for 1-methylethyl 4-hydroxy-3,5-bis(1-methylethyl)benzoate (propofol impurity P) is about 2.9, for 2,4-bis(1-methylethyl)phenol (propofol impurity A) is about 3.0, for 2-(1-methylethyl)phenol (propofol impurity C) is about 3.4, for propofol impurity E is about 4.0, for 3-(1-methylethyl)phenol (propofol impurity F) is about 5.8 and for 4-(1-methylethyl)phenol (propofol impurity H) is about 6.4.

Inject reference solution (a), (b), (c) and test solution (a). Run the chromatogram 7 times the retention time of the principal peak. In the chromatogram obtained with test solution (a), the area of peak due to propofol impurity G, multiplied with relative response factor 5.0 is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent); the area of the peak due to propofol impurity E, multiplied with correction factor 0.25 is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.01 per cent). The area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent) and the sum of the areas of all the secondary peaks is not more than 3 times the area of the

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principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent). Ignore any peak other than propofol impurity E with an area less than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.03 per cent).

Impurities J, K, L and O. Determine by gas chromatography (2.4.13).

Test solution. Dissolve 40 mg of the substance under examination in 10 ml of *dichloromethane*.

Reference solution (a). Dilute 1.0 ml of the test solution to 100 ml with *dichloromethane*. Further dilute 1.0 ml of the solution to 10 ml with *dichloromethane*.

Reference solution (b). Dilute 5 µl containing 5 mg of *propofol impurity J IPRS* to 100 ml with *dichloromethane*. Dilute 1.0 ml of the solution to 25 ml with *dichloromethane*.

Reference solution (c). Dissolve 4 mg of *propofol IPRS* in 1.0 ml of reference solution (b).

Chromatographic system
- a fused-silica capillary column 30 m x 0.32 mm, coated with polymethylphenylsiloxane (0.5 µm),
- temperature:
  Column 80° from 0 - 3 minutes, 80°-210° from 3-25 minutes and 210° from 25 - 40 minutes,
  Injection port at 100° and detector at 270°,
- a flame ionisation detector,
- flow rate: 1.7 ml per minute using nitrogen as carrier gas.

Inject 1 µl of reference solution (c). The test is not valid unless the peak- to- valley ratio is not less than 3.0 where H_p is the height above the baseline of the peak due to propofol impurity J and H_v is the height above the baseline of the lowest point of the curve separating this peak from the peak due to propofol. The relative retention time with reference to propofol for 1-(1-methylethoxy)-2-(1-methylethyl)benzene (propofol impurity K) is about 0.76, for 2,2-dimethyl-4-(1-methylethyl)-1,3-benzodioxole (propofol impurity L) is about 0.81 , for propofol impurity J is about 1.01 and for 2-(1-methylethyl)-6-propylphenol (propofol impurity O) is about 1.03.

Inject 1 µl of the test solution, reference solution (a) and (c). In the chromatogram obtained with the test solution the area of the peak due to propofol impurities J, K, L and O, for each impurity, is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Assay. Determine by liquid chromatography (2.4.14) as described under Related substances.

Inject reference solution (d) and test solution (b).

Calculate the content of $C_{12}H_{18}O$.

Storage. Store protected from light under an inert gas.

Propofol Injection

Propofol Injection is a sterile emulsion of Propofol in a suitable base.

Propofol Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of propofol, $C_{12}H_{18}O$.

Usual strength. 10 mg per ml.

Identification

A. Extract a volume of the injection containing 0.1 g of Propofol with three 25 ml quantities of *hexane* and filter the combined extracts using a glass fibre filter (such as Whatman GF/C). Extract the filtrate with two 20 ml quantities of *methanol* (90 per cent) and evaporate the combined extracts under reduced pressure. Dissolve the residue obtained in 2 ml of *ethanol* and evaporate to dryness under reduced pressure. Dry the residue at 50° over *phosphorus pentoxide* for 1 hour. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *propofol IPRS* or with the reference spectrum of propofol.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution (a).

Tests

pH (2.4.24). 6.0 to 8.5.

Propofol quinone and propofol dimer. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of injection containing 80 mg of Propofol in 100 ml of *propan-2-ol*.

Reference solution. A solution containing 0.0008 per cent w/v of *propofol IPRS*, 0.00008 per cent w/v of *2,6-bis(1-methylethyl)-1,4-benzoquinone IPRS* (*propofol impurity J IPRS*) and 0.0002 per cent w/v of *3,3',5,5'-tetrakis (1-methylethyl)biphenyl-4,4'-diol IPRS* (*propofol dimer IPRS*) in *propan-2-ol* containing 6.8 per cent v/v of *water*.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (such as Hypersil ODS),
- mobile phase: a mixture of 40 volumes of *tetrahydrofuran* and 60 volumes of *water*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention time with reference to propofol for propofol impurity J is about 0.8 and for propofol dimer is about 2.5.

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Inject the reference solution and the test solution. Run the chromatogram three times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of the peak due to propofol quinone (propofol impurity J) is not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent) and the area of the peak due to propofol dimer is not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.25 per cent).

Globule size. Not more than 5000 globules more than or equal to 2 µm per ml of a 0.01 per cent v/v dilution in a filtered 0.9 per cent w/v solution of *sodium chloride*. By means of suitable equipment, pass the solution through a 50 µm aperture and count the number of globules by monitoring the change in electrical current between two electrodes immersed on either side of the aperture (such as Coulter Counter). Repeat the procedure using the 0.9 per cent w/v solution of *sodium chloride* only to determine the background count.

Free fatty acid. Not more than 7 millimoles per litre, when determined by the following method.

To 5 ml of a well shaken injection add 25 ml of a mixture of 40 volumes of *propan-2-ol*, 10 volumes of *n-heptane* and 1 volume of *0.5 sulphuric acid* and shake for 1 minute. Allow to stand for 10 minutes, add 15 ml of *n-heptane* and 15 ml of *water*. Mix by inverting the container 10 times and allow to stand for 15 minutes. To 15 ml of the upper phase add 5 ml of *nile blue A solution* and pass a stream of *nitrogen*, previously passed through *0.1 M sodium hydroxide*, through the solution. Titrate with 0.02 M sodium hydroxide, using a microburette. Calculate the content of free fatty acid from a calibration curve prepared from quantities of 1, 2, 4, 6 and 8 ml of a 1.282 per cent w/v solution of *palmitic acid* in *n-heptane*, each diluted to 50 ml with *n- heptane*. Carry out the method described above, using 5 ml of each solution and beginning at the words 'add 25 ml of a mixture'.

Lysolecithin. Not more than 0.2 per cent w/v.

Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of injection containing 0.1 g of Propofol in 100 ml of the mobile phase.

Reference solution. A 0.01 per cent w/v solution of *lysolecithin* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Nucleosil C18),
- mobile phase: a mixture of 1.4 volumes of *orthophosphoric acid*, 40 volumes of *water* and 60 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 250 µl.

Inject the reference solution and the test solution.

Calculate the content of lysolecithin.

Bacterial endotoxins (2.2.3). Not more than 0.33 Endotoxin Unit per mg of propofol.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of injection containing 80 mg of Propofol to 100.0 ml with the *propan-2-ol*.

Reference solution (a). A 0.08 per cent w/v solution of *propofol IPRS* in in *propan-2-ol* containing 6.8 per cent v/v of *water*.

Reference solution (b). A solution containing 0.0008 per cent w/v of *propofol IPRS* and 0.00008 per cent w/v of *propofol impurity J IPRS* in *propan-2-ol* containing 6.8 per cent v/v of *water*.

The chromatographic system described under Propofol quinone and propofol dimer may be used but using a detection wavelength of 275 nm and injecting 20 µl of each solution.

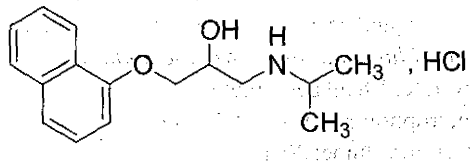
Inject reference solution (b). The test is not valid unless the resolution between the peaks due to propofol and propofol impurity J is not less than 2.5.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{12}H_{18}O$ in the injection.

Storage. Store at a temperature not exceeding 30°. It should not be allowed to freeze.

Propranolol Hydrochloride



$C_{16}H_{21}NO_2,HCl$ Mol. Wt. 295.8

Propranolol Hydrochloride is (*RS*)-1-[(1-methylethyl)amino]-3-(naphthalen-1-yloxy)propan-2-ol hydrochloride.

Propranolol Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{16}H_{21}NO_2,HCl$, calculated on the dried basis.

Category. Antihypertensive, antianginal and antiarrhythmic agent.

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Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *propranolol hydrochloride IPRS* or with the reference spectrum of propranolol hydrochloride.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution in *methanol* shows absorption maxima at about 290 nm, 306 nm and 319 nm.

C. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution in *methanol* is clear (2.4.1), and not more intensely coloured than degree 6 of the appropriate range of reference solution (2.4.1).

pH (2.4.24). 5.0 to 6.0, determined in a 1.0 per cent w/v solution.

Specific optical rotation (2.4.22). – 1.0° to + 1.0°, determined in a 4.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in 10.0 ml of the mobile phase.

Reference solution. Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 1.6 g of *sodium laurylsulphate* and 0.31 g of *tetrabutylammonium dihydrogen phosphate* in a mixture of 1 ml of *sulphuric acid*, 450 ml of *water* and 550 ml of *acetonitrile*, adjusted to pH 3.3 using *dilute sodium hydroxide solution*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 292 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution. Run the chromatogram 7 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (0.1 per cent) and the sum of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (0.4 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.25 g in 25 ml of *ethanol (95 per cent)* and titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25).

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.02958 g of $C_{16}H_{21}NO_2,HCl$.

Storage. Store protected from moisture.

Propranolol Prolonged-release Capsules

Propranolol Hydrochloride Prolonged-release Capsules; Propranolol Hydrochloride Sustained-release Capsules; Propranolol Sustained-release Capsules; Propranolol Hydrochloride Extended-release Capsules; Propranolol Extended-release Capsules.

Propranolol Prolonged-release Capsules manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufactures may not be the same.

Propranolol Prolonged-release Capsules contain Propranolol Hydrochloride.

Propranolol Prolonged-release Capsules contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of propranolol hydrochloride, $C_{16}H_{21}NO_2,HCl$.

Usual strengths. 40 mg; 60 mg and 80 mg.

Identification

A. When examined in the range 220 nm and 380 nm (2.4.7), the solution obtained in the Assay, shows two absorption maximum at 290 nm and 319 nm and a shoulder at 306 nm.

B. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 1 volume of *18 M ammonia* and 99 volumes of *methanol*.

Test solution. Shake a quantity of the powdered capsules containing 20 mg of Propranolol Hydrochloride with 10 ml of *methanol* for 5 minutes and filter.

Reference solution. A 0.2 per cent w/v solution of *propranolol hydrochloride IPRS* in *methanol*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at

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254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2). Complies with the test stated under Capsules.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered capsules containing 0.1 g of Propranolol Hydrochloride with 100 ml of *methanol* sonicate for 15 minutes and filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 500.0 ml with the mobile phase.

Reference solution (b). A 0.1 per cent w/v solution of *propranolol hydrochloride for performance test IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 20 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- mobile phase: a mixture of 1.15 g of *sodium dodecyl sulphate*, 10 ml of a mixture of 1 ml of *sulphuric acid* and 9 ml of *water*, 20 ml of a 1.7 per cent w/v solution of *tetrabutylammonium dihydrogen orthophosphate*, 370 ml of *water* and 600 ml of *acetonitrile*, the mixture adjusted to pH 3.3 with *2M sodium hydroxide*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 292 nm,
- injection volume: 10 µl.

Run the chromatogram 5 times the retention time of the principal peak.

Inject reference solution (b). The test is not valid unless the chromatogram obtained with reference solution (b) baseline separation is achieved between the peaks due to impurity A [(2*RS*)-3-(naphthalene-1-yloxy) propane-1,2-diol] and propranolol.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Weigh a quantity of the mixed contents of 20 capsules containing about 80 mg of Propranolol Hydrochloride, add 150 ml of *methanol*, heat to boiling and boil for 2 minutes, remove from the heat, shake for 20 minutes, cool to 20° and add sufficient *methanol* to produce 200.0 ml. Mix and filter, discard the first 20 ml of filtrate, and dilute 10.0 ml of the filtrate to 200.0 ml with *methanol*. Measure the absorbance of the resulting solution at the maximum at about 290 nm (2.4.7).

Calculate the content of $C_{16}H_{21}NO_2$,HCl taking 206 as the specific absorbance at 290 nm.

Propranolol Injection

Propranolol Hydrochloride Injection

Propranolol Injection is a sterile solution of Propranolol Hydrochloride in Water for Injections containing Citric Acid.

Propranolol Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of propranolol hydrochloride, $C_{16}H_{21}NO_2$,HCl.

Usual strength. 1 mg per ml.

Identification

A. Make alkaline with *1 M sodium hydroxide* a volume containing 10 mg of Propranolol Hydrochloride and extract with three quantities, each of 5 ml, of *ether*. Wash the combined *extracts* with *water* until the washings are free from alkali, dry with *anhydrous sodium sulphate*, filter, evaporate the filtrate to dryness and dry the residue at 50° at a pressure of 2 kPa for 1 hour.

On the residue determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *propranolol hydrochloride IPRS* treated in the same manner or with the reference spectrum of propranolol.

B. When examined in the range 230 nm to 360 nm (2.4.7), the solution obtained in the Assay shows absorption maxima at about 290 nm, 306 nm and 319 nm.

Tests

pH (2.4.24). 3.0 to 3.5.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of injection containing about 100 mg of Propranolol Hydrochloride in 100 ml of *acetonitrile*.

Reference solution. Dilute 1.0 ml of the test solution to 500.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 20 cm x 5 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),

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– mobile phase: a mixture of 1.15 g of *sodium dodecyl sulphate*, 10 ml of a mixture of 1 volume of *sulphuric acid* and 9 volumes of *water*, 20 ml of a 1.7 per cent w/v solution of *tetrabutylammonium dihydrogen orthophosphate*, 370 ml of *water* and 600 ml of *acetonitrile*, adjusted to pH 3.3 with *2 M sodium hydroxide*,
– flow rate: 1.8 ml per minute,
– spectrophotometer set at 292 nm,
– injection volume: 10 µl.

Inject the reference solution and the test solution. Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than four times the area of the principal peak in the chromatogram obtained with the reference solution (0.8 per cent).

Bacterial endotoxins (2.2.3). Not more than 55.6 Endotoxin Units per mg of propranolol hydrochloride.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. To a measured volume containing 2 mg of Propranolol Hydrochloride add sufficient *methanol* to produce 100.0 ml and measure the absorbance of the resulting solution at the maximum at about 290 nm (2.4.7). Calculate the content of $C_{16}H_{21}NO_2$, HCl taking 206 as the specific absorbance at 290 nm.

Storage. Store protected from light, in a single dose containers.

Propranolol Tablets

Propranolol Hydrochloride Tablets

Propranolol Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of propranolol hydrochloride, $C_{16}H_{21}NO_2$,HCl.

Usual strengths. 10 mg; 40 mg.

Identification

A. Suspend a quantity of the powdered tablets containing 0.1 g of Propranolol Hydrochloride in 20 ml of *water*, filter, make the filtrate alkaline with *1 M sodium hydroxide* and extract with three quantities, each of 10 ml, of *ether*. Wash the combined extracts with *water* until the washings are free from alkali, dry with *anhydrous sodium sulphate*, filter, evaporate the filtrate to dryness and dry the residue at 50° at a pressure of 2 kPa for 1 hour.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *propranolol hydrochloride IPRS* treated in the same manner or with the reference spectrum of propranolol.

B. When examined in the range 230 nm to 360 nm (2.4.7), the final solution obtained in the Assay shows absorption maxima at about 290 nm, 306 nm and 319 nm.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Dilute a suitable volume of the filtrate with the same solvent. Measure the absorbance of the resulting solution at the maximum at about 290 nm (2.4.7). Calculate the content of $C_{16}H_{21}NO_2$,HCl in the medium taking 206 as the specific absorbance at 290 nm.

Q. Not less than 75 per cent of the stated amount of $C_{16}H_{21}NO_2$, HCl.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing about 100 mg of Propranolol Hydrochloride in 100.0 ml of *methanol* and filter.

Reference solution. Dilute 1 ml of the test solution to 500 ml with the mobile phase.

Chromatographic system
– a stainless steel column 20 cm x 5 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
– mobile phase: a mixture of 1.15 g of *sodium dodecyl sulphate*, 10 ml of a mixture of 1 volume of *sulphuric acid* and 9 volumes of *water*, 20 ml of a 1.7 per cent w/v solution of *tetrabutylammonium dihydrogen orthophosphate*, 370 ml of *water* and 600 ml of *acetonitrile*, adjusted to pH 3.3 with *2 M sodium hydroxide*,
– flow rate: 1.8 ml per minute,
– spectrophotometer set at 292 nm,
– injection volume: 10 µl.

Inject the reference solution and the test solution. Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than four times the area of the principal peak

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in the chromatogram obtained with the reference solution (0.8 per cent).

Uniformity of content. Complies with the test stated under Tablets.

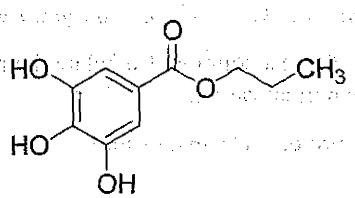
Transfer one tablet to a 100-ml volumetric flask, add 5 ml of *dilute hydrochloric acid* and allow to stand, swirling occasionally, until it is disintegrated. Add about 70 ml of *methanol* and shake well for about 1 minute. Dilute to volume with *methanol*, mix and centrifuge an aliquot of the solution. Dilute a suitable volume of the clear solution with *methanol* to produce a solution containing 20 µg of Propranolol Hydrochloride per ml. Measure the absorbance of the resulting solution at the maximum at about 290 nm (2.4.7), using *methanol* as the blank. Calculate the content of $C_{16}H_{21}NO_2,HCl$ taking 206 as the specific absorbance at 290 nm.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 20 mg of Propranolol Hydrochloride and shake with 20 ml of *water* for 10 minutes. Add 50 ml of *methanol*, shake for a further 10 minutes, add sufficient *methanol* to produce 100.0 ml and filter. Dilute 10.0 ml of the filtrate to 50.0 ml with *methanol* and measure the absorbance of the resulting solution at the maximum at about 290 nm (2.4.7). Calculate the content of $C_{16}H_{21}NO_2,HCl$ taking 206 as the specific absorbance at 290 nm.

Storage. Store protected from light and moisture.

Propyl Gallate



$C_{10}H_{12}O_5$ Mol. Wt. 212.2

Propyl Gallate is propyl 3,4,5-trihydroxybenzoate.

Category. Pharmaceutical aid (antioxidant).

Description. A white or creamy white, crystalline powder.

Identification

A. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *methanol* shows an absorption maximum only at about 275 nm; absorbance at about 275 nm, about 0.49.

B. Dissolve 10 mg in 50 ml of *water*, cool and add 5 ml of *dilute ammonia solution*; a red colour is produced which becomes brown on standing and is restored on shaking.

C. Dissolve 5 mg in 50 ml of *water* and add 0.05 ml of *ferric chloride test solution*; a bluish black colour is produced.

Tests

Chlorides (2.3.12). Shake 1.0 g with 50 ml of *water* for 5 minutes and filter. 25 ml of the filtrate complies with the limit test for chlorides (500 ppm).

Sulphates (2.3.17). Shake 0.8 g with 100 ml of *water* for 5 minutes and filter. 15 ml of the filtrate complies with the limit test for sulphates (0.12 per cent).

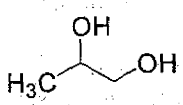
Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Storage. Store protected from light and moisture, in non-metallic containers.

Propylene Glycol

1,2-Propanediol; propane-1,2-diol



$C_3H_8O_2$ Mol. Wt. 76.1

Propylene Glycol is propane-1,2-diol.

Propylene Glycol contains not less than 99.5 per cent of $C_3H_8O_2$.

Category. Pharmaceutical aid (humectant; solvent).

Description. A clear, colourless, viscous liquid; hygroscopic.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *propylene glycol IPRS* or with the reference spectrum of propylene glycol.

B. Diethylene glycol and Ethylene glycol. (see Tests)

C. In the test for Diethylene glycol and Ethylene glycol, the retention time of propylene glycol peak obtained with the test solution corresponds to the propylene glycol peak in the chromatogram obtained with the reference solution.

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Tests

Acidity. Mix 1 ml of *phenolphthalein solution* with 50 ml of *water* and add *0.1 M sodium hydroxide* until the solution is pink for 30 seconds. Add 10 ml of Propylene Glycol and titrate with *0.1M sodium hydroxide* until the original pink colour returns and remains for 30 seconds, not more than 0.2 ml of *0.1 M sodium hydroxide* consumed.

Relative density (2.4.29). 1.035 to 1.037.

Diethylene glycol and Ethylene glycol. Determine by gas chromatography (2.4.13).

Test solution. A solution containing 5.0 per cent w/v of Propylene Glycol and 0.01 per cent w/v of 2,2,2-trichloroethanol (internal standard) in *methanol.*

Reference solution. A solution containing 0.2 per cent w/v of *propylene glycol IPRS*, 0.005 per cent w/v of *ethylene glycol*, 0.005 per cent w/v of *diethylene glycol* and 0.01 per cent w/v of 2,2,2-trichloroethanol (internal standard) in *methanol.*

Chromatographic system

- a capillary column 30 m × 0.53 mm, packed with 6.0 per cent cyanopropylphenyl and 94 per cent dimethylpolysiloxane (film thickness 3.0 µm) (Such as DB-624),
- temperature:
  column. 100° hold for 4 minutes, 100° to 120° @ 50° per minute and hold for 10 minutes, 120° to 220° @ 50° per minute and hold for 6 minutes,
  inlet port at 220° and detector at 250°,
- split ratio: 10:1,
- flame ionization detector,
- flow rate: 4.5 ml per minute, using nitrogen as the carrier gas,
- injection volume: 1µl.

Name	Relative retention time
Ethylene glycol	0.8
Propylene glycol (Retention time about 4 minutes)	1.0
2,2,2-trichloroethanol	1.7
Diethylene glycol	2.4

Inject the reference solution. The test is not valid unless the resolution between the peaks due to ethylene glycol and propylene glycol is not less than 5.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the peak response ratio, each of, ethylene glycol and diethylene glycol relative to 2,2,2-trichloroethanol, is not more than the peak response ratio for ethylene glycol and diethylene glycol relative to 2,2,2-trichloroethanol in the chromatogram obtained with the reference solution (0.1 per cent).

Heavy metals (2.3.13). Dilute 5 ml of Propylene Glycol to 25 ml with *water.* The solution complies with the limit test for heavy metals, Method D (5 ppm), using 10 ml of *lead standard solution (1 ppm Pb).*

Chlorides (2.3.12). 15 ml of 23.8 per cent w/v solution of Propylene Glycol complies with the limit test for chlorides (70 ppm).

Sulphates (2.3.17). 10 ml of 25 per cent w/v solution of Propylene Glycol complies with the limit test for sulphates (60 ppm).

Sulphated ash (2.3.18). Not more than 0.007 per cent w/w, determined by heating 50 g of Propylene Glycol in 100 ml shallow dish until it burns, and ignite.

Water (2.3.43). Not more than 0.2 per cent, determined on 5.0 g.

Assay. Determine by gas chromatography (2.4.13).

Test solution. Propylene Glycol.

Chromatographic system

- a glass column 1 m × 4.0 mm, packed with support (40-60 mesh) high molecular weight tetrafluorethylene polymer coated with polyethylene glycol compound (avg. mol. wt. about 15,000), a high molecular weight compound of polyethylene glycol with a diepoxide linker,
- temperature:
  column. 120° to 200° @ 5° per minute,
  inlet port 240° and detector 250°,
- a thermal-conductivity detector,
- using nitrogen as the carrier gas,

NOTE — Retention time of propylene glycol is about 5.7 minutes, and of three isomers of dipropylene glycol, when present, is about 8.2, 9.0, and 10.2 minutes, respectively.

Inject 10 µl of the test solution. Calculate the percentage of $C_3H_8O_2$ by area normalization.

Storage. Store protected from moisture.

Propylene Glycol Monocaprylate

Propylene Glycol Monooctanoate

Octanoic acid, monoester with 1,2- propane diol; Caprylic acid, monoester with propane-1,2-diol.

Propylene glycol monocaprylate is a mixture of the propylene glycol monoesters and diesters of fatty acids composed predominately of caprylic acid. The requirements for monoesters and diester content differ for the two types of propylene glycol monocaprylate.

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	Content of Monoesters (per cent)	Content of Diesters (per cent)
Type I	not less than 55 and not more than 80	not less than 20 and not more than 45
Type II	not less than 90	not more than 10

Category. Pharmaceutical aid (humectant; solvent).

Description. A clear, colourless, or slightly yellow, oily liquid at 20°.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *propylene glycol monocaprylate IPRS* or with the reference spectrum of propylene glycol *monocaprylate* .

B. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *ether*, 30 volumes of *hexane*.

Test solution. Dissolve 5 g of the substance under examination in 100.0 ml of *methylene chloride* .

Reference solution. A 5 per cent w/v solution of *propylene glycol monocaprylate type-I IPRS* or *propylene glycol monocaprylate type-II IPRS* in *methylene chloride.*

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 15 cm and, dry the plate in a currene of air, spray with a 0.01 per cent w/v solution of *rhodamine 6G* in *ethanol* and examine under ultraviolet light at 365 nm. The test is not valid unless the Rf value of the principal spot obtained with the test solution corresponds to the spot in the chromatogram obtained with reference solution.

C. Complies with the test of Fatty Acid Composition by Gas Chromatography (2.3.50) and exhibits the following composition profile of fatty acids.

Carbon-Chain length	No. of Double bonds	Percentage
8	0	≥ 90.0
10	0	≤ 3.0
12	0	≤ 3.0
14	0	≤ 3.0
16	0	≤ 1.0

Tests

Acid value (2.3.23). Not more than 1.5.

Saponification value (2.3.37). 285 to 315 for type-I and 270 to 295 for type-II.

Iodine value (2.3.28). Not more than 1.0.

Peroxide value (2.3.35). Not more than 6.0.

Limit of Propylene Glycol. Determine by liquid chromatography (2.4.14).

Use the chromatographic system and test solution as described under Assay.

Reference solution (a). A 0.4 per cent w/v solution of *propylene glycol IPRS* in the *tetrahydrofuran.*

Reference solution (b). Take four 5-ml of volumetric flasks , dilute respectively 0.25, 0.5, 1.0 and 2.5 ml of reference solution (a) dilute with *tetrahydrofuran*. In a fifth 5-ml volumetric flask, take 5.0 ml of reference solution (a).

Inject reference solution (b) with defferent strength and the test solution. Prepare a standard curve of peak area versus concentration, in mg per ml, of propylene glycol in reference solution (b). Obtain the concentration of propylene glycol in the test solution from the standard curve.

Calculate the percentage of free propylene glycol in the portion of propylene glycol monocaprylate using the following equation.

$$\frac{c}{c_u} \times 100$$

where, c = concentration of propylene glycol in the sample solution from the standard curve,

c_u = concentration of the sample solution (mg/ml).

Acceptance criteria. Propylene glycol monocaprylate type-I and Propylene glucol monocaprylate type-II not more than 1.5 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on a mixture of equal volume of *methanol* and *methylene chloride.*

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 4.0 g of the substance under examination in mobile phase and dilute to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 60 cm x 7.0 mm, packed with octadecylsilane bonded to styrene-divinylbenzene copolymer in the hydrogen form (5 µm),
- column temperature: 40°,

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- mobile phase: tetrahydrofuran,
- flow rate: 1 ml per minute,
- refractive index detector (Detector cell temperature: 40°)
- injection volume: 40 µl.

The relative retention time with reference to propylene glycol for diesters is about 0.85 and for monoesters is about 0.9.

Inject the test solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent for the monoester peak.

Inject the test solution. Calculate the percentage of monoesters or diesters by area nomalization procedure. In the portion of propylene glycol monocaprylate using following equation.

$$\frac{r_u}{r_t} \times (100 - D)$$

where, r_u = peak response for monoesters or diesters,
r_t = sum of the peak responses of the monoesters and diesters,
D = sum of the per centage content of propylene glycol and the per centage content of free fatty acid.

Inject the test solution. Calculate the percentage of free fatty acids using following equation.

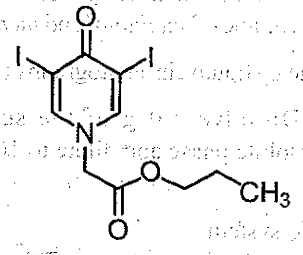
$$D = \frac{A}{561.1} \times 144$$

where, A = acid value.

Storage. Store protected from moisture and keep in well-closed container.

Labelling. The label states that the contents indicate the type (Type-I or Type-II).

Propyliodone



$C_{10}H_{11}I_2NO_3$ Mol. Wt. 447.0

Propyliodone is Propyl 3,5-diiodo-4-oxo-1(4*H*)pyridylacetate.

Propyliodone contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{10}H_{11}I_2NO_3$, calculated on the dried basis.

Description. A white to off-white crystalline powder.

Identification

A. Heat 100 mg with a few drops of *sulphuric acid*; a violet vapours are evolved.

B. Reflux 1 g with 10 ml of *1 M sodium hydroxide* for 30 minutes, add 10 ml of *water*, and acidify to litmus paper with *hydrochloric acid*; the precipitate of *3,5-diiodo-4-oxo-1(4H)-pyridineacetic acid*, after being washed with *water* and dried at 105°, melts at about 245°.

Tests

Melting range (2.4.21). 187° to 190°.

Acidity. Dissolve 1.0 g in 40 ml of hot *n-propyl alcohol* previously neutralised to *phenolphthalein solution*, cool, and allow to stand in an ice bath for 15 minutes with frequent shaking. Filter, wash the residue with neutralised *n-propyl alcohol*, combine the filtrate and washings, add *phenolphthalein solution*, and titrate with *0.05 M sodium hydroxide* to a pink colour that persists for 15 seconds; not more than 0.15 ml of *0.05 M sodium hydroxide* is required for neutralisation.

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Iodine and iodide. Shake 2.4 g with 30 ml of *water* for 15 minutes, filter. To 10 ml of filtrate add 1 ml of *2 M nitric acid*, 1 ml of 0.2 per cent v/v solution of *sodium nitrite solution*, and 2 ml of *chloroform*, shake, and centrifuge. Any purple colour in the chloroform layer is not darker than that obtained with a mixture of 6 ml of *water* and 4 ml of 0.0026 per cent w/v solution of *potassium iodide* treated in the same manner (0.01 per cent of I).

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g. by drying in an oven at 105°.

Assay. Weigh 15 mg in a mixture of 10 ml of 1 per cent w/v solution of *sodium hydroxide* and 1 ml of freshly prepared 1 per cent w/v solution of *sodium bisulphate*. Titrate with *0.02 M sodium thiosulphate*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.02 M sodium thiosulphate* is equivalent to 0.000745 g of $C_{10}H_{11}I_2NO_3$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

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Propyliodone Injectable Oil Suspension

Propyliodone Injectable Oil Suspension is a sterile suspension of Propyliodone in Peanut Oil.

Propyliodone Injectable Oil Suspension contains not less than 57.0 per cent and not more than 63.0 per cent of $C_{10}H_{11}I_2NO_3$.

Identification

Mix 1 g of Injectable Oil Suspension with 20 ml of *hexane*, filter the diluted suspension through a fine-porosity, sintered-glass crucible, and wash the residue free from peanut oil with the *hexane*. The residue complies with the following tests.

A. Heat 100 mg with a few drops of *sulphuric acid*; a violet vapour appears.

B. To 1 g with 10 ml of *1 M sodium hydroxide* for 30 minutes, add 10 ml of *water*, and acidify to litmus paper with *hydrochloric acid*; the precipitate of *3,5-diiodo-4-oxo-1(4H)-pyridineacetic acid*, after being washed with *water* and dried at 105°, melts between 187° to 190°.

Tests

Weight per ml (2.4.29). 1.236 g to 1.276 g.

Iodine and iodide. Disperse 3.3 ml in 125 ml of *alcohol-free chloroform*, add 25 ml of 0.04 per cent w/v solution of *sodium hydroxide*, and shake. Separate the aqueous layer, shake it with 125 ml of *alcohol-free chloroform*, and discard the *chloroform*. To 10 ml of filtrate add 1 ml of *2 M nitric acid*, 1 ml of 0.2 per cent v/v solution of *sodium nitrite solution*, and 2 ml of *chloroform*, shake, and centrifuge. Any purple colour in the chloroform layer is not darker than that obtained with a mixture of 6 ml of *water* and 4 ml of 0.0026 per cent w/v solution of *potassium iodide solution* treated in the same manner (0.01 per cent of I).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

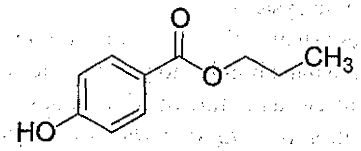
Assay. Transfer the content of 1 container containing 30 mg of Injectable Oil Suspension to a tared combustion capsule and weigh. Dissolve in a mixture of 10 ml of 1 per cent w/v solution of *sodium hydroxide* and 1 ml of freshly prepared 1 per cent w/v solution of *sodium bisulphate*. Titrate with *0.02 M sodium thiosulphate*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.02 M sodium thiosulphate* is equivalent to 0.000745 g of $C_{10}H_{11}I_2NO_3$.

Storage. Store protected from light.

Propylparaben

Propyl Hydroxybenzoate



$C_{10}H_{12}O_3$ Mol. Wt. 180.2

Propylparaben is propyl 4-hydroxybenzoate.

Propylparaben contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{10}H_{12}O_3$, calculated on the dried basis.

Category. Pharmaceutical aid (antimicrobial preservative).

Description. A white or almost white, crystalline powder or colourless crystals.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *propylparaben IPRS* or with the reference spectrum of propylparaben.

B. Melting point (2.4.21). 96° to 99°.

Tests

Appearance of solution. A 10.0 per cent w/v solution in *ethanol (95 per cent)* is clear (2.4.1), and not more intensely coloured than reference solution BYS6 (2.4.1).

Acidity. Dissolve 1.0 g in sufficient *ethanol (95 per cent)* to produce 10 ml. To 2 ml of the solution add 3 ml of *ethanol (95 per cent)*, 5 ml of *carbon dioxide-free water* and 0.1 ml of *bromocresol green solution*. Not more than 0.1 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 2.5 ml of *methanol* and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of *4-hydroxybenzoic acid* (propylparaben impurity A), 5 mg of *ethyl parahydroxybenzoate* (propylparaben impurity C) and 5 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 50 mg of *propylparaben IPRS* in 2.5 ml of *methanol* and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

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Reference solution (c). Dilute 1.0 ml of the test solution to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of 0.68 per cent w/v solution of *potassium dihydrogen phosphate*, and 65 volumes of *methanol*,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 272 nm,
- injection volume: 10 µl.

The relative retention time with reference to propylparaben for 4-hydroxybenzoic acid (propylparaben impurity A) is about 0.3, for ethyl parahydroxybenzoate (propylparaben impurity C) is about 0.7.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to propylparaben and propylparaben impurity C is not less than 3.0.

Inject reference solution (c) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any peak corresponding to propylparaben impurity A multiplied by 1.4 is not more than the area the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent). Ignore the peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

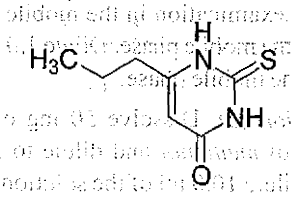
Assay. Determine by liquid chromatography (2.4.14) as described under Related substances.

Inject reference solution (b) and the test solution.

Calculate the content of $C_{10}H_{12}O_3$.

Storage. Store protected from moisture.

Propylthiouracil



$C_7H_{10}N_2OS$ Mol. Wt. 170.2

Propylthiouracil is 2,3-dihydro-6-propyl-2-thioxopyrimidin-4(1*H*)-one.

Propylthiouracil contains not less than 98.0 per cent and not more than 100.5 per cent of $C_7H_{10}N_2OS$, calculated on the dried basis.

Category. Antithyroid.

Description. A white or pale cream-coloured crystals or crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *propylthiouracil IPRS* or with the reference spectrum of propylthiouracil.

B. Examine the chromatograms obtained in the test for Related substances under ultraviolet light at 254 nm before exposure of the plate to iodine vapour. The principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (b).

C. To about 20 mg add 8 ml of *bromine water* and shake for a few minutes. Boil until the mixture is decolorised, allow to cool and filter. Add 2 ml of *barium chloride solution*; a white precipitate is produced. Add 5 ml of *2 M sodium hydroxide*; the precipitate does not become violet.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 100 volumes of *chloroform*, 12 volumes of *2-propanol* and 0.2 volume of *glacial acetic acid*.

Test solution (a). Dissolve 0.1 g of the substance under examination in 10 ml of *methanol*.

Test solution (b). Dissolve 0.1 g of the substance under examination in 100 ml of *methanol*.

Reference solution (a). A 0.01 per cent w/v solution of the substance under examination in *methanol*.

Reference solution (b). A 0.1 per cent w/v solution of *propylthiouracil IPRS* in *methanol*.

Reference solution (c). A 0.0005 per cent w/v solution of *thiourea* in *methanol*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Expose the plate to iodine vapour for 10 minutes. By

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both methods of visualisation, any spot corresponding to thiourea in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (c) and any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (a).

Arsenic (2.3.10). Dissolve 2.0 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (5 ppm).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method C (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in oven at 105°.

Assay. Weigh 0.3 g, add 30 ml of *water* and 30.0 ml (n_1 ml) of *0.1 M sodium hydroxide*, boil and shake until solution is complete. Add 50 ml of *0.1 M silver nitrate* with stirring, boil gently for 5 minutes, cool and titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25) (n_2 ml). Record the total volume ($n_1 + n_2$ ml) of *0.1 M sodium hydroxide* added.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.008511 g of $C_7H_{10}N_2OS$.

Storage. Store protected from light and moisture.

Propylthiouracil Tablets

Propylthiouracil Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of propylthiouracil, $C_7H_{10}N_2OS$.

Usual strength. 50 mg.

Identification

A. Shake a quantity of the powdered tablets containing 50 mg of Propylthiouracil with 20 ml of *methanol* for 10 minutes, filter and evaporate to dryness.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *propylthiouracil IPRS* or with the reference spectrum of propylthiouracil.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium. 900 ml of *water*,

Speed and time. 100 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter and dilute a suitable volume of the filtrate with the same solvent. Measure the absorbance of the resulting solution at the maximum at about 274 nm (2.4.7). Calculate the content of $C_7H_{10}N_2OS$ in the medium from the absorbance obtained from a solution of known concentration of *propylthiouracil IPRS*.

Q. Not less than 85 per cent of the stated amount of $C_7H_{10}N_2OS$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 100 volumes of *chloroform*, 12 volumes of *2-propanol* and 0.2 volume of *glacial acetic acid*.

Test solution. Shake a quantity of the powdered tablets containing 50 mg of Propylthiouracil with 5 ml of *methanol* for 15 minutes, filter and use the filtrate.

Reference solution (a). Dilute 1 volume of the test solution to 100 volumes with *methanol*.

Reference solution (b). A 0.001 per cent w/v solution of thiourea in *methanol*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Expose the plate to iodine vapour for 10 minutes. By both methods of visualisation, any spot corresponding to thiourea in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (b) and any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (a).

Other tests. Comply with the tests stated under Tablets

Assay. Determine by liquid chromatography (2.4.14),

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of Propylthiouracil in 10 ml of *methanol*, add 50 ml of *water*, shake mechanically for about 20 minutes and dilute to 100.0 ml with *water*, filter. Dilute 10.0 ml of the solution to 100.0 ml with *water*.

Reference solution. Dissolve 25 mg of *propylthiouracil IPRS* in 5 ml of *methanol*, add 25 ml of *water*, shake mechanically for about 15 minutes and dilute to 50.0 ml with *water*. Dilute 10.0 ml of the solution to 100.0 ml with *water*.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 80 volumes of a buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen*

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phosphate in 1000 ml of *water*, adjusted to pH 4.6 with *orthophosphoric acid* or *0.1 M sodium hydroxide* and 20 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 272 nm,
- injection volume: 20 µl.

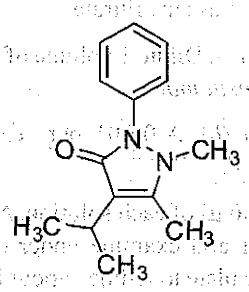
Inject the reference solution. The test is not valid unless the column efficiency is not less than 3500 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_7H_{10}N_2OS$.

Storage. Store protected from light and moisture.

Propyphenazone



$C_{14}H_{18}N_2O$ Mol. Wt. 230.3

Propyphenazone is 4-isopropyl-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one.

Propyphenazone contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{14}H_{18}N_2O$, calculated on the dried basis.

Category. Analgesic; antipyretic.

Description. A white or slightly yellowish, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *propyphenazone IPRS* or with the reference spectrum of propyphenazone.

B. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (b).

C. Dissolve 2 g in sufficient of a mixture of equal volumes of *ethanol (95 per cent)* and *carbon dioxide-free water* to produce 50 ml (solution A). To 1 ml of solution A add 0.1 ml of *ferric chloride solution*; a brownish red colour is produced which becomes yellow on addition of 1 ml of *2 M hydrochloric acid*.

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. To 10 ml of solution A add 0.1 ml of *phenolphthalein solution*; the solution is colourless. Add 0.2 ml of *0.01 M sodium hydroxide*; the solution is pink. Add 0.4 ml of *0.01 M hydrochloric acid*; the solution becomes colourless. Add 0.2 ml of *methyl red solution*; the solution is orange or red.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Mobile phase. A mixture of 45 volumes of *cyclohexane*, 45 volumes of *ethyl acetate* and 10 volumes of *1-butanol*.

Test solution (a). An 8 per cent w/v solution of the substance under examination in *methanol*.

Test solution (b). A 1.6 per cent w/v solution of the substance under examination in *methanol*.

Reference solution (a). A 0.016 per cent w/v solution of the substance under examination in *methanol*.

Reference solution (b). A 1.6 per cent w/v solution of *propyphenazone IPRS* in *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in a current of hot air for 15 minutes and examine under ultraviolet light at 254 nm. Spray the plate with a mixture of equal volumes of *potassium ferricyanide solution* and *ferric chloride solution*. By both methods of visualisation, any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a).

Arsenic (2.3.10). Mix 1.0 g with 10 ml of a 2 per cent w/v solution of *magnesium nitrate* in *ethanol* in a silica or platinum dish, evaporate on a water-bath and heat gradually in order to incinerate. If the material remains incompletely carbonised, moisten with a small quantity of *nitric acid* and ignite again. Cool, add 3 ml of *hydrochloric acid* and heat on a water-bath to dissolve the residue. The resulting solution complies with the limit test for arsenic (10 ppm).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

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Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 60° over *phosphorus pentoxide* at a pressure of 1.5 to 2.5 kPa for 4 hours.

Assay. Weigh 0.2 g of the dried material, dissolve in 30 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02303 g of $C_{14}H_{18}N_2O$.

Storage. Store protected from moisture.

Protamine Sulphate

Protamine Sulphate is a purified mixture of the sulphates of basic peptides prepared from the sperm or mature testes of suitable species of fish. It binds with heparin in solution, inhibiting its anticoagulant activity. It is prepared in conditions designed to minimise the degree of Microbial contamination.

Each mg of Protamine Sulphate precipitates not less than 100 Units of *heparin sodium IPRS*, calculated on the dried basis.

Category. Heparin antidote.

Description. A white or almost white powder; hygroscopic.

Identification

A. Produces a precipitate under the conditions of the Assay.

B. Dissolve 0.2 g in 5 ml of *water* and dilute to 10 ml with the same solvent (solution A). To 0.5 ml of solution A add 4.5 ml of *water*, 1 ml of a 10 per cent w/v solution of *sodium hydroxide* and 1 ml of a 0.02 per cent w/v solution of *1-naphthol* and mix. Cool to 5° and add 0.5 ml of *alkaline sodium hypobromite solution*; an intense red colour is produced.

C. Heat 2 ml of solution A on a water-bath at 60°, add 0.1 ml of *mercuric sulphate solution* and mix; no precipitate is produced. Cool the mixture in ice; a white precipitate is produced.

D. It gives reaction (A) of sulphates (2.3.1).

Tests

Appearance of solution. To 2.5 ml of solution A add 7.5 ml of *water*. The resulting solution is not more opalescent than opalescence standard OS2 (2.4.1), and not more intensely coloured than reference solution BYS6 (2.4.1).

Specific optical rotation (2.4.22). −85.0° to −65.0°, determined at 20° in a 1.0 per cent w/v solution in *0.1 M hydrochloric acid*.

Light absorption (2.4.7). Dilute 2.5 ml of solution A to 5.0 ml with *water*. Absorbance of the resulting solution at 260 to 280 nm is not more than 0.1.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Iron (2.3.14). Dissolve 2.0 g in *water* with the aid of heat and dilute to 20 ml with *water*. The resulting solution complies with the limit test for iron (20 ppm).

Mercury. Add 20 ml of a mixture of equal volumes of *nitric acid* and *sulphuric acid* to 2.0 g in a 250-ml flask fitted with a ground-glass stopper, boil under a reflux condenser for 1 hour, cool and carefully dilute with *water*. Boil until nitrous fumes are no longer evolved. Cool, carefully dilute the solution to 200 ml with *water*, mix and filter. Transfer 50 ml of the filtrate to a separating funnel. Shake with successive small quantities of *chloroform* until the *chloroform* layer remains colourless. To the aqueous layer add 25 ml of *1 M sulphuric acid*, 115 ml of *water* and 10 ml of a 20 per cent w/v solution of *hydroxylamine hydrochloride*. Titrate with *dithizone solution*; after each addition, shake the mixture 20 times and towards the end-point of the titration allow to separate and discard the chloroform layer. Titrate until a greenish blue colour is produced. Calculate the content of mercury using the equivalent of mercury in µg per ml of titrant determined in the standardisation of the dithizone solution (10 ppm).

Nitrogen (2.3.30). 21.0 to 26.0 per cent, calculated on the dried basis, determined by Method C.

Sulphates. 16 to 24 per cent, determined by the following method. Dissolve 0.15 g in 15 ml of *water* in a beaker, add 5 ml of *2 M hydrochloric acid* and heat to boiling. Slowly add to the boiling solution 10 ml of *barium chloride solution*. Cover and heat on a *water*-bath for 1 hour. Filter and wash the precipitate several times with small quantities of hot *water*. Dry and ignite the residue to constant weight at 600°.

1 g of the residue is equivalent to 0.4117 g of SO_4.

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Prepare solutions of the substance under examination in *water* containing (1) 0.015 per cent w/v (2) 0.01 per cent w/v and (3) 0.005 per cent w/v. Titrate each of the solutions in duplicate with a 174 IU per ml or a suitable dilution of *heparin sodium IPRS* using the following procedure. Introduce a measured volume of the solution to be titrated, for example 1.5 ml, into the cell of a suitable spectrophotometer, set the instrument at a suitable wavelength (none is critical) in the visible range and add the titrant in small volumes until there is a sharp increase in the absorbance. Note the volume of titrant added.

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Carry out three independent assays. For each individual titration, calculate the number of Units of heparin titrated per mg of the substance under examination. Calculate the result of the assay as the average of the 18 values. Test the linearity of the response by standard statistical methods. The assay is not valid unless the standard deviations calculated for the results obtained with each test solution are less than 5 per cent of the average result.

Protamine Sulphate intended for use in the manufacture of Parenteral Preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 7.0 Endotoxin Units per mg of protamine sulphate.

Protamine Sulphate intended for use in the manufacture of Parenteral Preparations without a further appropriate sterilisation procedure complies with the following additional requirement.

Sterility. Complies with the test for sterility (2.2.11).

Storage. Store protected from moisture. If it is intended for use in the manufacture of Parenteral Preparations, the container should be sterile and sealed so as to exclude micro-organisms.

Labelling. The label states whether or not the contents are intended for use in the manufacture of Parenteral Preparations.

Protamine Sulphate Injection

Protamine Sulphate Injection is a sterile solution of Protamine Sulphate in Water for Injections.

Protamine Sulphate Injection contains not less than 80.0 per cent of the stated amount of protamine sulphate.

Usual strengths. 50 mg in 5 ml; 100 mg in 10 ml.

Identification

A. Produces a precipitate under the conditions of the Assay.

B. Dilute a suitable volume with *water* to give a solution containing 0.2 per cent w/v solution of Protamine Sulphate. To 5 ml of the solution add 1 ml of a 10 per cent w/v solution of *sodium hydroxide* and 1 ml of a 0.02 per cent w/v solution of *1-naphthol* and mix. Cool to 5° and add 0.5 ml of *alkaline sodium hypobromite solution*; an intense red colour is produced.

C. Heat 2 ml on a water-bath at 60°, add 0.1 ml of *mercuric sulphate solution* and mix; no precipitate is produced. Cool the mixture in ice; a white precipitate is produced.

D. It gives reaction (A) of sulphates (2.3.1).

Tests

pH (2.4.24). 2.5 to 3.5.

Optical rotation (2.4.22). – 0.68° to – 0.52°, determined in a solution prepared by diluting the injection with *0.5 M hydrochloric acid* so as to contain 0.8 per cent w/v of Protamine Sulphate.

Light absorption. Dilute the injection, if necessary, with *water* to produce a solution containing 1 per cent w/v solution of Protamine Sulphate. Absorbance of the resulting solution at 260 to 280 nm, not more than 0.1 (2.4.7).

Bacterial endotoxins (2.2.3). Not more than 7.0 Endotoxin Units per mg of protamine sulphate.

Abnormal toxicity. Complies with the test for abnormal toxicity (2.2.1), using a volume containing 0.5 mg of Protamine Sulphate.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Prepare solutions of the substance under examination in *water* containing (1) 0.015 per cent w/v (2) 0.01 per cent w/v and (3) 0.005 per cent w/v. Titrate each of the solutions in duplicate with a 174 IU per ml or a suitable dilution of *heparin sodium IPRS* using the following procedure. Introduce a measured volume of the solution to be titrated, for example 1.5 ml, into the cell of a suitable spectrophotometer, set the instrument at a suitable wavelength (none is critical) in the visible range and add the titrant in small volumes until there is a sharp increase in the absorbance. Note the volume of titrant added.

Carry out three independent assays. For each individual titration, calculate the number of Units of heparin titrated per mg of the substance under examination. Calculate the result of the assay as the average of the 18 values. Test the linearity of the response by standard statistical methods. The assay is not valid unless the standard deviations calculated for the results obtained with each test solution are less than 5 per cent of the average result.

Storage. Store protected from light, in single dose containers.

Labelling. The label states (1) that the dose is calculated from the results of determinations of the amount required to produce an acceptable blood-clotting time in the patient; (2) the approximate number of Units of heparin activity 1 ml is capable of neutralising.

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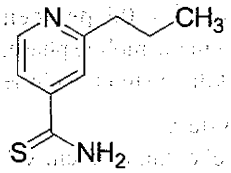
Prothionamide



$C_9H_{12}N_2S$ Mol. wt. 180.3

Prothionamide is 2-propyl-4-pyridinecarbothioamide.

Prothionamide contains not less than 99.0 per cent and not more than 101.0 per cent of $C_9H_{12}N_2S$, calculated on the dried basis.

Category. Antitubercular.

Description. A yellow, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prothionamide IPRS* or with the reference spectrum of prothionamide.

B. When examined in the range 230 nm to 350 nm (2.4.7), a 0.002 per cent w/v solution in *ethanol (95 per cent)* shows an absorption maximum only at about 291 nm. The absorbance at 291 nm is about 0.78.

Tests

Acidity. Dissolve 2.0 g in 20 ml of *methanol*, heating to about 50°, and add 20 ml of *water*. Cool slightly, shake until crystallisation occurs, if any and allow to cool to room temperature. Add 60 ml of *water* and titrate with *0.1 M sodium hydroxide* using 0.2 ml of *cresol red solution* as indicator. Not more than 0.2 ml of *0.1 M sodium hydroxide* is required to change the colour of the indicator to red.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 100 ml with the mobile phase.

Reference solution. A 0.025 per cent w/v solution of *prothionamide IPRS* in the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: 60 volumes of a buffer solution prepared by mixing 2.0 ml of *triethylamine* with 1000 ml of *water*, adjusted to pH 6.0 with *dilute orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 290 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any individual impurity is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent).

Heavy Metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14) as described under Related substances using the following solutions.

Test solution. Dissolve 50.0 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 5.0 ml of the solution to a 50.0 ml with the mobile phase.

Reference solution. A 0.05 per cent w/v solution of *prothionamide IPRS* in the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency is not less than 5000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_9H_{12}N_2S$.

Storage. Store protected from light and moisture.

Prothionamide Tablets

Prothionamide Tablets contain not less than 90.0 per cent not more than 110.0 per cent of the stated amount of prothionamide, $C_9H_{12}N_2S$.

Usual strength. 250 mg.

Identification

A. Extract a quantity of the powdered tablets containing 25 mg of Prothionamide with 5 ml of *methanol*, filter and

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evaporate the filtrate to dryness. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *prothionamide IPRS* or with the reference spectrum of prothionamide.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium. 900 ml *0.1 M hydrochloric acid,*

Speed and time. 100 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtered solution, suitably diluted with the dissolution medium if necessary, at the maximum at about 290 nm (2.4.7). Calculate the content of $C_9H_{12}N_2S$ in the medium from the absorbance obtained from a solution of known concentration of *prothionamide IPRS* in the same medium.

Q. Not less than 75 per cent of the stated amount of $C_9H_{12}N_2S$.

Related substances. Determine by liquid chromatography (2.4.14) as described under Assay using the following solutions.

Test solution. Weigh a quantity of the powdered tablets containing 50 mg of Prothionamide disperse in the mobile phase, shake, dilute to 100.0 ml with the mobile phase and filter.

Reference solution. A solution containing 0.025 per cent w/v of *prothionamide IPRS* in the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution the area of any individual impurity peak is not more than the area of the peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all such impurities is not more than twice the area of the peak in the chromatogram obtained with the reference solution (1.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 50 mg of Prothionamide, disperse in the mobile phase, shake and dilute to 100.0 ml with the mobile phase. Dilute 5.0 ml of the resulting solution to 50.0 ml with the mobile phase.

Reference solution. A 0.05 per cent w/v solution of *prothionamide IPRS* in the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by mixing 2.0 ml of *triethylamine* with 1000 ml of *water* and adjusted to pH 6.0 with *dilute orthophosphoric acid*, and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 290 nm,
- injection volume: 20 µl.

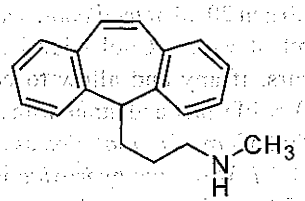
Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 the column efficiency is not less than 5000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_9H_{12}N_2S$ in the tablets.

Storage. Store protected from light and moisture.

Protriptyline Hydrochloride



$C_{19}H_{21}N,HCl$ Mol. Wt. 299.8

Protriptyline Hydrochloride is 3-(5*H*-dibenzo[*a*,*d*]cycloheptan-5-yl)propyl(methyl)amine hydrochloride.

Protriptyline Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{19}H_{21}N,HCl$, calculated on the dried basis.

Category. Tricyclic antidepressant.

Description. A white to yellowish white powder.

Identification

A. Dissolve 0.1 g in 10 ml of *water*, make alkaline with *1M sodium hydroxide*, extract with 5 ml of *chloroform*, dry with *anhydrous sodium sulphate* and evaporate the solvent using a current of nitrogen. On the oily residue, determine by the

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infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *protriptyline hydrochloride IPRS* treated in the same manner or with the reference spectrum of protriptyline.

B. It gives reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 5.0 to 6.5, determined in a 1.0 per cent w/v solution.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 60° at a pressure not exceeding 0.7 kPa.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Dissolve 0.7 g in 0.5 ml of *acetic anhydride* and 20 ml of *anhydrous glacial acetic acid*, add 10 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1M perchloric acid* is equivalent to 0.02998 g of $C_{19}H_{21}N,HCl$.

Protriptyline Tablets

Protriptyline Hydrochloride Tablets

Protriptyline Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of protriptyline hydrochloride, $C_{19}H_{21}N,HCl$.

Usual strength. 5 mg.

Identification

A. When examined in the range 230 to 350 nm (2.4.7), a solution obtained in the Assay exhibits a maximum only at 292 nm.

B. Shake a quantity of the powdered tablets containing about 5 mg of Protriptyline Hydrochloride with 20 ml of *methanol* and filter. To 1 ml of the filtrate add 1 ml of a 2.5 per cent w/v solution of *sodium hydrogen carbonate*, 1 ml of a 2 per cent w/v solution of *sodium periodate* and 1 ml of a 0.3 per cent w/v solution of *potassium permanganate*. Allow to stand for 15 minutes, acidify with *1 M sulphuric acid* and extract with 10 ml of *2,2,4-trimethylpentane*, washing the extract with 10 ml of *0.25 M sulphuric acid*. The absorbance of trimethylpentane solution does not exhibit a maxima at 265 nm (2.4.7) (distinction from amitriptyline and nortriptyline).

C. Triturate a quantity of the powdered tablets containing about 0.1 g of Protriptyline Hydrochloride with 10 ml of *chloroform*, filter and evaporate the filtrate to dryness. Dissolve part of the residue in 3 ml of *water* and add 0.05 ml of a 2.5 per cent w/v solution of *quinhydrone* in *methanol*. A red colour develops.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of *water*,
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the medium, if necessary, at the maxima at about 290 nm (2.4.7). Calculate the content of $C_{19}H_{21}N,HCl$ in the medium from the absorbance obtained from a solution of known concentration of *protriptyline hydrochloride IPRS* in dissolution medium.

Q. Not less than 75 per cent of the stated amount of $C_{19}H_{21}N,HCl$.

Uniformity of content. Disperse one tablet in 50 ml of a solution prepared by mixing 1 volume of *1 M hydrochloric acid* and 9 volumes of *methanol* and dilute to 100.0 ml with the same solution. Shake well and filter, discard the first few ml of filtrate and dilute a volume of the filtrate containing 1 mg of protriptyline hydrochloride to 100 ml with the same solution and measure the absorbance at the maximum at 292 nm (2.4.7). Calculate the content of $C_{19}H_{21}N,HCl$ taking 465 as the specific absorbance at 292 nm.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14),

Buffer solution. Dissolve 11 g of *monobasic sodium phosphate* and 1.9 g of *sodium 1-hexanesulphonate*, in 1000 ml of *water*, adjusted to pH 2.9 with *orthophosphoric acid*.

Solvent mixture. Dilute 200 ml of *ethanol* and 40 ml of *2.5 M hydrochloric acid* and dilute to 1000 ml with *water*.

Test solution. Disperse 5 intact tablets in the solvent mixture and dilute to obtain a solution containing 0.02 per cent w/v protriptyline hydrochloride in the solvent mixture.

Reference solution. A 0.02 per cent w/v solution of *protriptyline hydrochloride IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature:30°
- mobile phase: A. a mixture of 83 volumes of the buffer solution and 17 volumes of *acetonitrile*,
  B. a mixture of 50 volumes of the buffer solution and 50 volumes of *acetonitrile* ,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

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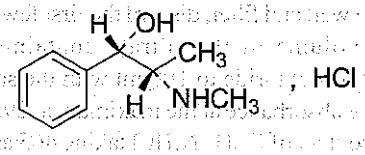
Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
15	0	100
20	0	00
25	100	0
30	100	0

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{21}N,HCl$ in the tablets.

Pseudoephedrine Hydrochloride



$C_{10}H_{15}NO,HCl$ Mol. Wt. 201.7

Pseudoephedrine Hydrochloride is (1*S*,2*S*)-2-methylamino-1-phenylpropan-1-ol hydrochloride.

Pseudoephedrine Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{10}H_{15}NO$, HCl, calculated on the dried basis.

Category. Sympathomimetic.

Description. A white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pseudoephedrine hydrochloride IPRS* or with the reference spectrum of pseudoephedrine hydrochloride.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.1 per cent w/v solution shows absorption maxima at about 251 nm, 257 nm and 263 nm; absorbances at the maxima, about 0.75, about 0.98 and about 0.78, respectively.

C. A 5 per cent w/v solution gives the reactions of chlorides (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution is not more than very faintly opalescent and colourless (2.4.1).

pH (2.4.24). 4.6 to 6.0, determined in a 5.0 per cent w/v solution.

Specific optical rotation (2.4.22). +61.0° to +62.5°, determined in a 5.0 per cent w/v solution using a 2-dm tube.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 50 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 20 mg of *ephedrine hydrochloride IPRS* (*pseudoephedrine impurity A IPRS*) in 20.0 ml of the mobile phase. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (c). Dissolve 10 mg of *ephedrine hydrochloride IPRS* (*pseudoephedrine impurity A IPRS*) in 5 ml of the test solution and dilute to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with phenylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 6 volumes of *methanol* and 94 volumes of 1.2 per cent w/v solution of *ammonium acetate*, adjusted to pH 4.0 with *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 257 nm,
- injection volume: 20 µl.

Inject reference solution (a), (b), (c) and the test solution. Run the chromatogram 1.5 times the retention time of pseudoephedrine. The relative retention time with reference to pseudoephedrine for pseudoephedrine impurity A is about 0.89. The test is not valid unless the resolution between the peaks due to pseudoephedrine impurity A and pseudoephedrine is not less than 2.0. In the chromatogram obtained with the test solution the area of the peak corresponding to pseudoephedrine impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of all the secondary peaks other than pseudoephedrine impurity A is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

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Assay. Dissolve 0.5 g in a mixture of 50 ml of *anhydrous glacial acetic acid* and 10 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02017 g of $C_{10}H_{15}NO$, HCl.

Storage. Store protected from light and moisture.

Pseudoephedrine Syrup

Pseudoephedrine Hydrochloride Syrup

Pseudoephedrine Syrup is a solution of Pseudoephedrine Hydrochloride in a suitable flavoured vehicle.

Pseudoephedrine Syrup contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pseudoephedrine hydrochloride, $C_{10}H_{15}NO$, HCl.

Usual strength. 30 mg per 5 ml.

Identification

A. Shake a quantity of the syrup containing 120 mg of Pseudoephedrine Hydrochloride with two quantities, each of 30 ml, of *ether*, and discard the ether layer. Add 4 ml of *1 M sodium hydroxide* to the aqueous layer and extract with two quantities, each of 10 ml, of *ether*. Dry the combined ether extracts with *anhydrous sodium sulphate*, filter and evaporate the filtrate to dryness.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pseudoephedrine hydrochloride IPRS* treated in the same manner or with the reference spectrum of pseudoephedrine.

B. The residue obtained in test A melts at about 118° (2.4.21).

C. Dissolve 50 mg of the residue obtained in test A in 10 ml of *0.1 M hydrochloric acid*; it is dextro-rotatory.

Tests

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. To a measured volume of the syrup containing about 0.12 g of Pseudoephedrine Hydrochloride, add 50 ml of *0.1 M hydrochloric acid* mix, add sufficient *0.1 M hydrochloric acid* to produce 100.0 ml, filter and use the filtrate.

Reference solution. A 0.12 per cent w/v solution of *pseudoephedrine hydrochloride IPRS* in *0.1 M hydrochloric acid*.

Chromatographic system
- a stainless steel column 25 cm x 4.2 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 85 volumes of *ethanol* and 15 volumes of a 0.4 per cent w/v solution of *ammonium acetate*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution and record the chromatogram. The test is not valid unless the relative standard deviation is not more than 2.0 per cent and the tailing factor is not more than 1.5.

Inject the reference solution and the test solution.

Storage. Store protected from light and moisture.

Pseudoephedrine Tablets

Pseudoephedrine Hydrochloride Tablets

Pseudoephedrine Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of pseudoephedrine hydrochloride, $C_{10}H_{15}NO$, HCl.

Usual strength. 60 mg.

Identification

A. Shake a quantity of the powdered tablets containing 60 mg of Pseudoephedrine Hydrochloride with 10 ml of *water* and filter. Shake the filtrate with 10 ml of *ether*, discard the ether layer. Add 1 ml of *1 M sodium hydroxide* to the aqueous layer and extract with two quantities, each of 10 ml, of *ether*. Dry the combined ether extracts with *anhydrous sodium sulphate*, filter and evaporate the filtrate to dryness.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pseudoephedrine hydrochloride IPRS* treated in the same manner or with the reference spectrum of pseudoephedrine.

B. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (b).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

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Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *pseudoephedrine hydrochloride IPRS* in the dissolution medium to obtain a solution having a known concentration similar to the test solution.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with porous silica (5 µm),
- mobile phase: a mixture of 3 volumes of 0.4 per cent w/v solution of *ammonium acetate* and 17 volumes of *ethanol*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{15}NO,HCl$, in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{10}H_{15}NO,HCl$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G.*

Mobile phase. A mixture of 40 volumes of *butyl acetate*, 20 volumes of *acetone*, 20 volumes of *1-butanol*, 10 volumes of *5 M ammonia* and 10 volumes of *methanol.*

Test solution (a). Add 25 ml of *methanol* to a quantity of the powdered tablets containing 0.5 g of Pseudoephedrine Hydrochloride, shake for 5 minutes, filter, wash the filter with *methanol* and evaporate the combined filtrate and washings to dryness. Dissolve the residue as completely as possible in 5 ml of *methanol*, centrifuge and use the supernatant liquid.

Test solution (b). Dilute 1 volume of the test solution to 10 volumes with *methanol.*

Reference solution (a). Dilute 1 volume of the test solution to 100 volumes with *methanol.*

Reference solution (b). A 1.0 per cent w/v solution of *pseudoephedrine hydrochloride IPRS* in *methanol.*

Apply to the plate 10 µl of each solution. After development, dry the plate in a current of warm air, spray with a solution containing 0.3 g of *ninhydrin* in a mixture of 100 ml *1-butanol* and 3 ml of *glacial acetic acid* and heat at 120° for 20 minutes. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a). Ignore any yellow spot near the line of application.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. To a quantity of the powdered tablets containing about 0.12 g of Pseudoephedrine Hydrochloride, add 50 ml of *0.1 M hydrochloric acid* mix with the aid of ultrasound for 15 minutes, add sufficient *methanol* to produce 100.0 ml, filter and use the filtrate.

Reference solution. A 0.12 per cent w/v solution of *pseudoephedrine hydrochloride IPRS* in *methanol (50 per cent).*

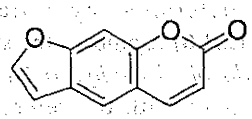
Chromatographic system
- a stainless steel column 20 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: *0.005 M dioctyl sodium sulphosuccinate* in a mixture of 65 volumes of *methanol*, 35 volumes of *water* and 1 volume of *glacial acetic acid,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 258 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{15}NO,HCl$ in the tablets.

Storage. Store protected from light and moisture.

Psoralen



$C_{11}H_6O_3$ Mol. Wt. 186.2

Psoralen is 7*H*-furo[3,2-*g*][1]benzopyran-7-one, obtained from the fruits of *Psoralea carylifolia* Linn. (Fam. Legumi-nosae) and from the leaves of *Ficus carica* (Fam. Urticaceae) or prepared by synthesis.

Psoralen contains not less than 95.0 per cent and not more than 101.0 per cent of $C_{11}H_6O_3$, calculated on the dried basis.

Category. Topical pigmenting agent.

Description. Colourless needles.

Identification

A. Dissolve 1 mg in 5 ml of *ethanol (95 per cent)* and add 15 ml of a mixture containing 43 volumes of *water*, 5 volumes of *acetic acid* and 3 volumes of *propylene glycol*; a blue fluorescence is visible under ultraviolet light at 365 nm.

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B. Dissolve 1 mg in 2 ml of *ethanol (95 per cent)* and add 0.1 ml of *0.1 M sodium hydroxide*; a yellow fluorescence is visible under ultraviolet light at 365 nm.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 90 volumes of *benzene* and 10 volumes of *ethyl acetate*.

Test solution. Dissolve 0.2 g of the substance under examination in 10 ml of *chloroform*.

Reference solution. Dilute 1 volume of the test solution to 100 volumes with *chloroform*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 365 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

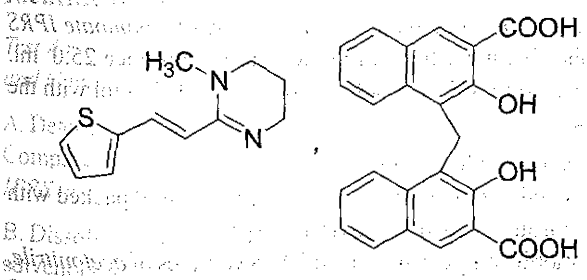
Assay. Dissolve 0.1 g in sufficient *methanol* to produce 100.0 ml. Dilute 2.0 ml of the solution to 100.0 ml with *methanol*. Further dilute 1.0 ml of the solution to 10.0 ml with *methanol* and measure the absorbance of the resulting solution at the maximum at about 247 nm (2.4.7).

Calculate the content of $C_{11}H_6O_3$ from the absorbance obtained by repeating the operation using a final solution of 2 µg per ml of *psoralen IPRS* in *methanol* in place of the substance under examination.

Storage. Store protected from light and moisture.

Pyrantel Pamoate

Pyrantel Embonate



$C_{11}H_{14}N_2S, C_{23}H_{16}O_6$ Mol. Wt. 594.7

Pyrantel Pamoate is 1,4,5,6-tetrahydro-1-methyl-2-[(*E*)-2-(2-thienyl)vinyl]pyrimidine hydrogen; 4,4′-methylene bis(3-hydroxynaphthalene-2-carboxylate).

Pyrantel Pamoate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{34}H_{30}N_2O_6S$, calculated on the dried basis.

Category. Anthelmintic.

Description. A pale yellow or yellow powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pyrantel pamoate IPRS* or with the reference spectrum of pyrantel pamoate.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE— Prepare the solutions immediately before use and protected from light.

Solvent mixture. Mix 25 volumes of *glacial acetic acid*, 25 volumes of *water* and 10 volumes of *diethylamine* with cooling.

Test solution. Dissolve 80 mg of the substance under examination in 7 ml of the solvent mixture and dilute to 100.0 ml with mobile phase.

Reference solution (a). Dissolve 10.0 mg of *1-methyl-2-[(Z)-2-(thiophen-2-yl)-1,4,5,6-tetrahydropyrimidine IPRS* (*pyrantel impurity A IPRS*) in the solvent mixture, add 2.5 ml of the test solution and dilute to 50.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with silica gel (5 µm),
- mobile phase: 7.2 volumes of the solvent mixture and 92.8 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 288 nm,
- injection volume: 20 µl.

The relative retention times with reference to pyrantel pamoate are about 0.5 for pamoic acid, about 1.3 for pyrantel impurity A and about 1.8 for ((*E*)-*N*-{methylamino)propyl]-3-(thiophen-2-yl)prop-2-enamide) pyrantel impurity B.

Inject reference solution (a). Run the chromatogram for 4 times the retention time of pyrantel. The test is not valid unless the resolution between the peaks due to pyrantel and pyrantel impurity A is at least 4.0.

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Inject reference solution (a), (b) and the test solution. For the calculation of content, multiply the peak area of pyrantel impurity B by 0.4. In the chromatogram obtained with the test solution, the area of the peak due to pyrantel impurity A is not more than the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the area of the peak due to pyrantel impurity B is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent); the area any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with the reference solution (b) (0.1 per cent) and the sum of the areas of all the secondary peaks other than those of pyrantel impurities A and B is not more than 0.6 times the area of the principal peak in the chromatogram obtained with the reference solution (b) (0.3 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Chlorides (2.3.12). To 0.7 g, add 10 ml of *dilute nitric acid* and 30 ml of *water*. Heat on water-bath for 5 minutes and cool. The resulting solution complies with the limit test for chlorides (360 ppm).

Sulphates (2.3.17). To 0.5 g, add 2.5 ml of *dilute nitric acid* and dilute to 50 ml with *water*. Heat on water bath for 5 minutes. 15 ml complies with the limit test for sulphates (0.1 per cent).

Iron (2.3.14). Ignite 0.54 g at 800° for 2 hours. Dissolve the residue in 2.5 ml of *dilute hydrochloric acid* with gentle heating for 10 minutes and cool. The resulting solution complies with the limit test for iron (75 ppm).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in vacuum at 60° for 3 hours.

Assay. Weigh 0.5 g, add 10 ml of *acetic anhydride* and 50 ml *glacial acetic acid*. Heat at 50° and stir for 10 minutes. Allow to cool (a clear solution is not obtained). Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.05947 g of $C_{34}H_{30}N_2O_6S$.

Storage. Store protected from light.

Pyrantel Pamoate Oral Suspension

Pyrantel Pamoate Oral Suspension is a suspension of Pyrantel Pamoate in a suitable flavoured aqueous vehicle.

Pyrantel Pamoate Oral Suspension contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pyrantel, $C_{11}H_{14}N_2S$.

Usual strength. 50 mg per ml

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H*.

Mobile phase. The upper layer of a mixture obtained by shaking 20 volumes of *methyl isobutyl ketone*, 10 volumes of *formic acid* and 10 volumes of *water*.

Test solution. Dilute a known volume of the oral suspension with sufficient *0.05 M methanolic ammonia* to produce a solution containing about 8 mg of pyrantel per ml. Shake the mixture by mechanical means and centrifuge. Use the clear supernatant liquid.

Reference solution. A solution of *pyrantel pamoate IPRS* in *0.05 M methanolic ammonia* containing 0.008 per cent w/v of *pyrantel*.

Apply to the plate 100 µl of each solution. Allow the mobile phase to rise to about 17 cm. Dry the plate in air and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. In the Assay, the major peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.5 to 6.0

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Determine by liquid chromatography (2.4.14).

NOTE— Carry out the test protected from light.

Test solution. Weigh a quantity of the oral suspension containing about 60 mg of pyrantel, disperse in 60 ml of *water* and add sufficient *water* to produce 100.0 ml. Dilute 1.0 ml of the well-stirred suspension to 25.0 ml with the mobile phase to obtain a clear solution.

Reference solution. Weigh 20 mg of *pyrantel pamoate IPRS* and dissolve in sufficient mobile phase to produce 25.0 ml. Dilute 10.0 ml of the resulting solution to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with porous silica particles (5 to 10 µm),
- mobile phase: a mixture of 92.8 volumes of *acetonitrile*, 3 volumes of *acetic acid*, 3 volumes of *water* and 1.2 volumes of *diethylamine*,

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– flow rate: 1 ml per minute,
– spectrophotometer set at 288 nm,
– injection volume: 20 µl.

Inject the reference solution. The resolution between pyrantel and pamoic acid is not less than 10.0, the column efficiency for the pyrantel peak is not less than 8000 theoretical plates, the tailing factor for the pyrantel peak is not greater than 1.3 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

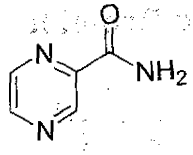
Inject the reference solution and the test solution. Continue the chromatography for a period not less than 2.5 times the retention times of pyrantel base. The relative retention times for pamoic acid and pyrantel base are about 0.6 and 1.0 respectively.

Determine the weight per ml (2.4.29) of the suspension and calculate the content of $C_{11}H_{14}N_2S$, weight in volume.

Storage. Store protected from light at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of pyrantel.

Pyrazinamide



$C_5H_5N_3O$ Mol. Wt. 123.1

Pyrazinamide is pyrazine-2-carboxamide.

Pyrazinamide contains not less than 99.0 per cent and not more than 100.5 per cent of $C_5H_5N_3O$, calculated on the anhydrous basis.

Category. Antitubercular.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pyrazinamide IPRS* or with the reference spectrum of pyrazinamide.

B. Dissolve 50 mg in *water* and dilute to 100 ml with the same solvent (solution A). Dilute 1 ml of the solution A to 10 ml. When examined in the range 290 nm to 360 nm (2.4.7), the resulting solution shows an absorption maximum at about 310 nm. Dilute 2 ml of solution A to 100 ml with *water*. When examined in the range 230 nm to 290 nm, the solution shows an absorption maximum at about 268 nm; absorbance at about 268 nm, between 0.64 and 0.68.

C. Boil 20 mg with 5 ml of *sodium hydroxide solution*; ammonia, recognisable by its odour, is evolved.

Tests

Appearance of solution. A 1.0 per cent w/v solution in *carbon dioxide-free water* (solution B) is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. To 25 ml of solution B add 0.05 ml of *phenolphthalein solution* and 0.2 ml of *0.01 M sodium hydroxide*; the solution is red. Add 1 ml of *0.01 M hydrochloric acid*; the solution is colourless. Add 0.15 ml of *methyl red solution*; the solution is red.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *1-butanol*, 20 volumes of *glacial acetic acid* and 20 volumes of *water*.

Test solution. Dissolve 0.1 g of the substance under examination in 10 ml of a mixture of 90 volumes of *chloroform* and 10 volumes of *methanol*.

Reference solution. A 0.002 per cent w/v solution of the substance under examination in a mixture of 90 volumes of *chloroform* and 10 volumes of *methanol*.

Apply to the plate 20 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.5 per cent, determined on 2.0 g.

Assay. Weigh 0.3 g and transfer to the flask of an ammonia distillation apparatus. Add 200 ml of *water* and 75 ml of *sodium hydroxide solution*. Boil gently for 20 minutes, collecting the distillate in 50.0 ml of *0.05 M sulphuric acid*. Boil vigorously to complete the distillation of the ammonia and titrate the excess of acid with *0.1 M sodium hydroxide*, using *methyl red solution* as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of acid required to neutralise the ammonia formed.

1 ml of *0.05 M sulphuric acid* is equivalent to 0.01231 g of $C_5H_5N_3O$.

Storage. Store protected from moisture.

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Pyrazinamide Tablets

Pyrazinamide Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of pyrazinamide, $C_5H_5N_3O$.

Usual strengths. 250 mg; 500 mg; 750 mg; 1000 mg.

Identification

A. Shake a quantity of the powdered tablets containing 0.25 g of Pyrazinamide with 20 ml of *ethanol*, filter, evaporate the filtrate to dryness and dry the residue at 105° for 30 minutes.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pyrazinamide IPRS* or with the reference spectrum of pyrazinamide.

B. Shake a quantity of the powdered tablets containing 50 mg of Pyrazinamide with 50 ml of *water*, dilute to 100 ml with *water* and filter (solution A). Dilute 1 ml of solution A to 10 ml with *water*. When examined in the range 290 nm to 360 nm (2.4.7), the resulting solution shows an absorption maximum at about 310 nm. Dilute 2 ml of solution A to 100 ml with *water*. When examined in the range 230 nm to 290 nm, the resulting solution shows an absorption maximum at about 268 nm; absorbance at 268 nm, between 0.64 and 0.68.

C. Boil a quantity of the powdered tablets containing 20 mg of Pyrazinamide with 5 ml of *sodium hydroxide solution*; ammonia, recognisable by its odour, is evolved.

Tests

Dissolution (2.5.2).
Apparatus No. 2 (Paddle),
Medium: 900 ml of *water*,
Speed and time. 50 rpm for 45 minutes.

Withdraw a suitable volume of the medium and filter, rejecting the first few ml of filtrate. Dilute a suitable volume of the filtrate with the medium, if necessary. Measure the absorbance of the resulting solution at the maximum at about 268 nm (2.4.7). Calculate the content of pyrazinamide, $C_5H_5N_3O$ in the medium from the absorbance obtained from a solution of known concentration of *pyrazinamide IPRS* in the dissolution medium.

Q. Not less than 75 per cent of the stated amount of $C_5H_5N_3O$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *1-butanol*, 20 volumes of *glacial acetic acid* and 20 volumes of *water*.

Test solution. Shake a quantity of the powdered tablets containing 0.1 g of Pyrazinamide with 50 ml of a mixture of 90 volumes of *chloroform* and 10 volumes of *methanol*, filter, evaporate to dryness and dissolve the residue in sufficient of the same solvent mixture to produce 10 ml.

Reference solution. Dilute 1 volume of the test solution to 500 volumes with a mixture of 90 volumes of *chloroform* and 10 volumes of *methanol*.

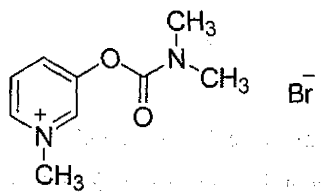
Apply to the plate 20 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.1 g of Pyrazinamide, add 200 ml of *water*, allow to stand for 10 minutes, swirling occasionally, mix with the aid of ultrasound for 10 minutes and dilute to 500.0 ml with *water*. Filter and discard the first 20 ml of the filtrate. Dilute 5.0 ml of the filtrate to 100.0 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 268 nm (2.4.7). Calculate the content of $C_5H_5N_3O$ taking 650 as the specific absorbance at 268 nm.

Storage. Store protected from moisture.

Pyridostigmine Bromide



$C_9H_{13}BrN_2O_2$ Mol. Wt. 261.1

Pyridostigmine Bromide is 3-(Dimethylcarbamoyl)oxy-1-methylpyridinium bromide.

Pyridostigmine Bromide contains not less than 98.5 per cent and not more than 100.5 per cent of $C_9H_{13}BrN_2O_2$, calculated on the dried basis.

Category. Anticholinesterase.

Description. A white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pyridostigmine bromide IPRS* or with the reference spectrum of pyridostigmine bromide.

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B. When examine in the range 200 nm to 400 nm (2.4.7), a 0.0035 per cent w/v solution in *0.1 M hydrochloric acid* gives absorption maxima at 269 nm.

C. To 100 mg in a test-tube, add 0.6 ml of *1 M sodium hydroxide*; an orange colour develops. When the mixture is heated, the colour changes to yellow and a strip of moistened red litmus paper held over the top of the test-tube turns blue.

D. A 2.0 per cent w/v solution gives the reactions of bromide (2.3.1).

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *cellulose with fluorescent indicator*.

Mobile phase. A mixture of equal volumes of *methanol* and *water*.

Test solution. Dissolve 100 mg of the substance under examination in 10 ml of *methanol*.

Reference solution. A 0.02 per cent w/v solution of *pyridostigmine bromide IPRS* in *methanol*.

Apply to the plate 20 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm and 365 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (2.0 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 2.0 per cent, determined on 1 g by drying under vacuum over *phosphorus pentoxide* at 100° for 4 hours.

Assay. Dissolve 850 mg of the substance under examination in 80 ml of *glacial acetic acid*, add 25 ml of *mercuric acetate TS* and 2 drops of *quinaldine red TS*, and titrate with *0.1 M perchloric acid* in *dioxane VS* to a colourless endpoint. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02611 g of $C_9H_{13}BrN_2O_2$.

Storage. Store protected from moisture.

Pyridostigmine Injection

Pyridostigmine Bromide Injection

Pyridostigmine Injection is a sterile solution of Pyridostigmine Bromide in a suitable medium.

Pyridostigmine Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pyridostigmine bromide, $C_9H_{13}BrN_2O_2$.

Usual strength. 5 mg per ml.

Identification

A. When examined in the range 200 nm to 400 nm (2.4.7), the solution prepared in the Assay exhibits maxima and minima that corresponds to that of the reference solution.

B. To 2 ml of injection, add 1 ml of *2 M nitric acid*; the solution gives the reactions for bromide (2.3.1).

Tests

pH (2.4.24). 4.5 to 5.5.

Bacterial endotoxins (2.2.3). Not more than 17.0 Endotoxin Units per mg of pyridostigmine bromide.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Transfer a volume of injection containing 20 mg of Pyridostigmine Bromide to a suitable separator. Add 10 ml of *1 M hydrochloric acid* and mix. Extract with four 20-ml portions and one 15-ml portion of *ethyl ether*, and discard the extracts. Transfer the aqueous layer to a 100-ml volumetric flask, using about 25 ml of *water* to aid the transfer. Place the flask on a steam-bath and warm, with the aid of a stream of nitrogen, to evaporate any residual ether, then cool the flask to room temperature, dilute with water to volume, and mix. Dilute 20.0 ml of the solution to 100.0 ml with *0.1 M hydrochloric acid*. Measure the absorbance at 269 nm (2.4.7). Calculate the content of $C_9H_{13}BrN_2O_2$ in the injection from the absorbance obtained from 0.004 per cent w/v solution of *pyridostigmine bromide IPRS* in *0.1 M hydrochloric acid*.

Storage. Store protected from light and in single-dose container, preferably of Type I glass.

Pyridostigmine Tablets

Pyridostigmine Bromide Tablets

Pyridostigmine Tablets contains Pyridostigmine Bromide.

Pyridostigmine Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of pyridostigmine bromide, $C_9H_{13}BrN_2O_2$.

Identification

Triturate a quantity of the powdered tablets containing 0.1 g of Pyridostigmine Bromide with two 5-ml quantities of *water* and filter. The filtrate complies with the following tests.

A. When examine in the range 200 nm to 400 nm (2.4.7), a 0.005 per cent w/v solution of Pyridostigmine Bromide solution in *water* gives absorption maximum at 270 nm (2.4.7).

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B. Determine by thin-layer chromatography (2.4.17) coating the plate with *silica gel G*.

Mobile phase. A mixture of 5 volumes of *water*, 10 volumes of *formic acid*, 35 volumes of *methanol* and 50 volumes of *chloroform*.

Test solution. Dilute 1.0 ml of the filtrate to 10 ml with *methanol*.

Reference solution. A 0.1 per cent w/v solution of *pyridostigmine bromide IPRS* in *methanol*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 20 cm. Dry the plate in air, spray with *dilute potassium iodobismuthate solution*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

C. The filtrate gives reactions of bromides (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *water*,

Speed and time. 50 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the medium, if necessary, at the maxima at about 270 nm (2.4.7). Calculate the content of $C_9H_{13}BrN_2O_2$ in the medium from the absorbance obtained from a solution of known concentration of *pyridostigmine bromide IPRS* in the dissolution medium.

Q. Not less than 80 per cent of the stated amount of $C_9H_{13}BrN_2O_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 30 volumes of *acetonitrile* and 70 volumes of *water*.

Test solution. Disperse a quantity of powdered tablets containing 0.1 g of Pyridostigmine Bromide with 100 ml of the solvent mixture with the aid of ultrasound for 30 minutes and filter.

Reference solution (a). Dilute 6.0 ml of the test solution to 100.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of the test solution to 250.0 ml with the solvent mixture.

Reference solution (c). A solution containing 0.0005 per cent w/v each of *pyridostigmine impurity A IPRS* and *pyridostigmine bromide IPRS* in the mobile phase.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 70 volumes of a 0.43 per cent w/v solution of *sodium dodecyl sulphate*, adjusted to pH 2.0 with *orthophosphoric acid* and 30 volumes of *acetonitrile*,
- flow rate: 1.1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Name	Relative retention time
Pyridostigmine impurity B[1]	0.75
Pyridostigmine impurity A[2]	0.92
Pyridostigmine(Retetion time: about 32 minutes)	1.0

[1]pyridin-3-yl dimethylcarbamate,

[2]3-hydroxy-1-methylpyridinium.

Inject reference solution (c). The test is not valid unless the resolution between the peaks corresponding to pyridostigmine impurity A and pyridostigmine bromide is not less than 1.5.

Inject reference solution (a), (b), (d) and the test solution. Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to pyridostigmine impurity B is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (6.0 per cent), the area of any peak corresponding to pyridostigmine impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent), the sum of the areas of any other secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.04 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 11.2 g of *orthophosphoric acid* in 500 ml of *water*, adjusted to pH 7.0 with 50 per cent w/v solution of *sodium hydroxide* and dilute to 1000 ml with *water*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 50 mg of Pyridostigmine Bromide in 100 ml of the solvent mixture, shake for 30 minutes and dilute to 200.0 ml with the solvent mixture, mix and centrifuge. Use the supernatant liquid.

Reference solution. A 0.25 per cent w/v solution of *pyridostigmine bromide IPRS* in the solvent mixture.

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Chromatographic system
- a stainless steel column 30 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 1.0 g of *sodium 1-heptanesulfonate* in 500 ml of *water*, add 5.0 ml of *triethylamine* and 100 ml of *acetronitrile*, dilute to 1000 ml with *water*, adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

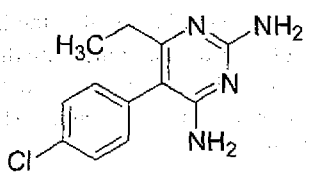
Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_9H_{13}BrN_2O_2$ in the tablets.

Storage. Store protected from light.

Pyrimethamine



$C_{12}H_{13}ClN_4$ Mol. Wt. 248.7

Pyrimethamine is 5-(4-chlorophenyl)-6-ethylpyrimidine-2,4-diamine.

Pyrimethamine contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{12}H_{13}ClN_4$, calculated on the dried basis.

Category. Antimalarial.

Description. Colourless crystals or an almost white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pyrimethamine IPRS* or with the reference spectrum of pyrimethamine.

B. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (b).

C. Dissolve 0.14 g in sufficient *ethanol* to produce 100 ml, dilute 10 ml of the solution to 100 ml with *0.1 M hydrochloric acid* and dilute 10 ml of the solution to 100 ml with the same solvent. When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows an absorption maximum at about 272 nm; absorbance at about 272 nm, 0.43 to 0.46.

Tests

Appearance of solution. Dissolve 0.25 g in sufficient of a mixture of 3 volumes of *dichloromethane* and 1 volume of *methanol* to produce 10 ml. The resulting solution is clear (2.4.1), and not more intensely coloured than reference solution BYS6 (2.4.1).

Acidity or alkalinity. Shake 1.0 g with 50 ml of *water* for 2 minutes and filter (solution A). To 10 ml of solution A add 0.05 ml of *phenolphthalein solution*; the solution is colourless and not more than 0.2 ml of *0.01 M sodium hydroxide* is required to change the colour to pink. Add 0.4 ml of *0.01 M hydrochloric acid* and 0.05 ml of *methyl red solution*; the solution is red or orange.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 76 volumes of *toluene*, 12 volumes of *glacial acetic acid*, 8 volumes of *1-propanol* and 4 volumes of *chloroform*.

Prepare the following solutions immediately before use.

Solvent mixture. 90 volumes of *chloroform* and 10 volumes of *methanol*.

Test solution (a). Dissolve 0.1 g of the substance under examination in 10 ml of the solvent mixture.

Test solution (b). Dissolve 0.1 g of the substance under examination in 100.0 ml of the solvent mixture.

Reference solution (a). A 0.0025 per cent w/v solution of the substance under examination in the solvent mixture.

Reference solution (b). A 0.1 per cent w/v solution of *pyrimethamine IPRS* in the solvent mixture.

Apply to the plate 20 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a).

Sulphates (2.3.17). 25 ml of Solution A complies with the limit test for sulphates. Use a mixture of 5.0 ml of *sulphate standard solution (10 ppm SO_4)* and 10 ml of *distilled water* to prepare the standard (100 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 4 hours.

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Assay. Dissolve 0.2 g in 25 ml of *anhydrous glacial acetic acid*, heating gently, cool. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02487 g of $C_{12}H_{13}ClN_4$.

Storage. Store protected from light and moisture.

Pyrimethamine Tablets

Pyrimethamine Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of pyrimethamine, $C_{12}H_{13}ClN_4$.

Usual strength. 25 mg.

Identification

A. Shake a quantity of the powdered tablets containing 50 mg of Pyrimethamine with 50 ml of *ethanol (95 per cent)* for 20 minutes, filter and evaporate the filtrate to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pyrimethamine IPRS* or with the reference spectrum of pyrimethamine.

B. Extract the powdered tablets with *1 M sulphuric acid*, filter and add *potassium tetraiodomercurate solution* to the filtrate. A creamy white precipitate is produced.

C. Extract a quantity of the powdered tablets containing 50 mg of Pyrimethamine with two 10 ml quantities of *chloroform* and evaporate the combined extracts to dryness. The residue melts at about 240° (2.4.21).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1M hydrochloric acid*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium, filter. Measure the absorbance of the filtrate, suitably diluted with the medium, if necessary at the maximum at about 273 nm (2.4.7). Calculate the content of $C_{12}H_{13}ClN_4$ in the medium from the absorbance obtained from a solution of known concentration of *pyrimethamine IPRS* in the dissolution medium.

Q. Not less than 75 per cent of the stated amount of $C_{12}H_{13}ClN_4$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Solvent mixture. 90 volumes of *chloroform* and 10 volumes of *methanol*.

Mobile phase. A mixture of 4 volumes of *chloroform*, 8 volumes of *1-propanol*, 12 volumes of *glacial acetic acid* and 76 volumes of *toluene*.

Test solution. Shake a quantity of the powdered tablets containing 50 mg Pyrimethamine with 5 ml of solvent mixture and filter.

Reference solution. Dilute 1 ml of the test solution to 400 ml with the solvent mixture.

Apply to the plate 20 µl of each solution. Allow the mobile phase to rise 10 cm. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.25 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.025g of Pyrimethamine, add 50 ml of hot *0.1 M hydrochloric acid* and heat on a water-bath for 10 minutes, swirling occasionally. Mix with the aid of ultrasound for 30 minutes, remove and cool to room temperature. Add sufficient *0.1 M hydrochloric acid* to produce 100 ml, filter and dilute 5 ml of the filtrate to 100 ml with *0.1 M hydrochloric acid* and measure the absorbance of the resulting solution at the maximum at about 272 nm (2.4.7). Calculate the content of $C_{12}H_{13}ClN_4$ taking 316 as the specific absorbance at 272 nm.

Pyrimethamine and Sulphadoxine Tablets

Pyrimethamine and Sulphadoxine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of pyrimethamine, $C_{12}H_{13}ClN_4$, and of sulphadoxine, $C_{12}H_{14}N_4O_4S$.

Usual strength. Pyrimethamine 25 mg and sulphadoxine 500 mg.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 4 volumes of *chloroform*, 4 volumes of *n-heptane*, 1 volume of *glacial acetic acid* and 4 volumes of a mixture of 1 volume of *methanol* and 19 volumes of *ethanol*.

Test solution. Shake a quantity of the powdered tablets containing 25 mg of Pyrimethamine with 50 ml of a 2 per cent w/v solution of *strong ammonia solution* in *methanol*.

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Reference solution (a). A 0.05 per cent w/v solution of *pyrimethamine IPRS* in *methanol*.

Reference solution (b). A 1.0 per cent w/v solution of *sulphadoxine IPRS* in *methanol*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. One of the principal spots in the chromatogram obtained with the test solution corresponds to the spot in the chromatogram obtained with reference solution (a) and the other corresponds to that in the chromatogram obtained with reference solution (b).

Tests

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the powdered tablets containing about 25 mg of Pyrimethamine and 500 mg of Sulphadoxine and shake with 35 ml of *acetonitrile* for 30 minutes in a 100-ml volumetric flask. Dilute to volume with the mobile phase, mix and filter. To 25.0 ml of the filtrate add 2.0 ml of solution A prepared by dissolving 0.1 g of *phenacetin* (internal standard) in 100 ml of *acetonitrile* and sufficient of the mobile phase to produce 50.0 ml.

Reference solution. Weigh 25 mg of *pyrimethamine IPRS* and 500 mg of *sulphadoxine IPRS*, add 35 ml of *acetonitrile* and sufficient of the mobile phase to produce 100.0 ml and mix. To 25.0 ml add 2.0 ml of solution A and add sufficient of the mobile phase to produce 50.0 ml and mix well.

Chromatographic system

- a stainless steel column 30 cm x 4 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 4 volumes of a 1 per cent v/v solution of *glacial acetic acid* and 1 volume of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention times with reference to phenacetin for sulphadoxine is about 0.7 and for pyrimethamine is about 1.3.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{12}H_{13}ClN_4$ and the content of $C_{12}H_{14}N_4O_4S$ in the tablets.

Storage. Store protected from light and moisture.

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Q

Quetiapine Fumarate 3409
Quetiapine Prolonged-release Tablets 3410
Quetiapine Tablets 3411
Quinalbarbitone Sodium 3412
Quinalbarbitone Tablets 3413
Quinapril Hydrochloride 3413
Quinapril and Hydrochlorothiazide Tablets 3414
Quinidine Sulphate 3416
Quinidine Tablets 3418
Quinine Bisulphate 3419
Quinine Bisulphate Tablets 3421
Quinine Dihydrochloride 3422
Quinine Dihydrochloride Injection 3423
Quinine Sulphate 3425
Quinine Tablets 3426
Quiniodochlor 3428
Quiniodochlor Cream 3429
Quiniodochlor Ointment 3429
Quiniodochlor Tablets 3430
Quiniodochlor and Hydrocortisone Cream 3431
Quiniodochlor and Hydrocortisone Ointment 3432

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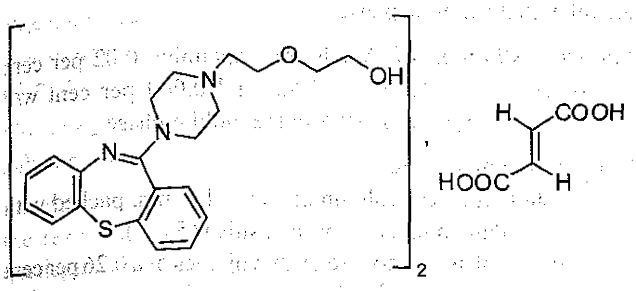
Quetiapine Fumarate



$C_{46}H_{54}N_6O_8S_2$ Mol. Wt. 883.1

Quetiapine Fumarate is Bis[2-[2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]ethoxy]ethanol] (2*E*)-but-2-enedioate.

Quetiapine Fumarate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{46}H_{54}N_6O_8S_2$, calculated on the dried basis.

Category. Antipsychotic.

Description. A white or almost white powder. It shows polymorphism (2.5.11).

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *quetiapine fumarate IPRS* or with the reference spectrum of quetiapine fumarate.

Tests

Fumaric acid. 12.5 to 13.8 per cent.

Dissolve 0.35 g of the substance under examination in 70 ml of a mixture of equal volumes of *methanol* and *water*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.005804 g of $C_4H_4O_4$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *acetonitrile* and *water*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 25.0 ml with the solvent mixture.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 10 cm × 2.1 mm, packed with end-capped, charged surface, ethylene-bridged phenylhexylsilyl bonded to porous silica (1.7 µm),
- column temperature: 50°,
- mobile phase: A. a mixture of 90 volumes of a buffer solution prepared by dissolving 3.85 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 9.0 with *ammonia solution* and 10 volumes of *methanol*,
  B. *acetonitrile*,
- flow rate: 0.5 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 240 nm,
- injection volume: 3 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
8	80	20
14.5	60	40
22.6	50	50
26	30	70
29	10	90
30	10	90
31	80	20
35	80	20

Name	Relative retention time	Correction factor
Fumaric acid	0.05	—
Quetiapine impurity G[1]	0.5	0.5
Quetiapine (retention time: about 13 minutes)	1.0	—
Quetiapine impurity N[2]	1.04	2.0

[1]dibenzo[*b*,*f*][1,4]thiazepin-11(10*H*)-one,

[2]2-[2-[4-[2-[2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]ethoxy]ethyl]piperazin-1-yl]ethoxy]ethanol,

Inject the reference solution. The test is not valid unless the signal-to-noise ratio for the principal peak is not less than 40.

Inject the reference solution and the test solution. In the chromatogram obtained with test solution, the area of any peak corresponding to quetiapine impurity G and quetiapine impurity N, each of, is not more than 1.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.15 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent). Ignore the peak due to fumaric acid and any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

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Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in oven at 105°.

Assay. Dissolve 0.17 g of the substance under examination in 40 ml of *glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02208 g of $C_{46}H_{54}N_6O_8S_2$.

Storage. Store protected from light.

Quetiapine Prolonged-release Tablets

Quetiapine Fumarate Prolonged-release Tablets

Quetiapine Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Quetiapine Prolonged-release Tablets contain quetiapine fumarate equivalent to not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of quetiapine, $C_{21}H_{25}N_3O_2S$.

Usual strengths. 100 mg; 200 mg; 300 mg; 400 mg.

Identification

A. Shake a quantity of the powdered tablets containing the equivalent of 40 mg of quetiapine with 10 ml of *acetonitrile*, filter and discard the filtrate. Extract the residue with 10 ml of *methanol*, filter and evaporate the filtrate to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *quetiapine fumarate IPRS* or with the reference spectrum of quetiapine fumarate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2). Complies with the test stated under Tablets.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and disperse a quantity of powdered tablets containing 12.5 mg of Quetiapine in 20 ml of the mobile phase, with the aid of ultrasound and dilute to 25.0 ml with the mobile phase, filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 5.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.02 per cent w/v of *quetiapine fumarate IPRS* and 0.004 per cent w/v of *quetiapine impurity I IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 39 volumes of a 0.26 per cent w/v solution of *diammonium hydrogen orthophosphate* in *water*, 54 volumes of *methanol* and 7 volumes of *acetonitrile*,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 30 µl.

Name	Relative retention time	Correction factor
Quetiapine impurity H[1]	0.5	—
Quetiapine impurity G[2]	0.6	0.7
Quetiapine impurity I[3]	0.9	—
Quetiapine (retention time: about 14 minutes)	1.0	—

[1]2-[2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)-1-oxidopiperazin-1-yl]ethoxy]ethanol,

[2]dibenzo[*b*,*f*][1,4]thiazepin-11(10*H*)-one,

[3]2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]ethanol.

Inject reference solution (b). The test is not valid unless the resolution between the peaks corresponding to quetiapine and quetiapine impurity I in the chromatogram obtained is not less than 2.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore the peak due to fumaric acid and any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 50 mg of Quetiapine in 200 ml of the mobile phase and dilute to 250.0 ml with the mobile phase, filter.

Reference solution (a). A 0.023 per cent w/v solution of *quetiapine fumarate IPRS* in the mobile phase.

Reference solution (b). A solution containing 0.02 per cent w/v of *quetiapine fumarate IPRS* and 0.004 per cent w/v of *quetiapine impurity I IPRS* in the mobile phase.

Use chromatographic system as described under Related substances.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to quetiapine and quetiapine impurity I is not less than 2.0 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 2.0 and the relative standard deviation of replicate injections is not more than 2.0 per cent for quetiapine in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{21}H_{25}N_3O_2S$ in the tablets.

1 mg of quetiapine fumarate $C_{46}H_{54}N_6O_8S_2$ is equivalent to 0.8686 mg of quetiapine, $C_{21}H_{25}N_3O_2S$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of quetiapine.

Quetiapine Tablets

Quetiapine Fumarate Tablets

Quetiapine Tablets contain quetiapine fumarate equivalent to not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of quetiapine, $C_{21}H_{25}N_3O_2S$.

Usual strengths. 25 mg; 50 mg; 100 mg; 200 mg; 300 mg.

Identification

A. Shake a quantity of the powdered tablets containing the equivalent of 40 mg of quetiapine with 10 ml of *acetonitrile*, filter and discard the filtrate. Extract the residue with 10 ml of *methanol*, filter and evaporate the filtrate to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *quetiapine fumarate IPRS* or with the reference spectrum of quetiapine fumarate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2).
Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.01 M hydrochloric acid*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with the dissolution medium if necessary, at the maximum at about 290 nm (2.4.7). Calculate the content of $C_{21}H_{25}N_3O_2S$ in the medium from the absorbance obtained from a solution of 0.0032 per cent w/v of *quetiapine fumarate IPRS* in the same medium.

Q. Not less than 75 per cent of the stated amount of $C_{21}H_{25}N_3O_2S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 25 mg of quetiapine in 20 ml of the mobile phase, with the aid of ultrasound and dilute to 25.0 ml with the mobile phase and filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 5.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.02 per cent w/v of *quetiapine fumarate IPRS* and 0.004 per cent w/v of *quetiapine impurity I IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 39 volumes of a 0.26 per cent w/v solution of *diammonium hydrogen orthophosphate* in *water*, 54 volumes of *methanol* and 7 volumes of *acetonitrile*,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 30 µl.

Name	Relative retention time	Correction factor
Quetiapine impurity H[1]	0.5	—
Quetiapine impurity G[2]	0.6	0.7
Quetiapine impurity I[3]	0.9	—
Quetiapine (retention time: about 14 minutes)	1.0	—

[1]2-[2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)-1-oxidopiperazin-1-yl]ethoxy]ethanol,

[2]dibenzo[*b*,*f*][1,4]thiazepin-11(10*H*)-one,

[3]2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]ethanol.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to quetiapine and quetiapine impurity I is not less than 2.0.

Inject reference solution (a) and the test solution. Run the chromatogram twice the retention time of the principal peak

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for test solution. The area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore the peak due to fumaric acid and any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14), using chromatographic system and reference solution (b) as described under Related substances.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 50 mg of quetiapine in 200 ml of the mobile phase, dilute to 250.0 ml with the mobile phase and filter.

Reference solution (a). A 0.023 per cent w/v of *quetiapine fumarate IPRS* in the mobile phase.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to quetiapine and quetiapine impurity I is not less than 2.0.

Inject reference solution (a) and test solution.

Calculate the content of $C_{21}H_{25}N_3O_2S$ in the tablets.

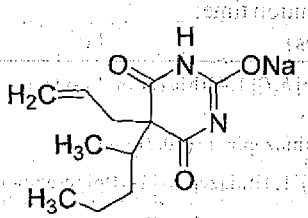
1 mg of quetiapine fumarate $C_{46}H_{54}N_6O_8S_2$ is equivalent to 0.8686 mg of quetiapine, $C_{21}H_{25}N_3O_2S$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of quetiapine.

Quinalbarbitone Sodium

Quinalbarbital Sodium; Secobarbital Sodium; Secobarbitone Sodium; Soluble Quinalbarbitone



$C_{12}H_{17}N_2NaO_3$ Mol.Wt.260.3

Quinalbarbitone Sodium is sodium (*RS*)-5-allyl-5-(1-methylbutyl)barbiturate.

Quinalbarbitone Sodium contains not less than 98.5 per cent and not more than 102.0 per cent of $C_{12}H_{17}N_2NaO_3$, calculated on the dried basis.

Category. Hypnotic.

Description. A white powder; hygroscopic.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests B, C and D may be omitted if tests A and E are carried out.

A. To 10 ml of a 10.0 per cent w/v solution in *ethanol (95 per cent)* add 120 ml of *water* and 5 ml of *2 M acetic acid*, stir vigorously, add 200 ml of *water* and boil until the precipitate dissolves and no oily particles remain on the surface of the liquid. Allow to cool until a haziness begins to appear in the solution, induce crystallisation, if necessary and allow the solution to stand for 12 hours. Wash the crystals with three quantities, each of 10 ml, of *water* and dry the residue at 80°.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *quinalbarbitone IPRS* treated in the same manner or with the reference spectrum of quinalbarbitone.

B. To 0.5 g, add 5 ml of *sodium carbonate solution* and 10 ml of *nitrobenzyl chloride solution*. Heat for 30 minutes on a water-bath under reflux and allow to stand for 1 hour. Filter, wash the precipitate successively with 10 ml of *dilute sodium hydroxide solution* and 50 ml of *water*; recrystallise from a mixture of equal volumes of *chloroform* and *ethanol (95 per cent)* and dry at 105°. The crystals melt at about 156° (2.4.21)

C. Complies with the test for identification of barbiturates (2.3.2).

D. It gives the reaction of non-nitrogen substituted barbiturates (2.3.1).

E. Ignite 1 g; the residue gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. A freshly prepared 10.0 per cent w/v solution in *ethanol (95 per cent)* is clear (2.4.1), and not more intensely coloured than reference solution YS7 (2.4.1).

pH (2.4.24). Not more than 11.0, determined on a 10.0 per cent w/v solution.

Heavy metals (2.3.13). 0.67 g dissolved in a mixture of 5 ml of *1 M sodium hydroxide* and 20 ml of *water* complies with the limit test for heavy metals, Method C (30 ppm).

Related substances. Complies with the test for related substances in barbiturates (2.3.4).

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Loss on drying (2.4.19). Not more than 3.0 per cent, determined on 0.5 g by drying in an oven at 105°.

Assay. Weigh 0.5 g, dissolve in 10 ml of *ethanol*, add 10 ml of *silver nitrate-pyridine reagent* and titrate with *0.1 M ethanolic sodium hydroxide* using 0.5 ml of *thymolphthalein solution* as indicator, until a pure blue colour is obtained. Carry out a blank titration.

1 ml of *0.1 M ethanolic sodium hydroxide* is equivalent to 0.02603 g of $C_{12}H_{17}N_2NaO_3$.

Storage. Store protected from moisture.

Quinalbarbitone Tablets

Quinalbarbital Sodium Tablets; Quinalbarbitone Sodium Tablets; Secobarbital Sodium Tablets; Secobarbitone Sodium Tablets

Quinalbarbitone Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of quinalbarbitone sodium, $C_{12}H_{17}N_2NaO_3$. The tablets are coated.

Usual strength. 100 mg.

Identification

A. Shake a quantity of the powdered tablets containing about 0.5 g of Quinalbarbitone Sodium with 10 ml of *water* and filter. To 10 ml of the filtrate add 5 ml of *sodium carbonate solution* and 10 ml of *nitrobenzyl chloride solution*. Heat for 30 minutes on a water-bath under reflux and allow to stand for 1 hour. Filter, wash the precipitate successively with 10 ml of *dilute sodium hydroxide solution* and 50 ml of *water*; recrystallise from a mixture of equal volumes of *chloroform and ethanol (95 per cent)* and dry at 105°. The crystals melt at about 156° (2.4.21).

B. Triturate a quantity of the powdered tablets containing 0.5 g of Quinalbarbitone Sodium with 10 ml of *water*, filter, acidify the filtrate with *acetic acid*; oily drops are formed which may eventually crystallise.

C. The powdered tablets give the reactions of sodium salts (2.3.1).

Tests

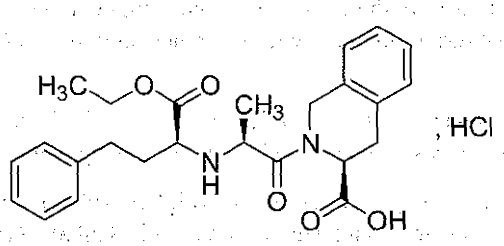
Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and digest 20 tablets with 50 ml of *water* until completely disintegrated and not more than a small residue remains. Add 5 ml of *1 M sodium hydroxide*, filter and wash the residue with sufficient *water* to produce 100.0 ml. Extract a volume of the solution containing 0.5 g of Quinalbarbitone Sodium with two quantities, each of 10 ml, of *ether*, washing each ether extract with the same 3 ml of *water*. Add the water to the aqueous liquid, acidify with *hydrochloric acid* and extract with successive quantities, each of 15 ml, of *ether* until complete extraction is effected. Wash the combined extracts with two quantities, each of 2 ml, of *water* and extract the combined washings with 10 ml of *ether*. Add the ether to the main ether layer, filter and wash the filter with *ether*. Evaporate the solvent and dry the residue to constant weight at 50°.

1 g of residue is equivalent to 1.092 g of $C_{12}H_{17}N_2NaO_3$.

Storage. Store protected from moisture.

Quinapril Hydrochloride



$C_{25}H_{30}N_2O_5,HCl$ Mol. Wt.474.9

Quinapril Hydrochloride is (*S*)-2-[(*S*)-*N*-[(*S*)-1-Carboxy-3-phenylpropyl]alanyl]-1,2,3,4-tetrahydro-3-isoquinoline-carboxylic acid, 1-ethyl ester, monohydrochloride.

Quinapril Hydrochloride contains not less than 98.5 per cent and not more than 101.5 per cent of $C_{25}H_{30}N_2O_5,HCl$, calculated on the anhydrous basis.

Category. Antihypertensive.

Description. A white to off-white powder, with a pink cast at times.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *quinapril hydrochloride IPRS* or with the reference spectrum of quinapril hydrochloride.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with reference solution (b).

Tests

Specific optical rotation (2.4.22). + 14.4° to + 15.4°, determined in a 2.0 per cent w/v solution in *methanol*.

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Related substances. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Reference solution (c). A solution containing 0.0005 per cent w/v each of *quinapril related compound A IPRS* and *quinapril related compound B IPRS* in the solvent mixture.

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution, the area of each peak due to quinapril related compound A and B is not more than twice the area of the corresponding peaks in the chromatogram obtained with reference solution (c) (0.5 per cent), the area of any secondary peak other than quinapril related compound A and B is not more than 0.8 times the area of the quinapril related compound A peak in the chromatogram obtained with reference solution (c) (0.2 per cent) and the sum of the areas of all other secondary peaks is not more than 8 times the area of the quinapril related compound A peak in the chromatogram obtained with reference solution (c) (2.0 per cent).

Chloride content. 7.2 to 7.6 per cent, calculated on the anhydrous basis, determined by the following method.

Dissolve 0.1 g of accurately weighed substance under examination in about 50 ml of *water*, add 10 ml of *ethanol (95 per cent)*, acidify with *nitric acid* and titrate with *0.01 M silver nitrate*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.01 M silver nitrate* is equivalent to 0.0003545 g of chloride.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method A (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.0 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. A mixture of 40 volumes of *acetonitrile* and 60 volumes of *0.025 M of monobasic ammonium phosphate*, adusted to pH 6.5.

Test solution. Dissolve 100.0 mg of the substance under examination in 50.0 ml of the solvent mixture.

Reference solution (a). A solution containing 0.2 per cent w/v of *quinapril hydrochloride IPRS* and 0.0005 per cent w/v each of *ethyl [3S-[(2R*), 3a, 11ab]]-1,3,4,6,11,11a-hexahydro-3-methyl-1,4-dioxo-a-(2-phenylethyl)-2H-pyrazino[1,2-b]isoquinoline-2-acetate IPRS (quinapril related compound A IPRS)* and *3-isoquinolinecarboxylic acid, 2-[2-[(1-carboxy-3-phenylpropyl)amino]-1-oxopropyl]-1,2,3,4-tetrahydro-, [3S-[2[R*(R*)],3R*]] IPRS (quinapril related compound B IPRS)* in the solvent mixture.

Reference solution (b). A 0.2 per cent w/v solution of *quinapril hydrochloride IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with nitrile groups chemically bonded to porous silica particles (5 µm),
- mobile phase: a mixture of 72 volumes of *water*, 28 volumes of *acetonitrile* and 0.1 volumes of *methanesulphonic acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the resolution between the peaks corresponding to quinapril and quinapril related compound A is not less than 1.75, the resolution between the peaks corresponding to quinapril and quinapril related compound B is not less than 3.5, the column efficiency is not less than 550 theoretical plates. The relative standard deviation for replicate injections is not more than 2.0 per cent for reference solution (b).

Inject reference solution (b) and the test solution.

Calculate the content of $C_{25}H_{30}N_2O_5,HCl$.

Storage. Store protected from moisture at a temperature not exceeding 30°.

Quinapril and Hydrochlorothiazide Tablets

Quinapril and Hydrochlorothiazide Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of quinapril, $C_{25}H_{30}N_2O_5$ and hydrochlorothiazide, $C_7H_8ClN_3O_4S_2$.

Usual strengths. Quinapril, 10 mg and Hydrochlorothiazide, 12.5 mg; Quinapril, 20 mg and Hydrochlorothiazide, 12.5 mg and Quinapril, 20 mg and Hydrochlorothiazide, 25 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution (b) correspond to the principal peaks in the chromatogram obtained with reference solution (c).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of *water*,
Speed and time. 100 rpm and 20 minutes.

Withdraw a suitable volume of the medium and filter through a 0.45 µm nylon filter.

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Determine by liquid chromatography (2.4.14) as described under Assay using the following solutions.

Test solution. Use the filtrate obtained as given above.

Reference solution (a). A 0.06 per cent w/v solution of *quinapril hydrochloride IPRS* in the solvent mixture.

Reference solution (b). A 0.055 per cent w/v solution of *hydrochlorothiazide IPRS* in the solvent mixture.

Reference solution (c). Dilute reference solution (a) and reference solution (b) in the dissolution medium to obtain a solution having similar concentration to that of the test solution.

Inject reference solution (c). The test is not valid unless the column efficiency for is not less than 2500 theoretical plates for each component, the tailing factor is not more than 2.0 for each component and the relative standard deviation of replicate injections is not more than 2.0 per cent for each component.

Inject reference solution (c) and the test solution.

Q. Not less than 80 per cent of the stated amount of $C_{25}H_{30}N_2O_5$ and $C_7H_8ClN_3O_4S_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of mobile phase A and mobile phase B.

Test solution. Disperse a quantity of the powder containing 62.5 mg of hydrochlorothiazide in 50 ml of the solvent mixture, with the aid of ultrasound for 10 minutes. Add 50 ml of *acetonitrile*, mix with the aid of ultrasound for 15 minutes with occasional shaking and dilute to 250.0 ml with the solvent mixture.

Reference solution. A solution containing 0.005 per cent w/v, each of, *quinapril hydrochloride IPRS, quinapril related compound A IPRS, quinapril related compound B IPRS, hydrochlorothiazide IPRS* and *benzothiadiazine related compound A IPRS* in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- sample temperature: 5°,
- mobile phase: A. a buffer solution prepared by dissolving 1.36 g of *monobasic potassium phosphate* in 1000 ml of *water*, add 2.0 ml of *triethylamine*, adjusted to pH 3.0 with *orthophosphoric acid*,
  B. *acetonitrile*,
- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
10	60	40
30	30	70
31	90	10
40	90	10

Name	Relative retention time
Benzothiadiazine related compound A[1]	0.42
Chlorothiazide*	0.45
Hydrochlorothiazide*	0.49
5-Chlorohydrochlorothiazide*	0.65
Quinapril related compound B[2]	0.74
Hydrochlorothiazide dimer*	0.78
Quinapril methyl ester*	0.91
Quinapril*	1.0
Quinapril isopropyl ester*	1.1
Hexahydroquinapril*	1.23
Quinapril related compound A[3]	1.59
Quinapril, benzyl ester*	1.94

*Process related impurity, monitored in the drug substance,

[1]4-Amino-6-chloro-1,3-benzenedisulfonamide,

[2]3-Isoquinolinecarboxylic acid, 2-[2-[(1-carboxy-3-phenylpropyl) amino]-1-oxopropyl]-1,2,3,4-tetrahydro-, [3*S*-[2[*R**(*R**)],3*R**]]],

[3]Ethyl[3*S*-[2(*R**),3a,11a beta]]-1,3,4,6,11,11a-hexahydro-3-methyl-1,4-dioxo-alpha-(2-phenylethyl)-2*H* -pyrazino[1,2-b]isoquinoline-2-acetate.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 5000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation of replicate injections is not more than 5.0 per cent for the peaks due to quinapril and hydrochlorothiazide.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to benzothiadiazine related compound A is not more than 5 times the area of the peak due to benzothiadiazine related compound A in the chromatogram obtained with the reference solution (1.0 per cent), the area of any peak corresponding to quinapril related compound A is not more than 5 times the area of the peak due to quinapril related compound A in the chromatogram obtained with the reference solution (1.0 per cent), the area of any peak corresponding to quinapril related compound B is not more than 15 times the area of the peak due to quinapril related compound B in the chromatogram obtained with the reference solution (3.0 per cent), the area of any other secondary peak is not more than the area of the peak due to quinapril in the

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chromatogram obtained with the reference solution (0.2 per cent) and the sum of the areas of all the secondary peaks other than quinapril related compound B and benzothiadiazine related compound A is not more than 10 times the area of the peak due to quinapril in the chromatogram obtained with the reference solution (2.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of mobile phase A and mobile phase B.

Test solution (a). Weigh and powder 20 tablets. Disperse an accurately of the powder equivalent to the average weight of five tablets into a 250-ml volumetric flask, add 50 ml of solvent mixture and disperse with the aid of ultrasound for 15 minutes. Add 50.0 ml of *acetonitrile* and mix with the aid of ultrasound for 15 minutes with occasional shaking. Add 50.0 ml of solvent mixture and mix with the aid of ultrasound for 15 minutes and dilute to volume with the solvent mixture.

Test solution (b). Dilute test solution (a) with the solvent mixture to obtain a solution containing 0.002 per cent w/v of quinapril.

Reference solution (a). A 0.0225 per cent w/v solution of *quinapril hydrochloride IPRS* in the solvent mixture.

Reference solution (b). A 0.025 per cent w/v solution of *hydrochlorothiazide IPRS* in the solvent mixture.

Reference solution (c). Dilute reference solution (a) and reference solution (b) in the solvent mixture to obtain a solution having similar concentration to that of the test solution.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: A. a buffer solution prepared by dissolving 1.36 g of *monobasic potassium phosphate* in 1000 ml of *water*, add 2.0 ml of *triethylamine*, adjusted to pH 3.0 with *orthophosphoric acid*,
  B. *acetonitrile*,
- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	75	25
8	40	60
12	40	60
13	75	25
18	75	25

Inject reference solution (c). The test is not valid unless the column efficiency is not less than 2500 theoretical plates for each component, the tailing factor is not more than 2.0 for each component and the relative standard deviation of replicate injections is not more than 2.0 per cent for each component.

Inject reference solution (c) and the test solution (b).

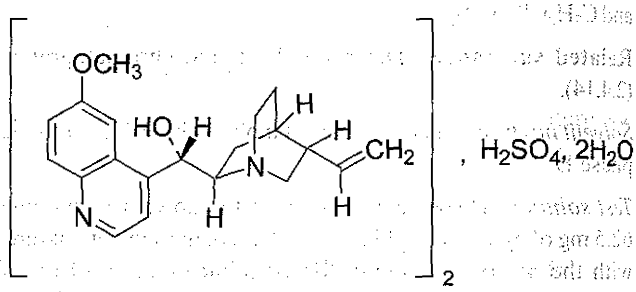
Calculate the content of $C_{25}H_{30}N_2O_5$ and $C_7H_8ClN_3O_4S_2$ in the tablet.

Storage. Store protected from light and moisture at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of equivalent amount of quinapril and hydrochlorothiazide.

Quinidine Sulphate

Quinidine Bisulphate



$(C_{20}H_{24}N_2O_2)_2,H_2SO_4,2H_2O$ Mol. Wt. 783.0

Quinidine Sulphate is (8*R*,9*S*)-6′-methoxycinchonan-9-ol sulphate dihydrate. The alkaloid is obtained from the bark of various species of *Cinchona* and from *Remijia pedunculata* Fluckiger (Fam. *Rubiaceae*) or prepared from quinine.

Quinidine Sulphate contains not less than 99.0 per cent and not more than 101.5 per cent of alkaloid monosulphates, calculated as $(C_{20}H_{24}N_2O_2)_2,H_2SO_4$ on the dried basis.

Category. Antiarrhythmic.

Description. A white or almost white, crystalline powder or needle-like crystals.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 60 volumes of *toluene*, 36 volumes of *ether* and 15 volumes of *diethylamine*.

Test solution. Dissolve 1 g of the substance under examination in 100 ml of *methanol*.

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Reference solution (a). A 1.0 per cent w/v solution of *quinidine sulphate IPRS* in *methanol*.

Reference solution (b). A 1.0 per cent w/v solution of each of *quinidine sulphate IPRS* and *quinine sulphate IPRS* in *methanol*.

Apply to the plate 4 µl of each solution. After development, dry the plate in air for 15 minutes and repeat the development. Dry the plate at 105° for 30 minutes, allow to cool and spray with *potassium iodoplatinate solution*. The principal spot in the chromatogram obtained with the test solution corresponds to the spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

B. To 5 ml of a 0.1 per cent w/v solution add 0.2 ml of *bromine solution* and 1 ml of *dilute ammonia solution*; an emerald-green colour is produced.

C. To a 0.5 per cent w/v solution add an equal volume of *dilute sulphuric acid*; a strong blue fluorescence is produced.

D. To 5 ml of a 1 per cent w/v solution add 1 ml of *silver nitrate solution* and stir with a glass rod; after a short interval, a white precipitate soluble in *nitric acid* is produced (distinction from many other alkaloids).

E. A 1 per cent w/v solution gives the reactions of sulphates (2.3.1).

Tests

Appearance of solution. A 2.0 per cent w/v solution in *0.1 M hydrochloric acid* is clear (2.4.1), and not more intensely coloured than reference solution GYS4 (2.4.1).

pH (2.4.24). 6.0 to 6.8, determined in a 1.0 per cent w/v solution.

Specific optical rotation (2.4.22). +275° to +290°, determined in a 2.0 per cent w/v solution in *0.1 M hydrochloric acid*.

Other cinchona alkaloids. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in 5 ml of the mobile phase. Heat gently, if necessary to dissolve the powder as completely as possible, cool, dilute to 10.0 ml with the mobile phase and mix.

Reference solution (a). Dissolve 20 mg of *quinine sulphate IPRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Prepare in the same manner as reference solution (a) but using *quinidine sulphate IPRS* in place of *quinine sulphate IPRS*.

Reference solution (c). Mix equal volumes of reference solution (a) and (b).

Reference solution (d). Dilute 1 volume of reference solution (a) to 10 volumes with the mobile phase and dilute 1 volume of the resulting solution to 50 volumes with the mobile phase.

Reference solution (e). A solution containing 0.1 per cent w/v of *thiourea* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS 5 µm),
- mobile phase: a solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* and 3.0 g of *hexylamine* in 700 ml of *water*, adjusted to pH 2.8 with *1 M orthophosphoric acid*, adding 60 ml of *acetonitrile* and diluting to 1000 ml with *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 250 nm for reference solution (e) and 316 nm for the other solutions,
- injection volume: 10 µl.

The test is not valid unless (a) in the chromatogram obtained with reference solution (c) the resolution between the peaks due to quinine and quinidine is at least 1.5 and the resolution between the peaks due to dihydroquinidine and quinine is at least 1.0 and (b) the signal-to-noise ratio of the principal peak in the chromatogram obtained with reference solution (d) is at least 5.

Inject reference solution (b) and (e). If necessary, adjust the concentration of acetonitrile in the mobile phase so that in the chromatogram obtained with reference solution (b) the capacity factor of the peak due to quinidine is 3.5 to 4.5, V_0 (the distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak of an unretained component) being calculated from the peak due to thiourea in the chromatogram obtained with reference solution (e).

Inject reference solution (a), (b), (c) and (d). The chromatogram obtained with reference solution (a) shows a principal peak due to quinine and a peak due to dihydroquinine with a retention time relative to quinine of about 1.4. The chromatogram obtained with reference solution (b) shows a principal peak due to quinidine and a peak due to dihydroquinidine, with a retention time relative to quinidine of about 1.2. The chromatogram obtained with reference solution (c) shows four peaks due to quinine, dihydroquinine, quinidine and dihydroquinidine which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solution (a) and (b).

Inject the test solution and allow the chromatography to proceed for 2.5 times the retention time of the principal peak. Calculate the percentage content of related substances by



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normalisation, ignoring any peaks the areas of which are less than that of the peak in the chromatogram obtained with reference solution (d) (0.2 per cent). The content of dihydroquinidine is not greater than 15 per cent, the content of any related substance eluting before quinidine is not greater than 5 per cent and the content of any other related substance is not greater than 2.5 per cent.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). 3.0 per cent to 5.0 per cent, determined on 1.0 g by drying in an oven at 130°.

Assay. Dissolve 0.2 g in a mixture of 10 ml of *chloroform* and 20 ml of *acetic anhydride*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02490 g of $(C_{20}H_{24}N_2O_2)_2,H_2SO_4$.

Storage. Store protected from light.

Quinidine Tablets

Quinidine Sulphate Tablets

Quinidine Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of quinidine sulphate, $(C_{20}H_{24}N_2O_2)_2,H_2SO_4,2H_2O$.

Usual strength. 200 mg.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 80 volumes of *toluene*, 20 volumes of *acetone* and 10 volumes of *diethylamine*.

Test solution. Extract a quantity of the powdered tablets containing 0.1 g of Quinidine Sulphate with 10 ml of a mixture of 2 volumes of *chloroform* and 1 volume of *ethanol (95 per cent)* and filter.

Reference solution. 1.0 per cent w/v solution of *quinidine sulphate IPRS* in a mixture of 2 volumes of *chloroform* and 1 volume of *ethanol (95 per cent)*.

Apply to the plate 2 µl of each solution. After development, dry the plate in air and spray with *0.05 M ethanolic sulphuric acid* and then with *dilute potassium iodobismuthate solution*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. Extract a quantity of the powdered tablets containing 0.1 g of Quinidine Sulphate with 20 ml of *water* and filter. The filtrate (solution A) is dextro-rotatory.

C. To 1 ml of solution A add 4 ml of *water*, 2 or 3 drops of *bromine solution* and 1 ml of *dilute ammonia solution*; an emerald green colour is produced.

D. Solution A gives the reactions of sulphates (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 100 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted if necessary, at the maximum at about 248 nm (2.4.7). Calculate the content of $(C_{20}H_{24}N_2O_2)_2,H_2SO_4,2H_2O$ in the medium from the absorbance obtained from a solution of known concentration of *quinidine sulphate IPRS*.

Q. Not less than 70 per cent of the stated amount of $(C_{20}H_{24}N_2O_2)_2,H_2SO_4,2H_2O$.

Other cinchona alkaloids. Determine by liquid chromatography (2.4.14).

Test solution. Mix a quantity of the powdered tablets containing 50 mg of Quinidine Sulphate with 20 ml of the mobile phase. Heat gently to dissolve the powder as completely as possible, cool, dilute to 25.0 ml with the mobile phase and filter, discarding the first few ml of filtrate.

Reference solution (a). Dissolve 20 mg of *quinine sulphate IPRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Prepare in the same manner as reference solution (a) but using *quinidine sulphate IPRS* in place of *quinine sulphate IPRS*.

Reference solution (c). Mix equal volumes of reference solution (a) and (b).

Reference solution (d). Dilute 1 volume of reference solution (a) to 10 volumes with the mobile phase and dilute 1 volume of the resulting solution to 50 volumes with the mobile phase.

Reference solution (e). A solution containing 0.1 per cent w/v of *thiourea* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- mobile phase: a solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* and 3.0 g of *hexylamine* in 700 ml of *water*, adjusted to pH 2.8 with *1 M orthophosphoric acid*, adding 60 ml of *acetonitrile* and diluting to 1000 ml with *water*,

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- flow rate: 1.5 ml per minute,
- spectrophotometer set at 250 nm for reference solution (e) and 316 nm for the other solutions,
- injection volume: 10 µl.

Inject reference solution (b) and (e). If necessary, adjust the concentration of acetonitrile in the mobile phase so that in the chromatogram obtained with reference solution (b) the capacity factor of the peak due to quinidine is 3.5 to 4.5, V_0 (the distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak of an unretained component) being calculated from the peak due to thiourea in the chromatogram obtained with reference solution (e).

The test is not valid unless (a) in the chromatogram obtained with reference solution (c) the resolution between the peaks due to quinine and quinidine is at least 1.5 and the resolution between the peaks due to dihydroquinidine and quinine is at least 1.0 and (b) the signal-to-noise ratio of the principal peak in the chromatogram obtained with reference solution (d) is at least 5.

Inject reference solution (a), (b), (c) and (d). The chromatogram obtained with reference solution (a) shows a principal peak due to quinine and a peak due to dihydroquinine with a retention time relative to quinine of about 1.4. The chromatogram obtained with reference solution (b) shows a principal peak due to quinidine and a peak due to dihydroquinidine, with a retention time relative to quinidine of about 1.2. The chromatogram obtained with reference solution (c) shows four peaks due to quinine, dihydroquinine, quinidine and dihydroquinidine which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solution (a) and (b).

Inject the test solution and allow the chromatography to proceed for 2.5 times the retention time of the principal peak. Calculate the percentage content of related substances by normalisation, ignoring any peaks the areas of which are less than that of the peak in the chromatogram obtained with reference solution (d) (0.2 per cent). The content of dihydroquinidine is not greater than 15 per cent, the content of any related substance eluting before quinidine is not greater than 5 per cent and the content of any other related substance is not greater than 2.5 per cent.

Other tests. Comply with the tests stated under Tablets.

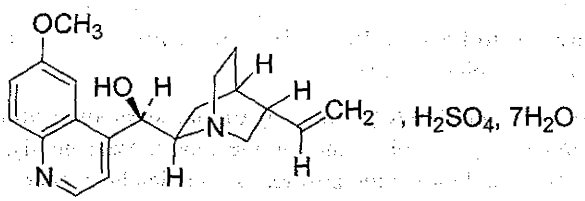
Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 0.4 g of Quinidine Sulphate, dissolve as completely as possible in 40 ml of *acetic anhydride* with the aid of heat and cool. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02610 g of $(C_{20}H_{24}N_2O_2)_2,H_2SO_4,2H_2O$.

Storage. Store protected from light.

Quinine Bisulphate

Quinine Acid Sulphate



$C_{20}H_{24}N_2O_2,H_2SO_4,7H_2O$ Mol. Wt. 548.6

Quinine Bisulphate is (8*S*,9*R*)-6′-methoxycinchonan-9-ol hydrogen sulphate heptahydrate. The alkaloid is obtained from the bark of various species of *Cinchona*.

Quinine Bisulphate contains not less than 98.5 per cent and not more than 101.5 per cent of alkaloid hydrogen sulphates, calculated as $C_{20}H_{24}N_2O_2,H_2SO_4$ on the anhydrous basis.

Category. Antimalarial.

Description. Colourless or faintly yellow crystals or a white or faintly yellow, crystalline powder; efflorescent in dry air.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 60 volumes of *toluene*, 36 volumes of *ether* and 15 volumes of *diethylamine*.

Test solution. Dissolve 1 g of the substance under examination in 100.0 ml *methanol*.

Reference solution (a). A 1.0 per cent w/v solution of *quinine sulphate IPRS* in *methanol*.

Reference solution (b). A 1.0 per cent w/v solution of each of *quinidine sulphate IPRS* and *quinine sulphate IPRS* in *methanol*.

Apply to the plate 4 µl of each solution. After development, dry the plate in air for 15 minutes and repeat the development. Dry the plate at 105° for 30 minutes, allow to cool and spray with *potassium iodoplatinate solution*. The principal spot in the chromatogram obtained with the test solution corresponds to the spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

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B. A 5.0 per cent w/v solution has a blue fluorescence.

C. A 5.0 per cent w/v solution gives the reactions of sulphates (2.3.1).

Tests

pH (2.4.24). 2.8 to 3.4, determined in a 1.0 per cent w/v solution.

Specific optical rotation (2.4.22). −216° to −208°, determined at 20° in a 3.0 per cent w/v solution in *0.1 M hydrochloric acid*.

Other cinchona alkaloids. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in 5 ml of the mobile phase. Heat gently, if necessary to dissolve the powder as completely as possible, cool, dilute to 10.0 ml with the mobile phase and mix.

Reference solution (a). Dissolve 20 mg of *quinine sulphate IPRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Prepare in the same manner as reference solution (a) but using *quinidine sulphate IPRS* in place of *quinine sulphate IPRS*.

Reference solution (c). Mix equal volumes of reference solution (a) and (b).

Reference solution (d). Dilute 1 volume of reference solution (a) to 10 volumes with the mobile phase and dilute 1 volume of the resulting solution to 50 volumes with the mobile phase.

Reference solution (e). A solution containing 0.1 per cent w/v of *thiourea* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- mobile phase: a solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* and 3.0 g of *hexylamine* in 700 ml of *water*, adjusted to pH 2.8 with *1 M orthophosphoric acid*, adding 60 ml of *acetonitrile* and diluting to 1000 ml with *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 250 nm for reference solution (e) and 316 nm for the other solutions,
- injection volume: 10 µl.

Inject reference solution (b) and (e). If necessary, adjust the concentration of acetonitrile in the mobile phase so that in the chromatogram obtained with reference solution (b) the capacity factor of the peak due to quinidine is 3.5 to 4.5; V_0 (the distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak of an unretained component) being calculated from the peak due to thiourea in the chromatogram obtained with reference solution (e).

Inject reference solution (a), (b), (c) and (d). The chromatogram obtained with reference solution (a) shows a principal peak due to quinine and a peak due to dihydroquinine with a retention time relative to quinine of about 1.4. The chromatogram obtained with reference solution (b) shows a principal peak due to quinidine and a peak due to dihydroquinidine, with a retention time relative to quinidine of about 1.2. The chromatogram obtained with reference solution (c) shows four peaks due to quinine, dihydroquinine, quinidine and dihydroquinidine which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solution (a) and (b).

The test is not valid unless (a) in the chromatogram obtained with reference solution (c) the resolution between the peaks due to quinine and quinidine is at least 1.5 and the resolution between the peaks due to dihydroquinidine and quinine is at least 1.0 and (b) the signal-to-noise ratio of the principal peak in the chromatogram obtained with reference solution (d) is at least 5.

Inject the test solution and allow the chromatography to proceed for 2.5 times the retention time of the principal peak. Calculate the percentage content of related substances by normalisation, ignoring any peaks the areas of which are less than that of the peak in the chromatogram obtained with reference solution (d) (0.2 per cent). The content of dihydroquinine is not greater than 10 per cent, the content of any related substance eluting before quinine is not greater than 5 per cent and the content of any other related substance is not greater than 2.5 per cent.

Titratable cation. 75.3 to 79.6 per cent, calculated on the anhydrous basis, determined by the following method. Titrate the combined aqueous solutions reserved in the Assay with *0.1 M hydrochloric acid* using 0.1 ml of *phenolphthalein solution* as indicator.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01632 g of $[C_{20}H_{26}N_2O_2]^{2+}$.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). 19.0 to 25.0 per cent, determined on 0.2 g.

Assay. Weigh 0.45 g, dissolve in 15 ml of *water*, add 25 ml of *0.1 M sodium hydroxide* and extract with three quantities, each of 25 ml, of *chloroform*. Wash each chloroform extract with the same 20 ml of *water*. Combine the aqueous solutions and reserve for the test for Titratable cation. Dry the chloroform extracts with *anhydrous sodium sulphate*, evaporate to dryness at a pressure of 2 kPa and dissolve the residue in 50 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

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1 ml of *0.1 M perchloric acid* is equivalent to 0.02113 g of $C_{20}H_{24}N_2O_2,H_2SO_4$.

Storage. Store protected from light.

Quinine Bisulphate Tablets

Quinine Acid Sulphate Tablets

Quinine Bisulphate Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of quinine bisulphate, $C_{20}H_{24}N_2O_2,H_2SO_4,7H_2O$. The tablets are coated.

Usual strength. 300 mg.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 80 volumes of *toluene*, 20 volumes of *acetone* and 10 volumes of *diethylamine*.

Test solution. Extract a quantity of the powdered tablets containing 0.1 g of Quinine Bisulphate with 10 ml of a mixture of 2 volumes of *chloroform* and 1 volume of *ethanol (95 per cent)* and filter.

Reference solution. A 1.0 per cent w/v solution of *quinine sulphate* in the same solvent mixture.

Apply to the plate 2 µl of each solution. After development, dry the plate in air and spray with *0.05 M ethanolic sulphuric acid* and then with *dilute potassium iodobismuthate solution*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. Extract a quantity of the powdered tablets containing 0.1 g of Quinine Bisulphate with 20 ml of *water* and filter (solution A). To 5 ml of solution A add 0.2 ml of *bromine solution* and 1 ml of *dilute ammonia solution*; an emerald-green colour is produced.

C. Solution A is *levo-rotatory*.

D. Solution A gives the reactions of sulphates (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted if necessary, at the maximum at about 348 nm (2.4.7). Calculate the content of $C_{20}H_{24}N_2O_2,H_2SO_4,7H_2O$ in the medium taking 99 as the specific absorbance at 348 nm.

Q. Not less than 70 per cent of the stated amount of $C_{20}H_{24}N_2O_2,H_2SO_4,7H_2O$.

Other cinchona alkaloids. Determine by liquid chromatography (2.4.14).

Test solution. Remove any coating from the tablets and mix a quantity of the powdered tablet cores containing 50 mg of Quinine Bisulphate with 20 ml of the mobile phase. Heat gently to dissolve the powder as completely as possible, cool, dilute to 25.0 ml with the mobile phase and filter, discarding the first few ml of the filtrate.

Reference solution (a). Dissolve 20 mg of *quinine sulphate IPRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10 ml with the mobile phase.

Reference solution (b). Prepare in the same manner as reference solution (a) but using *quinidine sulphate IPRS* in place of *quinine sulphate IPRS*.

Reference solution (c). Mix equal volumes of reference solution (a) and (b).

Reference solution (d). Dilute 1 volume of reference solution (a) to 10 volumes with the mobile phase and dilute 1 volume of the resulting solution to 50 volumes with the mobile phase.

Reference solution (e). A solution containing 0.1 per cent w/v of *thiourea* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- mobile phase: a solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* and 3.0 g of *hexylamine* in 700 ml of *water*, adjusted to pH 2.8 with *1 M orthophosphoric acid*, adding 60 ml of *acetonitrile* and diluting to 1000 ml with *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 250 nm for reference solution (e) and 316 nm for the other solutions,
- injection volume: 10 µl.

Inject reference solution (b) and (e). If necessary, adjust the concentration of acetonitrile in the mobile phase so that in the chromatogram obtained with reference solution (b) the capacity factor of the peak due to quinidine is 3.5 to 4.5, V_0 (the distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak of an unretained component) being calculated from the peak due to thiourea in the chromatogram obtained with reference solution (e).

Inject reference solution (a), (b), (c) and (d). The chromatogram obtained with reference solution (a) shows a principal peak due to quinine and a peak due to dihydroquinine with a

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retention time relative to quinine of about 1.4. The chromatogram obtained with reference solution (b) shows a principal peak due to quinidine and a peak due to dihydroquinidine, with a retention time relative to quinidine of about 1.2. The chromatogram obtained with reference solution (c) shows four peaks due to quinine, dihydroquinine, quinidine and dihydroquinidine which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solution (a) and (b).

The test is not valid unless (a) in the chromatogram obtained with reference solution (c) the resolution between the peaks due to quinine and quinidine is at least 1.5 and the resolution between the peaks due to dihydroquinidine and quinine is at least 1.0 and (b) the signal-to-noise ratio of the principal peak in the chromatogram obtained with reference solution (d) is at least 5.

Inject the test solution and allow the chromatography to proceed for 2.5 times the retention time of the principal peak. Calculate the percentage content of related substances by normalisation, ignoring any peaks the areas of which are less than that of the peak in the chromatogram obtained with reference solution (d) (0.2 per cent). The content of dihydroquinine is not greater than 10 per cent, the content of any related substance eluting before quinine is not greater than 5 per cent and the content of any other related substance is not greater than 2.5 per cent.

Other tests. Comply with the tests stated under Tablets.

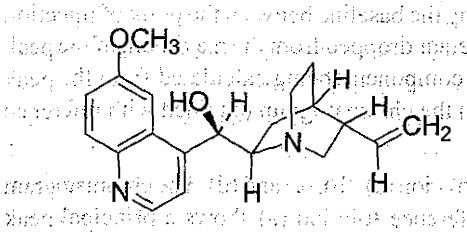
Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 0.6 g of Quinine Bisulphate, dissolve as completely as possible in 40 ml of *acetic anhydride* with the aid of heat and cool. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.05486 g of $C_{20}H_{24}N_2O_2,H_2SO_4,7H_2O$.

Storage. Store protected from light.

Quinine Dihydrochloride

Quinine Acid Hydrochloride



$C_{20}H_{24}N_2O_2,2HCl$ Mol. Wt. 397.3

Quinine Dihydrochloride is the (8*S*,9*R*)-6′–methoxycinchonan-9-ol dihydrochloride. The alkaloid is obtained from the bark of various species of *Cinchona*.

Quinine Dihydrochloride contains not less than 99.0 per cent and not more than 101.5 per cent of alkaloid dihydrochlorides, calculated as $C_{20}H_{24}N_2O_2,2HCl$ on the dried basis.

Category. Antimalarial.

Description. A white or almost white powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 60 volumes of *toluene*, 36 volumes of *ether* and 15 volumes of *diethylamine*.

Test solution. Dissolve 1 g of the substance under examination in 100 ml of *methanol*.

Reference solution (a). A 1.0 per cent w/v solution of *quinine sulphate IPRS* in *methanol*.

Reference solution (b). A 1.0 per cent w/v solution of each of *quinidine sulphate IPRS* and *quinine sulphate IPRS* in *methanol*.

Apply to the plate 4 µl of each solution. After development, dry the plate in air for 15 minutes and repeat the development. Dry the plate at 105° for 30 minutes, allow to cool and spray with *potassium iodoplatinate solution*. The principal spot in the chromatogram obtained with the test solution corresponds to the spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

B. To a 0.5 per cent w/v solution add an equal volume of *dilute sulphuric acid*; a strong blue fluorescence is produced.

C. A 1.0 per cent w/v solution gives the reactions of chlorides (2.3.1).

Tests

pH (2.4.24). 2.0 to 3.0, determined in a 3.0 per cent w/v solution.

Specific optical rotation (2.4.22). −229° to −223°, determined in a 3.0 per cent w/v solution in *0.1 M hydrochloric acid*.

Barium. To 15 ml of a 2 per cent w/v solution add 1 ml of *dilute sulphuric acid*; the solution remains clear for at least 15 minutes.

Other cinchona alkaloids. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in 5 ml of the mobile phase. Heat gently, if

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necessary to dissolve the powder as completely as possible, cool, dilute to 10.0 ml with the mobile phase and mix.

Reference solution (a). Dissolve 20 mg of *quinine sulphate IPRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Prepare in the same manner as reference solution (a) but using *quinidine sulphate IPRS* in place of *quinine sulphate IPRS*.

Reference solution (c). Mix equal volumes of reference solution (a) and (b).

Reference solution (d). Dilute 1 volume of reference solution (a) to 10 volumes with the mobile phase and dilute 1 volume of the resulting solution to 50 volumes with the mobile phase.

Reference solution (e). A solution containing 0.1 per cent w/v of *thiourea* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- mobile phase: a solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* and 3.0 g of *hexylamine* in 700 ml of *water*, adjusted to pH 2.8 with *1 M orthophosphoric acid*, adding 60 ml of *acetonitrile* and diluting to 1000 ml with *water*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 250 nm for reference solution (e) and 316 nm for the other solutions,
- injection volume: 10 µl.

Inject reference solution (b) and (e). If necessary, adjust the concentration of acetonitrile in the mobile phase so that in the chromatogram obtained with reference solution (b) the capacity factor of the peak due to quinidine is 3.5 to 4.5, V_0 (the distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak of an unretained component) being calculated from the peak due to thiourea in the chromatogram obtained with reference solution (e).

Inject reference solution (a), (b), (c) and (d). The chromatogram obtained with reference solution (a) shows a principal peak due to quinine and a peak due to dihydroquinine with a retention time relative to quinine of about 1.4. The chromatogram obtained with reference solution (b) shows a principal peak due to quinidine and a peak due to dihydroquinidine, with a retention time relative to quinidine of about 1.2. The chromatogram obtained with reference solution (c) shows four peaks due to quinine, dihydroquinine, quinidine and dihydroquinidine which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solution (a) and (b).

The test is not valid unless (a) in the chromatogram obtained with reference solution (c) the resolution between the peaks due to quinine and quinidine is at least 1.5 and the resolution between the peaks due to dihydroquinidine and quinine is at least 1.0 and (b) the signal-to-noise ratio of the principal peak in the chromatogram obtained with reference solution (d) is at least 5.

Inject the test solution and allow the chromatography to proceed for 2.5 times the retention time of the principal peak. Calculate the percentage content of related substances by normalisation, ignoring any peaks the areas of which are less than that of the peak in the chromatogram obtained with reference solution (d) (0.2 per cent). The content of dihydroquinine is not greater than 10 per cent, the content of any related substance eluting before quinine is not greater than 5 per cent and the content of any other related substance is not greater than 2.5 per cent.

Titratable cation. 79.7 to 84.2 per cent, calculated on the dried basis, determined by the following method. Weigh 0.4 g, dissolve in 10 ml of *water*, add 40 ml of *methanol* and titrate with *0.1 M sodium hydroxide* using *phenolphthalein solution* as indicator.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01632 g of $[C_{20}H_{26}N_2O_2]^{2+}$.

Sulphates (2.3.17). 0.125 g complies with the limit test for sulphates (0.12 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 3.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.3 g in 50 ml of *anhydrous glacial acetic acid* and add 20 ml of *acetic anhydride* and 10 ml of *mercuric acetate* solution. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent of 0.01987 g of $C_{20}H_{24}N_2O_2,2HCl$.

Storage. Store protected from light.

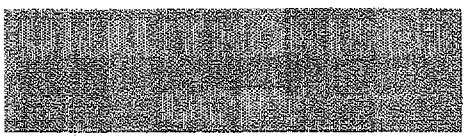
Quinine Dihydrochloride Injection

Quinine Acid Hydrochloride Injection

Quinine Dihydrochloride Injection is a sterile solution of Quinine Dihydrochloride in Water for Injections.

Quinine Dihydrochloride Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of quinine dihydrochloride, $C_{20}H_{24}N_2O_2,2HCl$.

Usual strength. 300 mg per ml.



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Description. A clear, almost colourless to light yellow solution.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 80 volumes of *toluene*, 20 volumes of *acetone* and 10 volumes of *diethylamine*.

Solvent mixture. 2 volumes of *chloroform* and 1 volume of *ethanol (95 per cent)*.

Test solution. Extract a volume of the injection containing 0.1 g of Quinine Dihydrochloride with 10.0 ml of the solvent mixture and filter.

Reference solution (a). A 1 per cent w/v solution of *quinine sulphate IPRS* in the solvent mixture.

Reference solution (b). A solution of 1 per cent w/v of each of *quinidine sulphate IPRS* and *quinine sulphate IPRS* in the solvent mixture.

Apply to the plate 2 µl of each solution. After development, dry the plate in air and spray with *0.05 M ethanolic sulphuric acid* and then with *dilute potassium iodobismuthate solution*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtain with the reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

B. Dilute 1 ml to 20 ml with *water*, add *0.1 M sodium hydroxide* dropwise until the solution becomed turbid. Add 1 or 2 drops of *0.05 M sulphuric acid* to obtain a clear solution. To this solution add an equal volume of *dilute sulphuric acid*; a strong blue fluorescence is produced.

C. It gives the reactions of chlorides (2.3.1).

Tests

pH (2.4.24). 1.5 to 3.0.

Other cinchona alkaloids. Determine by liquid chromatography (2.4.14).

Test solution. Dilute, if necessary a suitable volume of the injection with the mobile phase to produce a solution containing 0.2 per cent w/v of Quinine Dihydrochloride.

Reference solution (a). Dissolve 20 mg of *quinine sulphate IPRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Prepare in the same manner as reference solution (a) but using *quinidine sulphate IPRS* in place of *quinine sulphate IPRS*.

Reference solution (c). Mix equal volumes of reference solution (a) and (b).

Reference solution (d). Dilute 1 volume of reference solution (a) to 10 volumes with the mobile phase and dilute 1 volume of the resulting solution to 50 volumes with the mobile phase.

Reference solution (e). A solution containing 0.1 per cent w/v of *thiourea* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- mobile phase: a solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* and 3.0 g of *hexylamine* in 700 ml of *water*, adjusted to pH 2.8 with *1 M orthophosphoric acid*, adding 60 ml of *acetonitrile* and diluting to 1000 ml with *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 250 nm for reference solution (e) and 316 nm for the other solutions,
- injection volume: 10 µl.

Inject reference solution (b) and (e). If necessary, adjust the concentration of acetonitrile in the mobile phase so that in the chromatogram obtained with reference solution (b) the capacity factor of the peak due to quinidine is 3.5 to 4.5, V_0 (the distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak of an unretained component) being calculated from the peak due to thiourea in the chromatogram obtained with reference solution (e).

Inject reference solution (a), (b), (c) and (d). The chromatogram obtained with reference solution (a) shows a principal peak due to quinine and a peak due to dihydroquinine with a retention time relative to quinine of about 1.4. The chromatogram obtained with reference solution (b) shows a principal peak due to quinidine and a peak due to dihydroquinidine, with a retention time relative to quinidine of about 1.2. The chromatogram obtained with reference solution (c) shows four peaks due to quinine, dihydroquinine, quinidine and dihydroquinidine which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solution (a) and (b).

The test is not valid unless (a) in the chromatogram obtained with reference solution (c) the resolution between the peaks due to quinine and quinidine is at least 1.5 and the resolution factor between the peaks due to dihydroquinidine and quinine is at least 1.0 and (b) the signal-to-noise ratio of the principal peak in the chromatogram obtained with reference solution (d) is at least 5.

Inject the test solution and allow the chromatography to proceed for 2.5 times the retention time of the principal peak. Calculate the percentage content of related substances by normalisation, ignoring any peaks the areas of which are less

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than that of the peak in the chromatogram obtained with reference solution (d) (0.2 per cent). The content of dihydroquinine is not greater than 10 per cent, the content of any related substance eluting before quinine is not greater than 5 per cent and the content of any other related substance is not greater than 2.5 per cent.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

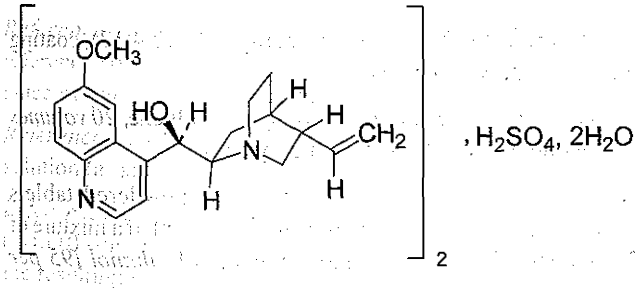
Assay. To a measured volume containing about 0.5 g of Quinine Dihydrochloride add 20 ml of *water* and 5 ml of *sodium hydroxide solution*. Extract with successive quantities, each of 10 ml, of *chloroform* until complete extraction of the alkaloid is effected, washing each extract with the same two quantities, each of 5 ml, of *water*. Remove the chloroform from the combined extracts, dissolve the residue in 50 ml of *anhydrous glacial acetic acid* and add 20 ml of *acetic anhydride*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01987 g of $C_{20}H_{24}N_2O_2,2HCl$.

Storage. Store protected from light.

Labelling. The label states that the solution must be diluted to a strength not exceeding 30 mg per ml before administration and that care should be taken to ensure slow intravenous injection.

Quinine Sulphate



$,H_2SO_4,2H_2O$

$(C_{20}H_{24}N_2O_2)_2,H_2SO_4,2H_2O$ Mol. Wt. 783.0

Quinine Sulphate is (8*S*,9*R*)-6′-methoxycinchonan-9-ol sulphate dihydrate. The alkaloid is obtained from the bark of various species of *Cinchona*.

Quinine Sulphate contains not less than 99.0 per cent and not more than 101.0 per cent of alkaloid monosulphates, calculated as $(C_{20}H_{24}N_2O_2)_2,H_2SO_4$ on the dried basis.

Category. Antimalarial.

Description. White or almost white, needle-like crystals or a crystalline powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 40 volumes of *toluene*, 24 volumes of *ether* and 10 volumes of *diethylamine*.

Test solution. Dissolve 1.0 g of the substance under examination in 100.0 ml of *methanol*.

Reference solution. A 1.0 per cent w/v solution of *quinine sulphate IPRS* in *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air for 15 minutes and repeat the development. Dry the plate at 105° for 30 minutes, allow to cool and spray with *potassium iodoplatinate solution*. The principal spot in the chromatogram obtained with the test solution corresponds to the spot in the chromatogram obtained with the reference solution.

B. To 5 ml of a 0.1 per cent w/v solution add 0.2 ml of *bromine solution* and 1 ml of *dilute ammonia solution*; an emerald-green colour is produced.

C. To a 0.5 per cent w/v solution add an equal volume of *dilute sulphuric acid*; a strong blue fluorescence is produced.

D. A 1 per cent w/v solution gives the reactions of sulphates (2.3.1).

Tests

Appearance of solution. A 2.0 per cent w/v solution in *0.1 M hydrochloric acid* is clear (2.4.1), and not more intensely coloured than reference solution GYS4 (2.4.1).

pH (2.4.24). 5.7 to 6.6, determined in a 1.0 per cent w/v suspension in *water*.

Specific optical rotation (2.4.22). −245° to −237°, determined in a 2.0 per cent w/v solution in *0.1 M hydrochloric acid*.

Other cinchona alkaloids. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in 5 ml of the mobile phase. Heat gently, if necessary to dissolve the powder as completely as possible, cool, dilute to 10.0 ml with the mobile phase and mix.

Reference solution (a). Dissolve 20 mg of *quinine sulphate IPRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Prepare in the same manner as reference solution (a) but using *quinidine sulphate IPRS* in place of *quinine sulphate IPRS*.

Reference solution (c). Mix equal volumes of reference solution (a) and (b).

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Reference solution (d). Dilute 1 volume of reference solution (a) to 10 volumes with the mobile phase and dilute 1 volume of the resulting solution to 50 volumes with the mobile phase.

Reference solution (e). A solution containing 0.1 per cent w/v of *thiourea* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS 5 µm),
- mobile phase: a solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* and 3.0 g of *hexylamine* in 700 ml of *water*, adjusted to pH 2.8 with *1 M orthophosphoric acid*, adding 60 ml of *acetonitrile* and diluting to 1000 ml with *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 250 nm for reference solution (e) and 316 nm for the other solutions,
- injection volume: 10 µl.

Inject reference solution (b) and (e). If necessary, adjust the concentration of acetonitrile in the mobile phase so that in the chromatogram obtained with reference solution (b) the capacity factor of the peak due to quinidine is 3.5 to 4.5, V_0 (the distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak of an unretained component) being calculated from the peak due to thiourea in the chromatogram obtained with reference solution (e).

Inject reference solution (a), (b), (c) and (d). The chromatogram obtained with reference solution (a) shows a principal peak due to quinine and a peak due to dihydroquinine with a retention time relative to quinine of about 1.4. The chromatogram obtained with reference solution (b) shows a principal peak due to quinidine and a peak due to dihydroquinidine, with a retention time relative to quinidine of about 1.2. The chromatogram obtained with reference solution (c) shows four peaks due to quinine, dihydroquinine, quinidine and dihydroquinidine which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with solution (a) and (b).

The test is not valid unless (a) in the chromatogram obtained with reference solution (c) the resolution between the peaks due to quinine and quinidine is at least 1.5 and the resolution between the peaks due to dihydroquinidine and quinine is at least 1.0 and (b) the signal-to-noise ratio of the principal peak in the chromatogram obtained with reference solution (d) is at least 5.

Inject the test solution and allow the chromatography to proceed for 2.5 times the retention time of the principal peak. Calculate the percentage content of related substances by normalisation, ignoring any peaks the areas of which are less than that of the peak in the chromatogram obtained with reference solution (d) (0.2 per cent). The content of dihydroquinine is not greater than 10 per cent, the content of any related substance eluting before quinine is not greater than 5 per cent and the content of any other related substance is not greater than 2.5 per cent.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). 3.0 to 5.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh 0.2 g, dissolve in a mixture of 10 ml of *chloroform* and add 20 ml of *acetic anhydride*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02490 g of $(C_{20}H_{24}N_2O_2)_2,H_2SO_4$.

Storage. Store protected from light.

Quinine Tablets

Quinine Sulphate Tablets

Quinine Sulphate Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of quinine sulphate, $(C_{20}H_{24}N_2O_2)_2,H_2SO_4,2H_2O$. The tablets are coated.

Usual strengths. 100 mg; 300 mg.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 80 volumes of toluene, *20 volumes of* acetone *and 10 volumes of* diethylamine.

Test solution. Extract a quantity of the powdered tablets containing 0.1 g of Quinine Sulphate with 10 ml of a mixture of 2 volumes of *chloroform* and 1 volume of *ethanol (95 per cent)* and filter.

Reference solution. A 1 per cent w/v solution of *quinine sulphate* in the same solvent mixture.

Apply to the plate 2 µl of each solution. After development, dry the plate in air and spray with *0.05 M ethanolic sulphuric acid* and then with *dilute potassium iodobismuthate solution*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. Extract a quantity of the powdered tablets containing 0.1 g of Quinine Sulphate with 20 ml of *water* and filter (solution A).

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To 5 ml of solution A add 0.2 ml of *bromine solution* and 1 ml of *dilute ammonia solution*; an emerald-green colour is produced.

C. Solution A is *levo-rotatory*.

D. Solution A gives the reactions of sulphates (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 100 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted if necessary, at the maximum at about 348 nm (2.4.7). Calculate the content of $C_{20}H_{24}N_2O_2, H_2SO_4, 2H_2O$ in the medium from a solution of known concentration of *quinine sulphate IPRS*.

Q. Not less than 70 per cent of the stated amount of $C_{20}H_{24}N_2O_2, H_2SO_4, 2H_2O$.

Other cinchona alkaloids. Determine by liquid chromatography (2.4.14).

Test solution. Remove any coating from the tablets and mix a quantity of the powdered tablet cores containing 50 mg of Quinine Sulphate with 20 ml of the mobile phase. Heat gently to dissolve the powder as completely as possible, cool, dilute to 25.0 ml with the mobile phase and filter, discarding the first few ml of the filtrate.

Reference solution (a). Dissolve 20 mg of *quinine sulphate IPRS*, with gentle heating if necessary, in 5.0 ml of the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Prepare in the same manner as reference solution (a) but using *quinidine sulphate IPRS* in place of *quinine sulphate IPRS*.

Reference solution (c). Mix equal volumes of reference solution (a) and (b).

Reference solution (d). Dilute 1 volume of reference solution (a) to 10 volumes with the mobile phase and dilute 1 volume of the resulting solution to 50 volumes with the mobile phase.

Reference solution (e). A solution containing 0.1 per cent w/v of *thiourea* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS 5 µm),
- mobile phase: a solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* and 3.0 g of *hexylamine* in 700 ml of *water*, adjusted to pH 2.8 with *1 M orthophosphoric acid*, adding 60 ml of *acetonitrile* and diluting to 1000 ml with *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 250 nm for reference solution (e) and 316 nm for the other solutions,
- injection volume: 10 µl.

Inject reference solution (b) and (e). If necessary, adjust the concentration of acetonitrile in the mobile phase so that in the chromatogram obtained with reference solution (b) the capacity factor of the peak due to quinidine is 3.5 to 4.5, V_0 (the distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak of an unretained component) being calculated from the peak due to thiourea in the chromatogram obtained with reference solution (e).

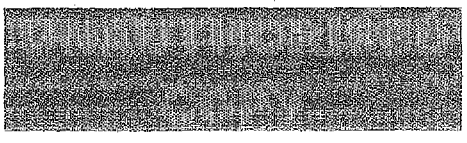
Inject reference solution (a), (b), (c) and (d). The chromatogram obtained with reference solution (a) shows a principal peak due to quinine and a peak due to dihydroquinine with a retention time relative to quinine of about 1.4. The chromatogram obtained with reference solution (b) shows a principal peak due to quinidine and a peak due to dihydroquinidine, with a retention time relative to quinidine of about 1.2. The chromatogram obtained with reference solution (c) shows four peaks due to quinine, dihydroquinine, quinidine and dihydroquinidine which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solution (a) and (b).

The test is not valid unless (a) in the chromatogram obtained with reference solution (c) the resolution between the peaks due to quinine and quinidine is at least 1.5 and the resolution between the peaks due to dihydroquinidine and quinine is at least 1.0 and (b) the signal-to-noise ratio of the principal peak in the chromatogram obtained with reference solution (d) is at least 5.

Inject the test solution and allow the chromatography to proceed for 2.5 times the retention time of the principal peak. Calculate the percentage content of related substances by normalisation, ignoring any peaks the areas of which are less than that of the peak in the chromatogram obtained with reference solution (d) (0.2 per cent). The content of dihydroquinine is not greater than 10 per cent, the content of any related substance eluting before quinine is not greater than 5 per cent and the content of any other related substance is not greater than 2.5 per cent.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.4 g of Quinine Sulphate, dissolve as completely as possible in 40 ml of *acetic anhydride* with the aid of heat and cool. Filter, if necessary through Whatman No.1 filter paper and rinse with an additional 40 ml of *acetic anhydride* in small volumes. Titrate with *0.1 M perchloric*



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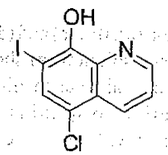
acid, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02610 g of $(C_{20}H_{24}N_2O_2)_2,H_2SO_4,2H_2O$.

Storage. Store protected from light.

Quiniodochlor

Clioquinol; Iodochlorhydroxyquinoline; Iodochlorhydroxyquin



C_9H_5ClINO Mol. Wt. 305.5

Quiniodochlor is 5-chloro-7-iodoquinolin-8-ol.

Quiniodochlor contains not less than 97.0 per cent and not more than 103.0 per cent of C_9H_5ClINO, calculated on the dried basis.

Category. Antiamoebic; topical and intestinal antiseptic.

Description. A yellowish white to brownish yellow powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *quiniodochlor IPRS* or with the reference spectrum of quiniodochlor.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.0005 per cent w/v solution in *3 M hydrochloric acid* shows an absorption maximum at about 267 nm.

C. Burn 20 mg by the oxygen-flask method (2.3.34), using 5 ml of *2 M sodium hydroxide* as the absorbing liquid and dilute to 25 ml with *water*. To 5 ml add 1 ml of *silver nitrate solution*; a yellow precipitate is produced. Add 5 ml of *5 M ammonia*, shake, filter and acidify the filtrate with *nitric acid*; a white precipitate is produced.

Tests

Acidity or alkalinity. Shake 0.5 g with 10 ml of *water* previously neutralised to *phenolphthalein solution*. The solution is colourless and not more than 0.05 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution to pink.

Free iodine. Shake 1.0 g with a solution of 1 g of *potassium iodide* in 20 ml of *water* for 30 seconds, allow to stand for 5 minutes and filter. To 10 ml of the filtrate add 1 ml of *1 M sulphuric acid* and 2 ml of *chloroform* and shake. Any colour in the chloroform layer is discharged on the addition of 0.1 ml of *0.005 M sodium thiosulphate*.

Halide ions. Shake 0.5 g with 25 ml of *water* for 1 minute and filter. To the filtrate add 0.5 ml of *2 M nitric acid* and 0.5 ml of *0.1 M silver nitrate* and allow to stand for 5 minutes. Any opalescence produced is not more intense than that obtained by adding 0.5 ml of *0.1 M silver nitrate* to 25 ml of *water* containing 0.5 ml of *2 M nitric acid* and 0.2 ml of *0.01 M hydrochloric acid* and allowing to stand for 5 minutes.

Related substances. Determine by gas chromatography (2.4.13).

Test solution. Add 0.5 ml of *N,O-bis (trimethylsilyl)acetamide* to 0.5 ml of a solution in *pyridine* containing 0.4 per cent w/v of each of *5-chloro-8- hydroxyquinoline, 5,7-dichloro-8-hydroxy- quinoline* and *5-7-diiodo-8-hydroxyquinoline* and 0.04 per cent w/v of the substance under examination, mix, allow to stand for 15 minutes and add 5 ml of a 0.05 per cent w/v solution of *dibutylphthalate* (internal standard) in *hexane*.

Reference solution (a). Add 0.5 ml of *N,O-bis (trimethylsilyl) acetamide* to a mixture of 0.1 g of the substance under examination and 0.5 ml of *pyridine*, mix, allow to stand for 15 minutes and add 5 ml of *hexane*.

Reference solution (b). Treat a mixture of 0.1 g of the substance under examination and 0.5 ml of *pyridine* as described for the test solution.

Chromatographic system
- a glass column 1.5 m x 4 mm, packed with silanised diatomaceous support (100 to 120 mesh) coated with 3 per cent w/w of methyl silicone gum,
- temperature:
  column 190°,
  inlet port and detector 240°,
- flame ionisation detector,
- flow rate: 30 ml per minute, using nitrogen as the carrier gas.

In the chromatogram obtained with the test solution the peaks following the solvent peak, in order of emergence, are due to (a) 5-chloro-8-hydroxyquinoline, (b) 5,7-dichloro-8-hydroxyquinoline, (c) the internal standard, (d) quiniodochlor and (e) 5,7-diiodo-8-hydroxyquinoline.

In the chromatogram obtained with reference solution (b) calculate the content of 5-chloro-8-hydroxy-quinoline, 5,7-dichloro-8-hydroxyquinoline and 5,7-diiodo-8-hydroxyquinoline by reference to the corresponding peaks in the chromatogram obtained with the test solution.

The total content of the named impurities does not exceed 3.0 per cent w/w, the content of any other impurity does not exceed 0.2 per cent w/w and the sum of the contents is not more than 4.0 per cent w/w.

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa for 5 hours.

Assay. Dissolve 0.3 g in 25 ml of *anhydrous pyridine*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M tetrabutylammonium hydroxide* is equivalent to 0.03055 g of C_9H_5ClINO.

Storage. Store protected from light.

Quiniodochlor Cream

Clioquinol Cream; Iodochlorhydroxyquinoline Cream; Iodochlorhydroxyquin Cream

Quiniodochlor Cream contains Quiniodochlor in a suitable cream base.

Quiniodochlor Cream contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of quiniodochlor, C_9H_5ClINO.

Usual strength. 4 per cent w/w.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution. The test solution and the reference solution prepared by using 1.0 ml of pyridine instead of the internal standard solution.

B. Weigh a quantity containing about 25 mg of quiniodochlor, in a 100-ml volumetric flask, add 75 ml of 25 per cent v/v solution of *hydrochloric acid*, heat on a steam bath to melt the cream, shake vigorously to extract the quiniodochlor. Cool under running water, and dilute to the volume with the same solvent. Dilute 3.0 ml of the filtrate to 100.0 ml with the same solvent.

When examined in the range 200 nm to 400 nm (2.4.7), the solution shows an absorption maximum at about 267 nm.

Tests

Other tests. Comply with the tests stated under Creams.

Assay. Determine by gas chromatography (2.4.13).

Internal standard solution. A 0.2 per cent w/v solution of *pyrene* in *pyridine*.

Solvent mixture. 80 volumes of *pyridine* and 20 volumes of *n-hexane*.

Test solution. Weigh a quantity containing about 150 mg of quiniodochlor, in a 60-ml separator. Place the separator on its side in a vacuum oven at a pressure of about 10 mm of mercury at 45° for 4 hours. Remove the separator from the oven, allow to cool, add 15 ml of the solvent mixture, insert a polytef stopper, and mix. Transfer the mixture to a 50-ml volumetric flask, and rinse the separator with two 15 ml portions of the solvent mixture, shaking each time for 30 seconds. Transfer both rinsings to the volumetric flask, dilute with the solvent mixture to volume, and mix. Transfer 1.0 ml to a screw-capped glass vial fitted with a septum, add 1.0 ml of *bis(trimethylsilyl)acetamide* and 1.0 ml of internal standard solution, attach the cap, and mix. Heat in a water-bath at 50° for 15 minutes, and cool.

Reference solution. A 0.3 per cent w/v solution of *quiniodochlor IPRS* in the solvent mixture. Transfer 1.0 ml to a screw-capped glass vial fitted with a septum, add 1.0 ml of *bis(trimethylsilyl)acetamide* and 1.0 ml of internal standard solution, attach the cap, and mix. Heat in a water-bath at 50° for 15 minutes, and cool.

Chromatographic system
- a glass column 1.83 m x 2 mm, packed with 3 per cent liquid phase G3 on 80 to 100-mesh support S1AB,
- temperature:
  column. 165°,
  inlet port 170° and detector 250°,
- flame ionisation detector,
- flow rate: 30 ml per minute, using nitrogen as the carrier gas.

Inject the reference solution. The test is not valid unless the resolution between the quiniodochlor and the internal standard peaks is not less than 3.0. The relative retention time with reference to pyrene for quiniodochlor is about 0.6.

Inject the reference solution and the test solution.

Calculate the content of C_9H_5ClINO in the Cream.

Storage. Store protected from light and moisture.

Quiniodochlor Ointment

Clioquinol Ointment; Iodochlorhydroxyquinoline Ointment; Iodochlorhydroxyquin Ointment

Quiniodochlor Ointment contains Quiniodochlor in a suitable base.

Quiniodochlor Ointment contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of quiniodochlor, C_9H_5ClINO.

Usual strength. 4 per cent w/w.

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Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution. The test solution and the reference solution prepared by using 1.0 ml of pyridine instead of the internal standard solution.

B. Weigh a quantity containing about 25 mg of quiniodochlor in a 100-ml volumetric flask, add 75 ml of 25 per cent v/v solution of *hydrochloric acid*, heat on steam bath to melt the ointment, shake vigorously to extract the quiniodochlor. Cool under running water, dilute to volume with the same solvent. Filter and dilute 3.0 ml of this solution to 100.0 ml with the same solvent.

When examined in the range 200 nm to 400 nm (2.4.7), the solution shows an absorption maximum at about 267 nm.

Tests

Other tests. Comply with the tests stated under Ointments.

Assay. Determine by gas chromatography (2.4.13).

Internal standard solution. A 0.2 per cent w/v solution of *pyrene* in *pyridine*.

Solvent mixture. 80 volumes of *pyridine* and 20 volumes of *n-hexane* .

Test solution. Transfer an weighed quantity of the ointment, containing about 150 mg of Quiniodochlor, to a suitable conical flask, add 75 ml of *n-hexane*, and mix. Add 15 ml of *dimethylformamide*, and mix for 1 minute. Allow the layers to separate, and transfer the lower layer to a 50-ml volumetric flask, repeat the extraction with separate 15 ml and 10 ml portions of *dimethylformamide*, and transfer the lower layers to the 50-ml volumetric flask. Dilute with *dimethylformamide* to volume, and mix. Transfer 1.0 ml of this solution to a screw-capped glass vial fitted with a septum, and evaporate at about 60° under a stream of nitrogen to dryness. Add 1.0 ml of a mixture of internal standard solution to the residue, add 1.0 ml of *bis(trimethylsilyl)acetamide* and 1.0 ml of internal standard solution, attach the cap, and mix. Heat in a water-bath at 50° for 15 minutes and cool.

Reference solution. A 0.3 per cent w/v solution of *quiniodochlor IPRS* in the solvent mixture. Transfer 1.0 ml to a screw-capped glass vial fitted with a septum, add 1.0 ml of *bis(trimethylsilyl)acetamide* and 1.0 ml of internal standard solution, attach the cap, and mix. Heat in a water-bath at 50° for 15 minutes, and cool.

Chromatographic system
- a glass column 1.83 m x 2 mm, packed with 3 per cent liquid phase G3 on 80 to 100-mesh support S1AB,
- temperature:
  column. 165°,
  inlet port 170° and detector 250°,
- flame ionization detector,
- flow rate: 30 ml per minute using nitrogen as the carrier gas.

Inject the reference solution. The test is not valid unless the resolution between the quiniodochlor and the internal standard peaks is not less than 3. The relative retention time with reference to pyrene for quiniodochlor is about 0.6.

Inject the reference solution and the test solution.

Calculate the content of C_9H_5ClINO in the Ointment.

Storage. Store protected from light and moisture.

Quiniodochlor Tablets

Clioquinol Tablets; Iodochlorhydroxyquinoline Tablets; Iodochlorhydroxyquin Tablets

Quiniodochlor Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of quiniodochlor, C_9H_5ClINO.

Usual strength. 250 mg.

Identification

Triturate a quantity of the powdered tablets containing about 250 mg of Quiniodochlor with 20 ml of *acetone*, filter and add 20 ml of *water* to the filtrate. Collect the precipitate formed on a filter and dry at 105°. The residue complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *quiniodochlor IPRS* or with the reference spectrum of quiniodochlor.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.0005 per cent w/v solution in *3 M hydrochloric acid* shows an absorption maximum at about 267 nm.

Tests

Disintegration (2.5.1). Maximum time, 30 minutes.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and finely powder 20 tablets. Weigh a quantity of the powder containing 0.125 g of Quiniodochlor, shake with 20 ml of hot *2-methoxyethanol*, decant the hot supernatant liquid through a fine filter. Repeat the extraction with two further quantities, of 20 ml and 10 ml, of *2-methoxyethanol*, combine the filtered extracts and dilute to 50.0 ml with *2-methoxyethanol*. To 5.0 ml of this solution add 1 ml of *water* and sufficient of a mixture of 24 volumes of *2-methoxyethanol* and 6 volumes of *water* to produce 50.0 ml. To 10.0 ml of the

solution add 10 ml of *2-methoxyethanol* and 2 ml of a solution prepared by dissolving 0.5 g of *ferric chloride hexahydrate* in 80 ml of *2-methoxyethanol* and adding 0.1 ml of *hydrochloric acid* and sufficient *2-methoxyethanol* to produce 100 ml. Dilute the solution to 25.0 ml with *2-methoxyethanol* and measure the absorbance of the resulting solution at the maximum at about 650 nm (2.4.7), using as blank a solution prepared by treating 10 ml of the aqueous *2-methoxyethanol* in the same manner beginning at the words "add 10 ml of *2-methoxyethanol*.....".

Calculate the content of C_9H_5ClINO from the absorbance obtained using 10.0 ml of a solution prepared in the following manner. Dissolve 0.125 g of *quiniodochlor IPRS* in sufficient *2-methoxyethanol* to produce 50.0 ml, warming to effect solution; add 1 ml of *water* to 5.0 ml of the solution and add sufficient of the mixture of 24 volumes of *2-methoxyethanol* and 6 volumes of *water* to produce 50.0 ml. Using 10.0 ml of this solution repeat the operation beginning at the words "add 10 ml of *2-methoxyethanol*...."

Storage. Store protected from light.

Quiniodochlor and Hydrocortisone Cream

Clioquinol and Hydrocortisone Cream; Iodochlorhydroxyquinoline and Hydrocortisone Cream; Iodochlorhydroxyquin and Hydrocortisone Cream

Quiniodochlor and Hydrocortisone Cream contains Hydrocortisone and Quiniodochlor in a suitable cream base.

Quiniodochlor and Hydrocortisone Cream contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of quiniodochlor, C_9H_5ClINO and not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of hydrocortisone, $C_{21}H_{30}O_5$.

Usual Strength. Quiniodochlor 4 per cent w/w and Hydrocortisone 1 per cent w/w.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silanised silica gel G*.

Mobile phase. A mixture of 77 volumes of *dichloromethane*, 15 volumes of *ether*, 8 volumes of *methanol* and 1.2 volumes of *water*.

Test solution. Disperse by warming and shaking, a quantity of the cream containing 2.5 g of Hydrocortisone in 10 ml of *ethanol (95 per cent)*, cool, allow to stand at 0° for 30 minutes, filter and use the filtrate.

Reference solution (a). A 0.25 per cent w/v solution of *hydrocortisone IPRS* in *ethanol (95 per cent)*.

Reference solution (b). Dissolve 12.5 mg of *hydrocortisone IPRS* in 5 ml of test solution.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and spray with *alkaline tetrazolium blue solution*. The principal spot in the chromatogram obtained with test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

B. In the Assay, the principal peak in the chromatogram due to hydrocortisone obtained with test solution (b) corresponds to the peak due to hydrocortisone in the chromatogram obtained with reference solution.

C. Fuse a quantity of the cream containing 0.1 g of quiniodochlor with *anhydrous sodium carbonate*, dissolve the fused mass in *water* and acidify with *2 M nitric acid*. Add *silver nitrate solution*; a pale yellow precipitate is produced which is insoluble in *5 M ammonia*. Add *5 M ammonia* until the solution becomes alkaline, boil gently, filter and acidify the filtrate with *2 M nitric acid* ; a white precipitate is produced which darkens on exposure to light.

Tests

Other tests. Comply with the tests stated under Creams.

Assay. *For hydrocortisone* — Determine by liquid chromatography (2.4.14).

Test solution (a). Add 30 ml of *2,2,4-trimethylpentane* to a quantity of the cream containing 10 mg of Hydrocortisone and warm on a water-bath until the preparation has melted. Extract the warm mixture with successive quantities of 30, 20 and 20 ml of *methanol (80 per cent)*, combine the aqueous methanolic layers, cool to about 20° and dilute to 100 ml with the same solvent.

Test solution (b). Prepare in the same manner as test solution (a) but adding 10 ml of a 4 per cent v/v solution of *bromobenzene* in *methanol* to the cooled methanolic extract and dilute to 100.0 ml with methanol (80 per cent).

Reference solution. Dissolve 5 mg of *hydrocortisone IPRS* in 5 ml of a 4 per cent v/v solution of *bromobenzene* (internal standard) in *methanol* and dilute to 50.0 ml with *methanol (80 per cent)*.

Chromatographic system
- a stainless steel column 25 cm x 5 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Spherisorb ODS 1),
- mobile phase: *methanol (65 per cent)*,

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– flow rate: 1 ml per minute;
– spectrophotometer set at 242 nm,
– injection volume: 10 µl.

Calculate the content of $C_{21}H_{30}O_5$ in the cream.

For quiniodochlor — Weigh a quantity of the cream containing about 25 mg of quiniodochlor, add 80 ml of a hot mixture of 6 volumes of *water* and 24 volumes of *2-methoxyethanol* and heat on a water-bath for 5 minutes. Cool in ice for 10 minutes, allow to warm to room temperature, dilute to 100 ml with a mixture of 6 volumes of *water* and 24 volumes of *2-methoxyethanol*, mix and filter. To 10 ml of the filtrate, add 10 ml of *2-methoxyethanol* and 2 ml of a solution prepared by dissolving 0.5 g of *iron(III) chloride hexahydrate* in 80 ml of *2-methoxyethanol* and adding 0.1 ml of *hydrochloric acid* and sufficient *2-methoxyethanol* to produce 100 ml. Dilute the solution to 25 ml with *2-methoxyethanol* and measure the absorbance of the resulting solution at the maximum at about 650 nm (2.4.7), using in the reference cell a solution prepared by treating 10 ml of the mixture of 6 volumes of *water* and 24 volumes of *2-methoxyethanol* in the same manner beginning at the words "add 10 ml of *2-methoxyethanol*.....".

Repeat the operation beginning at the words 'add 10 ml of *2-methoxyethanol* using 10 ml of a solution prepared in the following manner. Dissolve 0.125 g of *clioquinol IPRS* in sufficient *2-methoxyethanol* to produce 50 ml, warming to effect solution; add 1 ml of *water* to 5 ml of the solution and add sufficient of a mixture of 6 volumes of *water* and 24 volumes of *2-methoxyethanol* to produce 50 ml.

Calculate the content of C_9H_5ClINO in the cream.

Storage. Store protected from light.

Quiniodochlor and Hydrocortisone Ointment

Clioquinol and Hydrocortisone Ointment; Iodochlorhydroxyquinoline and Hydrocortisone Ointment; Iodochlorhydroxyquin and Hydrocortisone Ointment

Quiniodochlor and Hydrocortisone Ointment contains Quiniodochlor and Hydrocortisone in a suitable base.

Quiniodochlor and Hydrocortisone Ointment contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of quiniodochlor, C_9H_5ClINO and not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of hydrocortisone, $C_{21}H_{30}O_5$.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silanised silica gel G*.

Mobile phase. A mixture of 77 volumes of *dichloromethane*, 15 volumes of *ether*, 8 volumes of *methanol* and 1.2 volumes of *water*.

Test solution. Disperse by warming and shaking, a quantity of the ointment containing 2.5 g of Hydrocortisone in 10 ml of *ethanol (95 per cent)*, cool, and allow to stand at 0° for 30 minutes, filter and use the filtrate.

Reference solution (a). A 0.25 per cent w/v solution of *hydrocortisone IPRS* in *ethanol (95 per cent)*.

Reference solution (b). Dissolve 12.5 mg of *hydrocortisone IPRS* in 5 ml of test solution.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and spray with *alkaline tetrazolium blue solution*. The principal spot in the chromatogram obtained with test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

B. In the Assay, the principal peak in the chromatogram due to hydrocortisone obtained with test solution (b) corresponds to the peak due to hydrocortisone in the chromatogram obtained with reference solution.

C. Fuse a quantity of the ointment containing 0.1 g of Quiniodochlor with *anhydrous sodium carbonate*, dissolve the fused mass in *water* and acidify with *2 M nitric acid*. Add *silver nitrate solution*; a pale yellow precipitate is produced which is insoluble in *5 M ammonia*. Add *5 M ammonia* until the solution becomes alkaline, boil gently, filter and acidify the filtrate with *2 M nitric acid*; a white precipitate is produced which darkens on exposure to light.

Tests

Other tests. Comply with the tests stated under Ointments.

Assay. *For hydrocortisone* — Determine by liquid chromatography (2.4.14).

Test solution (a). Add 30 ml of *2,2,4-trimethylpentane* to a quantity of the ointment containing 10 mg of Hydrocortisone and warm on a water-bath until the preparation has melted. Extract the warm mixture with successive quantities of 30, 20 and 20 ml of *methanol (80 per cent)*, combine the aqueous methanolic layers, cool to about 20° and dilute to 100 ml with the same solvent.

Test solution (b). Prepare at the same manner as test solution (a) but adding 10 ml of a 4 per cent v/v solution of

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bromobenzene in *methanol* to the cooled methanolic extract and dilute to 100.0 ml with *methanol (80 per cent)*.

Reference solution. Dissolve 5 mg of *hydrocortisone IPRS* in 5 ml of a 4 per cent v/v solution of *bromobenzene* (internal standard) in *methanol* and dilute to 50 ml with *methanol (80 per cent)*.

Chromatographic system

- a stainless steel column 25 cm x 5 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Spherisorb ODS 1),
- mobile phase: *methanol (65 per cent)*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 242 nm,
- injection volume: 10 µl.

Calculate the content of $C_{21}H_{30}O_5$ in the ointment.

For quiniodochlor — Weigh a quantity of the ointment containing 25 mg of Clioquinol, add 80 ml of a hot mixture of 24 volumes of *2-methoxy-ethanol* and 6 volumes of *water* and heat on a water-bath for 5 minutes. Cool in ice for 10 minutes, allow to warm to room temperature, dilute to 100 ml with a mixture of 24 volumes of *2-methoxy-ethanol* and 6 volumes of *water*, mix and filter. To 10 ml of the filtrate, add 10 ml of *2-methoxyethanol* and 2 ml of a solution prepared by dissolving 0.5 g of *iron(III) chloride hexahydrate* in 80 ml of *2-methoxyethanol* and adding 0.1 ml of *hydrochloric acid* and sufficient *2-methoxyethanol* to produce 100 ml. Dilute the solution to 25 ml with *2-methoxyethanol* and measure the absorbance of the resulting solution at the maximum at 650 nm (2.4.7), using in the reference cell a solution prepared by treating 10 ml of a mixture of 24 volumes of *2-methoxy-ethanol* and 6 volumes of *water* in the same manner beginning at the words "add 10 ml of *2-methoxyethanol*.....".

Repeat the operation beginning at the words 'add 10 ml of *2-methoxyethanol* using 10 ml of a solution prepared in the following manner. Dissolve 0.125 g of *clioquinol IPRS* in sufficient *2-methoxyethanol* to produce 50 ml, warming to effect solution; add 1 ml of *water* to 5 ml of the solution and add sufficient of a mixture of 6 volumes of *water* and 24 volumes of *2-methoxyethanol* to produce 50 ml.

Calculate the content of C_9H_5ClINO in the ointment.

Storage. Store protected from light.

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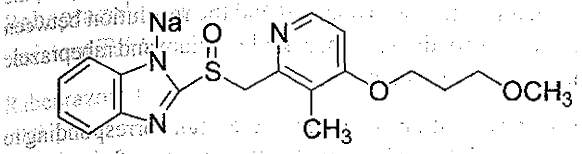
Rabeprazole Sodium



$C_{18}H_{20}N_3O_3S,Na$ Mol. Wt. 381.4

Rabeprazole sodium is 2-({[4-(3-methoxypropoxy)-3-methyl-2-pyridinyl]methyl}sulphinyl)-1*H*-benzimidazole sodium.

Rabeprazole sodium contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{18}H_{20}N_3O_3S,Na$, calculated on the anhydrous basis.

Category. Antiulcer.

Description. A white to light yellow, crystalline powder, hygroscopic.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rabeprazole sodium IPRS* or with the reference spectrum of rabeprazole sodium.

B. A 10 per cent w/v solution in *carbon dioxide-free water* gives reaction of sodium (2.3.1).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of *methanol*, 20 volumes of *water* and 0.1 volume of *diethylamine*.

Test solution. Dissolve 50 mg of the substance under examination in 100.0 ml with the solvent mixture.

Reference solution (a). A 0.05 per cent w/v solution of *rabeprazole sodium IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with solvent mixture.

Use chromatographic system as described under Assay.

Inject reference solution (b). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram three times of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than 1.5 times the area of the peak in the chromatogram obtained with the reference solution (b) (1.5 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not more than 7.0 per cent, determined on 0.3 g.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of *methanol*, 20 volumes of *water* and 0.1 volume of *diethylamine*.

Test solution. Dissolve 0.1 g of the substance under examination in 100.0 ml of solvent mixture. Dilute 5.0 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution. A 0.005 per cent w/v solution of *rabeprazole sodium IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octylsilane bonded to porous silica (5 µm) (Such as Hypersil keystone betabasic C8),
- column temperature 40°,
- mobile phase: a mixture of 72 volumes of *0.1 M phosphate buffer pH 7.0* and 28 volumes of *acetonitrile*,
- flow rate: 1.4 ml per minute,
- spectrophotometer set at 282 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the percentage content of $C_{18}H_{20}N_3O_3S,Na$.

Storage. Store protected from light and moisture, at a temperature not exceeding 25°.

Rabeprazole Injection

Rabeprazole Sodium Injection

Rabeprazole Injection is a sterile material consisting of Rabeprazole Sodium with or without excipients. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

The constituted solution complies with the requirements for the Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Rabeprazole Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of rabeprazole sodium, $C_{18}H_{21}N_3O_3SNa$.

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Usual strength. 20 mg.

Description. A white or off white powder.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 10.8 to 12.5, determine in a 0.8 per cent w/v of Rabeprazole Sodium solution.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use, protect from light.

Solvent mixture. Dissolve 2.725 g of *potassium dihydrogen orthophosphate* and 0.525 g of *dipotassium hydrogen orthophosphate* into 1000 ml of *water*, mix and adjusted to pH 10.0 with *1M potassium hydroxide.*

Test solution. Reconstitute 1 vial with 5.0 ml of *water*, shake. Reconstitute 4 more vials. Pool the contents of 5 vials to prepare a composite sample. Dilute a suitable volume of pooled sample with solvent mixture to obtain a solution containing 0.1 per cent w/v of rabeprazole sodium.

Reference solution (a). Weigh about 2.5 mg each of *rabeprazole sulphide IPRS* and *rabeprazole sulphone IPRS* dissolve in 2.5 ml of *methanol* and dilute to 25.0 ml with the solvent mixture.

Reference solution (b). Dissolve 50 mg of *rabeprazole sodium IPRS* in 30 ml of *water*, add 5.0 ml of reference solution (a) and dilute to 50.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5µm),
- sample temperature: 5°,
- mobile phase: a mixture of 30 volumes of *acetonitrile* and 70 volumes of buffer solution prepared by dissolving 2.725 g of *potassium dihydrogen orthophosphate* and 0.525 g of *dipotassium hydrogen orthophosphate* into 1000 ml of *water*, mix and adjusted to pH 7.0 with *1 M potassium hydroxide*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the resolution between the peak corresponding to rabeprazole sodium and rabeprazole sulphone is not less than 1.5.

Inject the test solution. The area of the peak corresponding to rabeprazole sulphide multiplied with correction factor 0.96 is not more than 1.0 per cent and the area of the peak corresponding to rabeprazole sulphone multiplied with correction factor 1.19 is not more than 1.0. per cent. The area of any secondary peak is not more than 0.5 per cent and the sum of areas of all secondary peaks is not more than 2.5 per cent, calculated by area normalization.

Water (2.3.43). Not more than 7.0 per cent, determined on 0.1 g.

Bacterial endotoxins (2.2.3). Not more than 5 Endotoxin Units per mg of rabeprazole sodium.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use and protect from light and air.

Test solution. Reconstitute 1 vial with 5.0 ml of *water*, shake. Reconstitute 4 more vials. Pool the contents of 5 vials and prepare a composite sample. Transfer an amount, equivalent to 20 mg of Rabeprazole Sodium, to a 100-ml volumetric flask, and dilute with *water* to volume.

Reference solution. A 0.02 per cent w/v solution of *rabeprazole sodium IPRS* in *water.*

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5µm),
- sample temperature: 5°,
- mobile phase: a mixture of 30 volumes of *acetonitrile* and 70 volumes of buffer solution prepared by dissolving 2.725 g of *potassium dihydrogen orthophosphate* and 0.525 g of *dipotassium hydrogen orthophosphate* into 1000 ml of *water*, mix and adjusted to pH 7.0 with *1 M potassium hydroxide*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{21}N_3O_3SNa$ in the injection.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

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Rabeprazole Gastro-resistant Tablets

Rabeprazole Tablets; Rabeprazole Sodium Gastro-resistant Tablets; Rabeprazole Sodium Tablets

Rabeprazole Gastro-resistant Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of rabeprazole sodium, $C_{18}H_{20}N_3O_3SNa$. They are made gastro-resistant by enteric-coating or by other means.

Usual strengths. 10 mg; 20 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

A. Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid,*
Speed and time. 50 rpm and 2 hours.

Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes *of methanol,* 20 volumes of *water* and 0.1 volume of *diethylamine.*

Test solution. Withdraw the medium completely and disperse the intact tablet in *0.1M sodium hydroxide* (20 volume of the total volume), with the aid of ultrasound and dilute to volume with the solvent mixture to obtain a solution containing 0.02 per cent w/v of Rabeprazole Sodium and filter.

Reference solution. Dissolve 20 mg of *rabeprazole sodium IPRS* in 20 ml of *0.1M sodium hydroxide* and dilute to 100.0 ml with the solvent mixture.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{20}N_3O_3S,Na$ released in the acid medium by subtracting the content of $C_{18}H_{20}N_3O_3S,Na$ in the test solution from the total content of Rabeprazole Sodium $C_{18}H_{20}N_3O_3S,Na$ determined in the Assay.

Complies with the acceptance criteria given under acid stage.

B. Apparatus No. 2 (Paddle),
Medium. 900 ml of *phosphate buffer pH 7.4,*
Speed and time. 75 rpm and 45 minutes.

Transfer another 6 tablets and run the apparatus for 2 hours in *0.1 M hydrochloric acid.* Decant the medium without losing the tablets, add phosphate buffer pH 7.4 and run the apparatus for 45 minutes. Withdraw a suitable volume of the medium, dilute if necessary, with the dissolution medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. To 5.0 ml of the filtrate, add immediately 1.0 ml of *0.1M sodium hydroxide.*

Reference solution. A 0.055 per cent w/v solution of *rabeprazole sodium IPRS* in *0.1M sodium hydroxide.* Dilute 2.0 ml of the solution to 100.0 ml with the dissolution medium. To 5.0 ml of the solution, add immediately 1.0 ml of *0.1M sodium hydroxide.*

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{20}N_3O_3S,Na$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{18}H_{20}N_3O_3S,Na$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of *methanol,* 20 volumes of *water* and 0.1 volume of *diethylamine.*

Test solution. Weigh a quantity of the powdered tablets containing 50 mg of Rabeprazole Sodium, disperse in 100 ml of solvent mixture and filter.

Reference solution (a). A 0.05 per cent w/v solution of *rabeprazole sodium IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with solvent mixture.

Use chromatographic system as described under Assay.

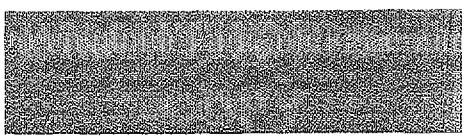
Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram three times of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than twice the area of the peak in the chromatogram obtained with reference solution (b) (2.0 per cent) and the sum of areas of all the secondary peaks is not more than 6 times the area of the peak in the chromatogram obtained with the reference solution (b) (6.0 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay using the following solutions.

Test solution. Disperse one tablet in *0.1M sodium hydroxide* (20 volume of the total volume), with the aid of ultrasound and dilute to volume with the solvent mixture to obtain a solution containing 0.02 per cent w/v of Rabeprazole Sodium and filter.



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Reference solution. Dissolve 20 mg of *rabeprazole sodium IPRS* in 20 ml of *0.1 M sodium hydroxide* and dilute to 100.0 ml with the solvent mixture.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{20}N_3O_3S$,Na in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of *methanol*, 20 volumes of *water* and 0.1 volume of *diethylamine*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of Rabeprazole Sodium in 20 ml of *0.1 M sodium hydroxide* and dilute to 100.0 ml with solvent mixture, filter.

Reference solution. Dissolve 25 mg of *rabeprazole sodium IPRS* in 10 ml of *0.1 M sodium hydroxide* and dilute to 50.0 ml with solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of 0.15 per cent w/v solution of *potassium dihydrogen phosphate* previously adjusted to pH 6.0 with *orthophosphoric acid* or *sodium hydroxide solution* and 35 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{20}N_3O_3SNa$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Rabeprazole Gastro-resistant and Itopride Prolonged-release Capsules

Rabeprazole Sodium Gastro-resistant and Itopride Hydrochloride Prolonged-release Capsules

Rabeprazole Sodium Gastro-resistant and Itopride Hydrochloride Prolonged release Capsules manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the itopride hydrochloride of different manufacturers may not be the same.

Rabeprazole Gastro-resistant and Itopride Prolonged release Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of rabeprazole sodium, $C_{18}H_{20}N_3O_3SNa$ and itopride hydrochloride, $C_{20}H_{26}N_2O_4HCl$.

Usual strength. Rabeprazole Sodium 20 mg and Itopride Hydrochloride 150 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

For Rabeprazole Sodium —

NOTE — Prepare the solutions immediately before use, protect the solutions from light.

A. Apparatus No. 2 (Paddle),
Medium. 750 ml of *0.1 M hydrochloric acid*,
Speed and time. 50 rpm and 2 hours.

Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of mobile phase A and B.

Test solution. Withdraw the medium completely without any loss of residue. Transfer the residue into 50-ml volumetric flask, disperse in 25 ml of mobile phase B, with the aid of ultrasound with intermittent shaking for 10 minutes, at a temperature not exceeding 20°. Add 15 ml of mobile phase A, further ultrasound for 25 minutes with intermittent shaking, at a temperature not exceeding 20°, allow to be at room temperature and dilute to volume with mobile phase A, filter. Dilute 5.0 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution. A 0.002 per cent w/v solution of *rabeprazole sodium IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as Hypersil BDS C8),
- sample temperature: 10°,
- mobile phase: A. a buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 7.3 with *triethylamine*,
  B. equal volumes of *acetonitrile* and *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 284 nm,
- injection volume: 20 µl.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	62	38
11	62	38
12	40	60
15	40	60
16	62	38
20	62	38

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{20}N_3O_3SNa$ released in the acid medium by subtracting the content of $C_{18}H_{20}N_3O_3SNa$ in the test solution from the total content of rabeprazole sodium $C_{18}H_{20}N_3O_3SNa$ determined in the Assay.

Complies with the acceptance criteria given under acid stage.

B. Apparatus No. 2 (Paddle),

Medium: 1000 ml of a buffer solution pH 8.0 prepared by dissolving 1.56 g of *anhydrous disodium hydrogen phosphate* in 900 ml of *water*, adjusted to pH 8.0 with *dilute orthophosphoric acid* and dilute to 1000 ml with *water*. Add 5 g of *sodium lauryl sulphate* and mix,

Speed and time: 100 rpm and 30 minutes.

Determine by liquid chromatography (2.4.14).

Transfer another 6 capsules and run the apparatus for 2 hours in *0.1 M hydrochloric acid*. Decant the medium without losing the residue, add the buffer solution pH 8.0 and run the apparatus for 30 minutes. Withdraw a suitable volume of the medium and filter.

Test solution. To 5.0 ml of the filtrate, add immediately 2.0 ml of *0.2M sodium hydroxide* and dilute to 10.0 ml with the dissolution media.

Reference solution. Dissolve 50 mg of *rabeprazole sodium IPRS* in 10 ml of *0.2 M sodium hydroxide*, with the aid of ultrasound, and dilute to 100.0 ml with the dissolution medium. To 1.0 ml of the solution, add 10 ml of *0.2 M sodium hydroxide* and diluteto 50.0 ml with the dissolution medium.

Use chromatographic system as described under test A, using following modification.

– sample temperature: 20°,

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{20}N_3O_3S,Na$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{18}H_{20}N_3O_3S,Na$.

For Itopride Hydrochloride — Complies with the test stated under capsules.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use, protect the solutions from light.

Solvent mixture. Equal volumes of mobile phase A and B.

Test solution. Disperse a quantity of the mixed powered content of the capsules containing 100 mg of Rabeprazole Sodium in 250 ml of mobile phase B, with the aid of ultrasound for 10 minutes with intermittent shaking, at a temperature not exceeding 20°. Add 100 ml of mobile phase A, further ultrasound for 20 minutes with intermittent shaking, at a temperature not exceeding 20°, allow to be at room temperature and dilute to 500.0 ml with mobile phase A, filter.

Reference solution. A solution containing 0.02 per cent w/v of *rabeprazole sodium IPRS* and 0.15 per cent w/v of *itopride hydrochloride IPRS* in the solvent mixture. Dilute 2.0 ml of the solution to 200.0 ml with the solvent mixture.

Use chromatographic system as described under Dissolution with the following modification.

– a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Kromasil C18),

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	75	25
60	50	50
70	50	50
72	75	25
80	75	25

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak at a relative retention time of about 0.21, 0.52 and 1.83 with respect to itopride peak, each of, is not more than 0.75 times the area of the itopride peak in the chromatogram obtained with the reference solution (0.75 per cent), the sum of the areas of all the secondary peaks related to itopride is not more than the area of the itopride peak in the chromatogram obtained with the reference solution (1.0 per cent). The area of any other secondary peak is not more than the area of the rabeprazole peak in the chromatogram obtained with the reference solution (1.0 per cent) and the sum of the areas of all the secondary peaks, excluding itopride impurities is not more

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than twice the area of the rabeprazole peak in the chromatogram obtained with the reference solution (2.0 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14), as described under Dissolution with the following modifications.

Test solution. Mix the contents of 20 capsules. Disperse a quantity of the mixed powdered contents equivalent to 100 mg of Rabeprazole Sodium in 125 ml of mobile phase B, with the aid of ultrasound with intermittent shaking for 10 minutes, at a temperature not exceeding 20°. Add 100 ml of mobile phase A, further ultrasound with intermittent shaking for 25 minutes,at a temperature not exceeding 20°. Allow to be at room temperature and dilute to 250.0 ml with mobile phase A, filter. Dilute 5.0 ml of the solution to 100.0 ml with the solvent mixture.

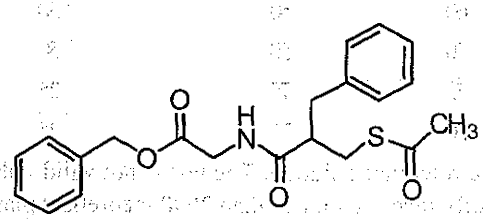
Reference solution. A solution containing 0.04 per cent w/v of *rabeprazole sodium IPRS* and 0.3 per cent w/v of *itopride hydrochloride IPRS* in the solvent mixture. Dilute 5.0 ml of the solution to 100.0 ml with the solvent mixture.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{20}N_3O_3SNa$ and $C_{20}H_{26}N_2O_4HCl$ in the capsules.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Racecadotril



$C_{21}H_{23}NO_4S$ Mol. Wt. 385.5

Racecadotril is (*RS*)-Benzyl-*N*-[3-(acetylthio)-2-benzylpropanoyl]glycinate.

Racecadotril contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{21}H_{23}NO_4S$, calculated on the dried basis.

Category. Antidiarrhoea.

Description. A white or almost white powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *racecadotril IPRS* or with the reference spectrum of racecadotril.

Tests

Appearance of solution. A 50 per cent w/v solution in *acetone* is clear (2.4.1) and not more intensely coloured than reference solution YS6 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of mobile phase A and mobile phase B.

Test solution (a). Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 25.0 ml with the solvent mixture.

Test solution (b). Dilute 5.0 ml of test solution (a) to 25.0 ml with the solvent mixture.

Reference solution (a). Dilute 1.0 ml of test solution (a) to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution (b). Dilute 500 µl of *racecadotril impurity A IPRS* in *acetonitrile* and dilute to 250.0 ml with *acetonitrile*. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution (c). A 0.01 per cent w/v solution of *racecadotril impurity G IPRS (benzyl [[(2RS)-2-benzyl-3-sulfanylpropanoyl]amino]acetate IPRS)* in the solvent mixture. To 5 ml of the solution add 1.0 ml of test solution (b) and dilute to 100.0 ml with the solvent mixture.

Reference solution (d). A 0.04 per cent w/v solution of *racecadotril IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. dissolve 1.0 g of *potassium dihydrogen phosphate* in *water*, adjust the pH to 2.5 with *orthophosphoric acid* and dilute to 1000 ml with *water*,

B. *acetonitrile*,

- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Time (in min.)	mobile phase A (per cent v/v)	mobile phase B (per cent v/v)
0	60	40
5	60	40
25	20	80
35	20	80
38	60	40

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Name	Relative retention time	Correction factor
Racecadotril impurity A[1]	0.2	—
Racecadotril impurity C[2]	0.3	1.4
Racecadotril impurity E[3]	0.5	0.6
Racecadotril impurity F[4]	0.9	0.7
Racecadotril (Retention time: about 16 minutes)	1.0	—

[1]thioacetic acid,

[2][[(2*RS*)-2-[(acetylsulfanyl)methyl]-3-phenylpropanoyl] amino]acetic acid,

[3]2-benzylacrylic acid,

[4]benzyl [(2-benzylprop-2-enoyl)amino]acetate.

Inject reference solution (c). The test is not valid unless the resolution between the peaks corresponding to racecadotril impurity G and racecadotril is not less than 1.5.

Inject reference solution (a), (b) and test solution (a). In the chromatogram obtained with test solution (a), the area of any peak due to racecadotril impurities C, E and F is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). The area of the peak corresponding to racecadotril impurity A is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent). The area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent). The sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in vacuum at 60° for 4 hours.

Assay. Determine by liquid chromatography (2.4.14) as described under test for Related substances with the following modification.

Inject reference solution (d) and test solution (b).

Calculate the content of $C_{21}H_{23}NO_4S$.

Racecadotril Capsules

Racecadotril Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of racecadotril, $C_{21}H_{23}NO_4S$.

Usual strength. 100 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle) with sinkers,

Medium. 900 ml of *0.05 M acetate buffer* in 1.0 per cent w/v solution of *sodium lauryl sulphate*, prepared by dissolving 6.804 g of *sodium acetate trihydrate* in sufficient *water* to produce 800 ml. Add 10 g of *sodium lauryl sulphate* and dissolve, adjusted to pH 4.5 with *glacial acetic* acid and dilute to 1000 ml with *water*,

Speed and time. 100 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14),

Solvent mixture. Equal volumes of *water* and *acetonitrile*.

Test solution. Use the filtrate and, if necessary, dilute with the dissolution medium.

Reference solution. A 0.11 per cent w/v solution of *racecadotril IPRS* in the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with dissolution medium.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 40 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000ml *water*, adjust to pH 3.0 with *orthophosphoric acid* and 60 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 231 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{23}NO_4S$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{21}H_{23}NO_4S$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Solvent mixture. Equal volumes of *water* and *acetonitrile*.

Test solution. Disperse a quantity of the mixed contents of 20 capsules containing 200 mg of Racecadotril in 50.0 ml of the

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solvent mixture with the aid of ultrasound for 30 minutes and dilute to 100.0 ml with the solvent mixture, filter.

Reference solution. A 0.05 per cent w/v solution of *racecadotril IPRS* in the solvent mixture. Dilute 10.0 ml of the solution to 25.0 ml with the solvent mixture. Further dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml *water*, adjust to pH 3.0 with *orthophosphoric acid*, 40 volumes of *acetonitrile* and 10 volumes of *methanol*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 231 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 5000 theoretical plates, the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. Run the chromatograms 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent). Ignore any peak up to retention time 2.5 minutes.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *water* and *acetonitrile*.

Test solution. Disperse a quantity of the mixed content of 20 capsules containing 400 mg of Racecadotril in 150 ml of the solvent mixture with the aid of ultrasound for 30 minutes and dilute to 200 ml with the solvent mixture, filter. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.02 per cent w/v solution of *racecadotril IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 40 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml *water*, adjust to pH 3.0 with *orthophosphoric acid* and 60 volumes of a mixture of equal volumes of *acetonitrile* and *methanol*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 231 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{23}NO_4S$ in the capsules.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Racecadotril Sachet

Racecadotril Sachet contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of racecadotril, $C_{21}H_{23}NO_4S$.

Usual strengths. 10 mg; 30 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.05 M acetate buffer* in 1.0 per cent w/v solution of *sodium lauryl sulphate*, prepared by dissolving 6.804 g of *sodium acetate trihydrate* in sufficient *water* to produce 800 ml. Add 10 g of *sodium lauryl sulphate* and dissolve, adjust the pH to 4.5 with *glacial acetic acid* and dilute to 1000 ml with *water*,

Speed and time. 100 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter,

Determine by liquid chromatography (2.4.14),

Solvent mixture. Equal volumes of *water* and *acetonitrile*.

Test solution. Use the filtrate and if necessary, dilute with the dissolution medium.

Reference solution. Dissolve a quantity of *racecadotril IPRS* in the solvent mixture and dilute with the dissolution medium to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 40 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 60 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 231 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{23}NO_4S$ in the sachet.

Q. Not less than 75 per cent of the stated amount of $C_{21}H_{23}NO_4S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powder containing 80 mg of Racecadotril in 70 ml *acetonitrile* with the aid of ultrasound for 30 minutes and dilute to 100.0 ml with *acetonitrile*, filter.

Reference solution. A 0.02 per cent w/v solution of *racecadotril IPRS* in *acetonitrile*. Dilute 1.0 ml of the solution to 25.0 ml with *acetonitrile*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 60 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 40 volumes of *acetonitrile*,

  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 231 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
15	85	15
20	60	40
50	60	40
55	85	15
60	85	15

Inject the reference solution. The test is not valid unless the column efficiency in not less than 3000 theoretical plates, the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent).

Other tests. Comply with the tests stated under Oral Powders.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *water* and *acetonitrile*.

Test solution. Disperse a quantity of powder containing 150 mg of Racecadotril in 150 ml of the solvent mixture with the aid of ultrasound for 30 minutes and dilute to 500.0 ml with the solvent mixture, filter.

Reference solution. A 0.03 per cent w/v solution of *racecadotril IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 40 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 60 volumes of a mixture of equal volumes of *acetonitrile* and *methanol*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 231nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

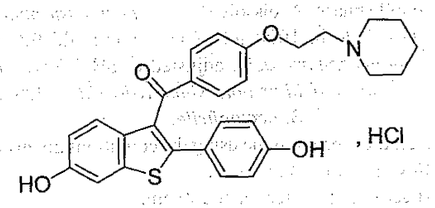
Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{23}NO_4S$ in the sachet.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Labelling. The label states, 'Should be administered orally, directly or mixed with water or beverage. While mixing with water stir well and swallow the total mixture immediately.'

Raloxifene Hydrochloride



$C_{28}H_{27}NO_4S,HCl$ Mol. Wt. 510.0

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Raloxifene Hydrochloride is [6-Hydroxy-2-(4-hydroxyphenyl) benzo[b]thien-3-yl][4-(2-piperidinoethoxy)phenyl]methanone hydrochloride.

Raloxifene Hydrochloride contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{28}H_{27}NO_4S,HCl$, calculated on the dried basis.

Description. A white to pale yellow, powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *raloxifene hydrochloride IPRS* or with the reference spectrum of raloxifene hydrochloride.

B. A solution in *methanol* gives reactions of chlorides (2.3.1).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 70 volumes of mobile phase A and 30 volumes of *acetonitrile*.

Test solution. Dissolve 30 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). A 0.0003 per cent w/v solution of *raloxifene hydrochloride IPRS* in the solvent mixture.

Reference solution (b). Dissolve 6 mg of *raloxifene hydrochloride IPRS* in the mixture of 15.0 ml of *acetonitrile*, 3.0 ml of *water* and 5.0 ml of 30 per cent *hydrogen peroxide* (unstabilized). Shake for 30 minutes and keep at 30° for minimum 6 hours and dilute to 50.0 ml with mobile phase A (Raloxifene hydrochloride is partly converted to raloxifene-*N*-oxide under these conditions).

Reference solution (c). Dissolve 15 mg of *raloxifene hydrochloride IPRS* in the solvent mixture and add 5.0 ml of reference solution (b) and dilute to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: A. dissolve 9.0 g of *monobasic potassium phosphate* in 1000 ml of *water* and add 0.6 ml of *orthophosphoric acid*, adjusted to pH 3.0 with *ortho phosphoric acid* or *potassium hydroxide* solution,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	75	25
9	75	25
40	50	50
42	75	25
49	75	25

The relative retention time with reference to raloxifene for raloxifene impurity I is about 0.74.

Inject reference solution (c). The test is not valid unless the resolution between the raloxifene-*N*-oxide and principal peak is not less than 3.0 and the tailing factor is not more than 2.0.

Inject reference solution (a) and the test solution. Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of raloxifene impurity I ([6-hydroxy-2-(4-hydroxyphenyl) benzo[b]thien-3,7-diyl]bis[4-[2-(1-piperidinyl)ethoxy] phenyl] methanone) is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with area less than 0.5 times the area of principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 5 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). A 0.005 per cent w/v solution of *raloxifene hydrochloride IPRS* in the mobile phase.

Reference solution (b). Dissolve 6 mg of *raloxifene hydrochloride IPRS* in the mixture of 15 ml of *acetonitrile*, 3 ml of *water* and 5 ml of 30 per cent *hydrogen peroxide* (unstabilized). Shake for 30 minutes and keep at 30° for minimum 6 hours and dilute to 50.0 ml with mobile phase A (Raloxifene hydrochloride is partly converted to raloxifene-*N*-oxide under these conditions).

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (3.5 µm),

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– column temperature: 35°,
– mobile phase: a mixture of 33 volumes of *acetonitrile* and 67 volumes of buffer solution prepared by dissolving 7.2 g of *monobasic potassium phosphate* in 1000 ml of *water* and add 1.5 ml of *orthophosphoric acid*, adjusted to pH 2.5 with *orthophosphoric acid* or *potassium hydroxide* solution,
– flow rate: 1.5 ml per minute,
– spectrophotometer set at 280 nm,
– injection volume: 10 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the raloxifene-N-oxide and principal peak is not less than 2.0 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 0.7 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{28}H_{27}NO_4S,HCl$.

Storage. Store protected from moisture.

Raloxifene Hydrochloride Tablets

Raloxifene Hydrochloride Tablets contain not less than 93.0 per cent and not more than 107.0 per cent of the stated amount of raloxifene hydrochloride, $C_{28}H_{27}NO_4S,HCl$.

Usual strength. 60 mg.

Identification

A. Disperse a quantity of powdered tablets containing 120 mg of Raloxifene Hydrochloride with 20 ml of *water*. Centrifuge, and discard the supernatant. Add 5 ml of *isopropyl alcohol*, shake to form a slurry, filter, and rinse the residue with *isopropyl alcohol*. Dry the residue at 105° for 30 minutes. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *raloxifene hydrochloride IPRS* or with the reference spectrum of raloxifene hydrochloride.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (b).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 1000 ml of 0.1 per cent w/v of polysorbate 80,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Triethylamine phosphate suspension. Add 2.0 ml of *triethylamine* to 500 ml of *acetonitrile*, adjusted to pH 4.0 with *orthophosphoric acid. (NOTE — Trimethylamine phosphate will precipitate, keep the suspension well mixed).*

Test solution. Dilute the filtrate, if necessary with the dissolution medium. Mix 1 volume of the solution with 1 volume of *triethylamine phosphate suspension.*

Reference solution. Dissolve a quantity of *raloxifene hydrochloride IPRS* in *methanol* and dilute with the dissolution medium to obtain a solution having known concentration similar to that of the test solution. Mix 1 volume of the solution with 1 volume of *triethylamine phosphate suspension.*

Chromatographic system
– a stainless column 15 cm x 4.6 mm, packed with nitrile group chemically bonded to porous silica (3.5µm),
– mobile phase: a mixture of 50 volumes of *acetonitrile*, 50 volumes of *water* and 0.2 volume of *triethylamine*, adjusted to pH 4.0 with *orthophosphoric acid*,
– flow rate: 2 ml per minute,
– spectrophotometer set at 290 nm,
– injection volume: 50 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_{28}H_{27}NO_4S,HCl$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{28}H_{27}NO_4S,HCl$.

Related substances. Determine by liquid chromatography (2.4.14).

Buffer solution. Dissolve 9.0 g of *monobasic potassium phosphate* in *water* and dilute to 1000 ml with *water*. Add 0.5 ml of *orthophosphoric acid*, adjusted to pH 3.0 with *orthophosphoric acid* or *potassium hydroxide solution.*

Solvent mixture. 60 volumes of *acetonitrile* and 40 volumes of buffer solution.

Test solution. Disperse a quantity of powdered tablets with the solvent mixture to obtain a solution containing 0.6 per cent w/v of Raloxifene Hydrochloride. Dilute 5.0 ml of the solution to 10.0 ml with mobile phase A to obtain a solution containing 0.3 per cent w/v of Raloxifene Hydrochloride and filter.

Reference solution (a). To 6 mg of *raloxifene hydrochloride IPRS*, add 15.0 ml of *acetonitrile*, 3.0 ml of *water* and 5.0 ml of

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30 per cent *hydrogen peroxide* (unstabilized). Mix and dissolve. Shake the solution for 30 minutes, followed by 30 minutes of ultrasound. Allow it to stand at 30° for 6 hours and then dilute to 50.0 ml with the solvent mixture. (*NOTE — Raloxifene hydrochloride is partly converted to raloxifene N-oxide under these conditions. The reaction time can be varied as necessary to achieve an appropriate level of raloxifene N-oxide*).

Reference solution (b). To 15 mg of *raloxifene hydrochloride IPRS*, add 5.0 ml of reference solution (a) and 20 ml of the solvent mixture and then dilute to 50.0 ml with mobile phase A.

Reference solution (c). A 0.006 per cent w/v solution of *raloxifene hydrochloride IPRS* in the solvent mixture.

Reference solution (d). Mix 5 ml of reference solution (c) and 45 ml of the solvent mixture and dilute to 100.0 ml with mobile phase A to obtain a solution containing 0.0003 per cent w/v of Raloxifene Hydrochloride.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: A. a mixture of 75 volumes of buffer solution and 25 volumes of *acetonitrile*,
  B. a mixture of 50 volumes of buffer solution and 50 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	100	0
36	0	100
38	100	0
48	100	0

Name	Relative retention time
Raloxifene	1.0
Raloxifene *N*-oxide[1]	1.17

[1]Methanone, [6-hydroxy-2-(4-hydroxyphenyl) benzo[*b*]thien-3-yl][4-[2-(1-oxido-1-piperidinyl)ethoxy]phenyl].

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to raloxifene and raloxifene *N*-oxide is not less than 3.0 and the tailing factor is not more than 2.0.

Inject reference solution (d) and the test solution. Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any peak corresponding to raloxifene N-oxide is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.3 per cent). The area of any other secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent). The sum of the areas of all the secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (d) (1.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Buffer solution. Dissolve 7.2 g of *monobasic potassium phosphate* in 1000 ml of *water*. Add 1.3 ml of *orthophosphoric acid*, adjusted to pH 2.5 with *orthophosphoric acid* or *potassium hydroxide solution*.

Solvent mixture. 60 volumes of *acetonitrile* and 40 volumes of buffer solution.

Test solution. Disperse a quantity of powdered tablets with the solvent mixture to obtain a solution having a concentration of 0.006 per cent w/v of Raloxifene Hydrochloride, filter.

Reference solution (a). Prepare as given in the related substances.

Reference solution (b). A 0.006 per cent w/v solution of *raloxifene hydrochloride IPRS* in the solvent mixture.

Chromatographic system

- a stainless column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 33 volumes of *acetonitrile* and 67 volumes of buffer solution,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the raloxifene-N-oxide and principal peak is not less than 2.0 in the chromatogram obtained with reference solution (a), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution.

Calculate the content of $C_{28}H_{27}NO_4S.HCl$ in the tablets.

Storage. Store protected from light and moisture.

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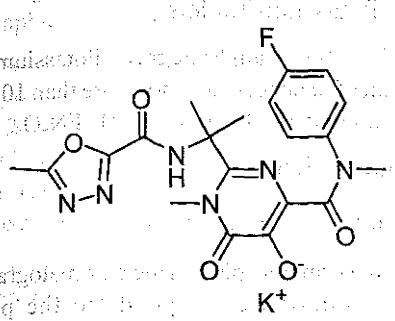
Raltegravir Potassium



$C_{20}H_{20}FKN_6O_5$ Mol. Wt. 482.5

Raltegravir Potassium is 4- pyrimidinecarboxamide,N-[(4-fluorophenyl) methyl]-1,6-dihydro-5-hydroxy-1-methyl-2-[1-methyl-1-[[(5-methyl-1,3,4-oxadiazol-2-yl) carbonyl] amino]ethyl]-6-oxo-monopotassium salt ; Potassium 4-[(4-fluorobenzyl) carbamoyl] -1-methyl-2-(1-methyl1-{[(5-methyl-1,3,4-oxadiazol-2-yl) carbonyl] amino} ethyl) -6-oxo-1,6-dihydropyrimidin-5-olate.

Raltegravir Potassium contains not less than 98.0 per cent and not more than 102. 0 per cent of $C_{20}H_{20}FKN_6O_5$, calculated on the anhydrous basis.

Category. Antiretroviral

Description. A white to off white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum obtained with *raltegravir potassium IPRS* or with the reference spectrum of raltegravir potassium.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

C. Mix 1 ml each of 10 per cent w/v solution of *sodium cobaltinitrite*, 4 per cent w/v solution of substance under examination and *dilute acetic acid*. A yellow or orange yellow precipitate is formed immediately.

Tests

Related substances. Determine by liquid chromatography (2.4.14), using the solvent mixture, test solution and chromatographic system as described under Assay.

Reference solution (a). A solution containing 0.01 per cent w/v of *raltegravir potassium IPRS* and 0.00002 per cent w/v of *raltegravir related compound E IPRS* in the solvent mixture.

Reference solution (b). A 0.00001 per cent w/v solution of *raltegravir potassium IPRS* in the solvent mixture.

Reference solution (c). A 0.2 per cent w/v solution of *raltegravir potassium IPRS* in *1 M sodium hydroxide*. Stir the solution for 2 hours at room temperature. To 5.0 ml of the solution, add 5 ml of *1 M hydrochloric acid* and dilute to 50.0 ml with the solvent mixture, (Degradation generates raltegravir amine and raltegravir oxalylacetohydrazide analog).

Name	Relative retention time
Raltegravir amine[1]	0.43
Raltegravir formididyl analog[2]	0.60
Raltegravir oxalylacetohydrazide analog[3]	0.72
Raltegravir related compound E[4]	0.94
Raltegravir	1.0
Raltegravir E-ethoxyacethydrazone analog[5]	1.1
Raltegravir Z-ethoxyacethydrazone analog[6]	1.2

[1]2-(2-Aminopropan-2-yl) –N- (4-fluorobenzyl)-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

[2](E)-2-(2-{[(Dimethylamino) methylidene] amino} proan-2-yl)-N-(4-fluorobenzyl)-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

[3]2-{2-[2-(2-Acetylhydrazinyl)-2-oxoacetamido]propan-2-yl}-N-(4-fluorobenzyl)-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

[4]N-{2-[4-(Benzylcarbamoyl)-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl]propan-2-yl}-5-methyl-1,3,4-oxadiazole-2-carboxamide,

[5]Ethyl(E)-N-{2-[(2-{4-[(4-fluorobenzyl)carbamoyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl}propan-2-yl)amino]-2-oxoacetyl}acetohydrazonate,

[6]Ethyl(Z)-N-{2-[(2-{4-[(4-fluorobenzyl)carbamoyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl}propan-2-yl)amino]-2-oxoacetyl}acetohydrazonate.

Inject reference solution (a). The test is not valid unless the resolution between raltegravir related compound E and raltegravir peak is not less than 1.5.

Inject reference solution (c) for peaks identification.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of peak due to raltegravir amine, raltegravir formididyl analog, raltegravir related compound E and raltegravir Z-ethoxyacethydrazone analog is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent), the area of peak due to raltegravir oxalylacetohydrazide analog and raltegravir E-ethoxyacethydrazone analog is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent). The sum of areas of all the secondary peaks is not more than 7 times the area of the principal peak in the

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chromatogram obtained with reference solution (b) (0.7 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). 1 g complies with limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not more than 0.6 per cent, using equal volumes of *methanol* and *formamide* as solvent. [*NOTE — Hydranal-Composite 2 to be used as a reagent*].

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 25 volumes of *acetonitrile* and 75 volumes of *water*.

Test solution. Dissolve 10 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *raltegravir potassium IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm packed with phenyl group bonded to porous silica (3.5 µm),
- column temperature: 15°,
- mobile phase: A. a 0.1 per cent v/v solution of *orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	75	25
2	75	25
5	60	40
10	55	45
19	10	90
22	5	95
25	75	25

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{20}FKN_6O_5$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Raltegravir Tablets

Raltegravir Potassium Tablets

Raltegravir Tablets contain Raltegravir Potassium equivalent to not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of raltegravir, $C_{20}H_{21}FN_6O_5$.

Usual strength. 400 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *water*,

Speed and time. 100 rpm and 15 minutes and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a suitable quantity of *raltegravir potassium IPRS* in minimum amount of *acetonitrile* (20 per cent) and dilute with the dissolution medium to obtain a solution having a known concentration similar to the concentration of the test solution.

Chromatographic system

- a stainless steel column 10 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Onyx monolithic C18),
- column temperature: 40°,
- mobile phase: a mixture of 62 volumes of a buffer solution prepared by dissolving 1.36 g of *monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 38 volumes of *acetonitrile*,
- flow rate: 5 ml per minute,
- spectrophotometer set at 303 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{21}FN_6O_5$ in the medium.

Not less than 15 per cent and not more than 45 per cent of the stated amount of $C_{20}H_{21}FN_6O_5$ is dissolved in 15 minutes.

Q. Not less than 70 per cent at 60 minutes of the stated amount of $C_{20}H_{21}FN_6O_5$.

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Related substances. Determine by liquid chromatography (2.4.14), using solvent mixture (a), (b), test solution and chromatographic system as described under Assay.

Reference solution (a). A 0.2 per cent w/v solution of *raltegravir potassium IPRS* in *1 M sodium hydroxide*, stir the solution for 2 hours. To 5.0 ml of the solution, add 5 ml of *1 M hydrochloric acid* and dilute to 50.0 ml with solvent mixture (b). (Degradation generates raltegravir amine and raltegravir oxalylacetohydrazide analog)

Reference solution (b). A solution containing 0.01 per cent w/v of *raltegravir potassium IPRS* and 0.00002 per cent w/v of *raltegravir related compound E IPRS* in solvent mixture (b).

Reference solution (c). A 0.000044 per cent w/v solution of *raltegravir potassium IPRS* in solvent mixture (b).

Name	Relative retention time	Correction factor
Raltegravir amine*	0.14	—
Raltegravir formididyl analog*	0.29	—
Raltegravir oxalyl analog[1]	0.33	—
Raltegravir oxalylacetohydrazide analog	0.48	1.33
Raltegravir related compound E*	0.83	—
Raltegravir	1.0	—

*This is a process impurity controlled in the drug substance and not monitored in the drug product,

[1]2-{(2-{4-[(4-Fluorobenzyl)carbamoyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl}propan-2-yl}amino}-2-oxoacetic acid.

Inject reference solution (b) and (c). The test is not valid unless the resolution between raltegravir related compound E and raltegravir peaks is not less than 1.5 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 10.0 per cent in the chromatogram obtained with reference solution (c).

Inject reference solution (a) for peak identification.

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution, the area of peak corresponding to raltegravir oxalyl analog is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent), the area of peak corresponding to raltegravir oxalylacetohydrazide analog is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent) and the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent). The sum of areas of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.8 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture (a). Equal volumes of *acetonitrile* and *water.*

Solvent mixture (b). 30 volumes of *acetonitrile* and 70 volumes of *water.*

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 500 mg of Raltegravir in 30 ml of solvent mixture (a), with the aid of ultrasound with intermittent shaking and dilute to 50.0 ml with solvent mixture (a). Centrifuge and use clear supernatant solution. Dilute 2.0 ml of the solution to 100.0 ml with solvent mixture (b).

Reference solution. A 0.022 per cent w/v solution of *raltegravir potassium IPRS* in solvent mixture (b).

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 71 volumes of a buffer solution prepared by dissolving 1.36 g of *monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 2.5 with *orthophosphoric acid* and 29 volumes of *acetonitrile,*
- flow rate: 2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 30 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

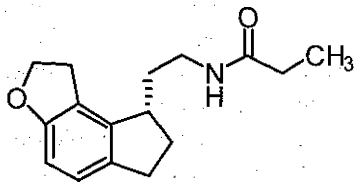
Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{21}FN_6O_5$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of raltegravir.

Ramelteon



$C_{16}H_{21}NO_2$ Mol Wt. 259.3

Ramelteon is (*S*)-*N*-[2-(1,6,7,8-tetrahydro-2*H*-indeno-[5,4-b]furan-8-yl)ethyl]propionamide.

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Ramelteon contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{16}H_{21}NO_2$, calculated on the anhydrous basis.

Category. Hypnotic, Sedative.

Description. A white to cream powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ramelteon IPRS* or with the reference spectrum of ramelteon.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solution A. Dissolve 3.4 g of *potassium dihydrogen orthophosphate* and 4.68 g of *sodium octanesulphonate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*.

Solvent mixture. 75 volumes of solution A and 25 volumes of *acetonitrile*.

Test solution. Dissolve 25 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). A 0.05 per cent w/v solution of *ramelteon IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v each of cyano impurity and amine impurity in the solvent mixture.

Reference solution(c). Weigh 12.5 mg of *ramelteon IPRS* and add 625 µl of reference solution (b) into 25-ml of volumetric flask and dilute with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 90 volumes of solution A and 10 volumes of *acetonitrile*,
  B. a mixture of 90 volumes of *acetonitrile* and 10 volumes of *water*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0.01	60	40
11	60	40
22	45	55
40	45	55
40.5	60	40
50	60	40

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to amine impurity and ramelteon is not less than 10, the column efficiency is not less than 3000 theoretical plates and tailing factor is not more than 2.0.

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution, the area of peak due to cyano impurity [(E)-(1,6,7,8-tetrahydro-2H-indeno [5,4-b]furan-8 ylidene) acetonitrile] is not more than 0.5 per cent, amine impurity [(S)-2-(1,6,7,8-tetrahydro-2H-indeno [5,4-b]furan-8 yl) ethyl amine hydrochloride] is not more than 0.5 per cent and any other secondary peak is not more than 0.3 per cent. The sum of areas of all the secondary peak is not more than 1.0 per cent.

R Isomer Impurity. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 12.5 mg of the substance under examination in 25 ml of the mobile phase.

Reference solution (a). A 0.05 per cent w/v solution of *ramelteon IPRS* in the mobile phase.

Reference solution (b). Weigh 5 mg of *ramelton R-isomer impurity IPRS* in a 50-ml volumetric flask and dilute with the mobile phase.

Reference solution(c). Weigh 12.5 mg of *ramelteon IPRS* and add 625 µl of reference solution (b) into 25-ml of volumetric flask and dilute with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with chiralcel OD-H (5 µm),
- mobile phase: a mixture of 90 volumes of *n-hexane*, 10 volumes of *ethanol* and 0.1 volume of *trifluoroacetic acid*,
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Inject reference solution(c). The test is not valid unless the resolution between ramelton R-isomer impurity and principal peak due to ramelton is not less than 1.

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Inject reference solution (a), and the test solution. In the chromatogram obtained with the test solution per cent area of peak due to R-isomer is not more than 0.5 per cent.

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 0.3 g.

Assay. Determine by liquid chromatography (2.4.14).

Solution A. Dissolve 3.4 g of *potassium dihydrogen orthophosphate* and 4.68 g of *sodium octanesulphonate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*.

Solvent mixture. 75 volumes of solution A and 25 volumes of *acetonitrile*.

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *ramelton IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 90 volumes of solution A and 10 volumes of *acetonitrile*,
  B. a mixture of 90 volumes of *acetonitrile* and 10 volumes of *water*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

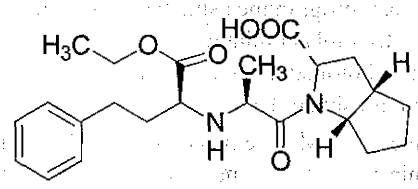
Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0.01	60	40
11	60	40
18	40	60
26	40	60
26.5	60	40
35	60	40

Inject the reference solution. The test is not valid unless, the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution and the test solution.

Calculate the content of $C_{16}H_{21}NO_2$.

Ramipril



$C_{23}H_{32}N_2O_5$ Mol. Wt. 416.5

Ramipril is (2*S*,3a*S*,6a*S*)-1-[(*S*)-2-[[(*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl] octahydrocyclopenta[*b*] pyrrole-2-carboxylic acid.

Ramipril contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{23}H_{32}N_2O_5$, calculated on the dried basis.

Category. Antihypertensive.

Description. A white or almost white, crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ramipril IPRS* or with the reference spectrum of ramipril.

Tests

Appearance of solution. A 1.0 per cent w/v solution in *methanol* is clear (2.4.1) and colourless (2.4.1).

Specific optical rotation (2.4.22). +32.0° to +38.0°, determined in 1.0 per cent w/v solution in *0.1 M methanolic hydrochloric acid*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in 25.0 ml of mobile phase B.

Reference solution (a). A 0.1 per cent w/v solution of *ramipril IPRS* in the mobile phase B.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with mobile phase B.

Chromatographic system
- a stainless steel column 25 cm x 4.0 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 65°,
- mobile phase: A. dissolve 2.0 g of *sodium perchlorate* in a mixture of 0.5 ml of *triethylamine* and 800 ml of *water;* adjusted to pH 3.6 with *orthophosphoric acid* and add 200 ml of *acetonitrile*,
  B. dissolve 2.0 g of *sodium perchlorate* in a mixture of 0.5 ml of *triethylamine* and 300 ml of

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water; adjusted to pH 2.6 with *orthophosphoric acid* and add 700 ml of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
6	90	10
7	75	25
20	65	35
30	25	75
40	25	75
45	90	10
55	90	10

Inject reference solution (a). The test is not valid unless the tailing factor is not more than 2.0 and the column efficiency in not less than 2000 theoretical plates.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than the area of the peak in the chromatogram obtained with the reference solution (b) (1.0 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.2 per cent, determined on 1.0 g by drying in an oven at 60°, under vacuum, for 4 hours.

Assay. Dissolve 0.3 gm in 25 ml of *methanol* and add 25 ml of *water*. Titrate with *0.1 M sodium hydroxide*. Determine the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.04165 gm of $C_{23}H_{32}N_2O_5$.

Storage. Store protected from light and moisture.

Ramipril Capsules

Ramipril Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ramipril, $C_{23}H_{32}N_2O_5$.

Usual strengths. 1.25 mg; 2.5 mg; 5 mg; 10 mg.

Identification

Shake a quantity of the content of the capsules containing 25 mg of Ramipril with 50 ml of *acetone*, centrifuge for 10 minutes, filter. Evaporate the filtrate to dryness at 60° for 3 hours. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ramipril IPRS* or with the reference spectrum of ramipril.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 500 ml of *0.1 M hydrochloric acid*,

Speed and time. 75 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Dilute the filtrate to get 0.00025 per cent w/v solution of Ramipril with *0.1 M hydrochloric acid*.

Reference solution. A 0.00025 per cent w/v solution of *ramipril IPRS* in *0.1 M hydrochloric acid*.

Chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{32}N_2O_5$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{23}H_{32}N_2O_5$.

Uniformity of content. Complies with the test stated under Capsules.

Determine by liquid chromatography (2.4.14).

Test solution. Disperse one capsule in 100 ml of *0.1 M hydrochloric acid*, sonicate for 15 minutes. Dilute if necessary, to produce 0.00025 per cent w/v solution of Ramipril in *0.1 M hydrochloric acid*.

Reference solution. A 0.00025 per cent w/v solution of *ramipril IPRS* in *0.1 M hydrochloric acid*.

Chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{32}N_2O_5$.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the content of capsules containing 25 mg of Ramipril, disperse in 100.0 ml of *0.1 M hydrochloric acid*, mix and centrifuge. Dilute 1.0 ml of the resulting solution to 100.0 ml with *0.1 M hydrochloric acid*.

Reference solution. A 0.00025 per cent w/v solution of *ramipril IPRS* in *0.1 M hydrochloric acid*.

Chromatographic system
- a stainless steel column 12.5 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),

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– mobile phase: a mixture of 42 volumes of *acetonitrile* and 58 volumes of a solution containing 1.4 per cent w/v solution of *sodium perchlorate* and 0.58 per cent w/v solution of *orthophosphoric acid* adjusted to pH 2.5 with *triethylamine*, adjust the pH of the mixture to 2.1 with *orthophosphoric acid*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 210 nm,
– injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{32}N_2O_5$.

Storage. Store protected from light and moisture.

Ramipril Tablets

Ramipril Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ramipril, $C_{23}H_{32}N_2O_5$.

Usual strengths. 1.25 mg; 2.5 mg; 5 mg; 10 mg.

Identification

Shake a quantity of the powdered tablets containing 25 mg of Ramipril with 50 ml of *acetone*, centrifuge for 10 minutes, filter. Evaporate the filtrate to dryness at 60° for 3 hours. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ramipril IPRS* or with the reference spectrum of ramipril.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 500 ml of *0.1 M hydrochloric acid*,
Speed and time. 75 rpm and 45 minutes.
Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Dilute the filtrate to get 0.00025 per cent w/v solution of Ramipril with *0.1 M hydrochloric acid*.

Reference solution. A 0.00025 per cent w/v solution of *ramipril IPRS* in *0.1 M hydrochloric acid*.

Chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{32}N_2O_5$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{23}H_{32}N_2O_5$.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay.

Test solution. Take one tablet, add 5 ml of *0.1 M hydrochloric acid*, sonicate for 10 minutes, dilute, if necessary, with sufficient *0.1 M hydrochloric acid* to produce a solution containing 0.00025 per cent w/v of Ramipril, centrifuge and use the supernatant liquid.

Calculate the content of $C_{23}H_{32}N_2O_5$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of powdered tablets containing 25 mg of Ramipril, disperse in 100.0 ml of *0.1 M hydrochloric acid* and centrifuge. Dilute 1.0 ml of the resulting solution to 100.0 ml with *0.1 M hydrochloric acid*.

Reference solution. A 0.00025 per cent w/v solution of *ramipril IPRS* in *0.1 M hydrochloric acid*.

Chromatographic system
– a stainless steel column 12.5 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
– mobile phase: a mixture of 42 volumes of *acetonitrile* and 58 volumes of a solution containing 1.4 per cent w/v solution of *sodium perchlorate* and 0.58 per cent w/v solution of *orthophosphoric acid*, adjusted to pH 2.5 with *triethylamine*, adjust the pH of the mixture to 2.1 with *orthophosphoric acid*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 210 nm,
– injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{32}N_2O_5$.

Storage. Store protected from light and moisture.

Ramipril and Hydrochlorothiazide Tablets

Ramipril and Hydrochlorothiazide Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of ramipril, $C_{23}H_{32}N_2O_5$ and hydrochlorothiazide, $C_7H_8ClN_3O_4S_2$.

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Usual strength. Ramipril 2.5 mg and Hydrochlorothiazide 12.5 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution corresponds to the principal peaks in the chromatogram obtained with reference solution (c).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium 750 ml of *0.1 M hydrochloric acid,*
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Dilute the filtrate, if necessary, with the dissolution medium.

Reference solution (a). Dissolve a quantity of *ramipril IPRS* in the mobile phase and dilute with dissolution medium to obtain a solution having a known concentration similar to the test solution.

Reference solution (b). Dissolve a quantity of *hydrochlorothiazide IPRS* in mobile phase and dilute with dissolution medium to obtain a solution having a known concentration similar to the test solution.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Thermo quest Hypersil),
- mobile phase: a mixture of 55 volumes of *water*, 45 volumes of *acetonitrile* and 0.1 volume of *triethylamine*, adjusted to pH 3.0 with *orthophoshoric acid,*
- flow rate: 0.7 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject reference solution (a) and (b). The relative standard deviation for replicate injections for each peak is not more than 2.0 per cent.

Inject reference solution (a), (b) and the test solution.

Calculate the content of $C_{23}H_{32}N_2O_5$ and $C_7H_8ClN_3O_4S_2$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{23}H_{32}N_2O_5$ and $C_7H_8ClN_3O_4S_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing about 10 mg of Ramipril in mobile phase A, sonicate for 15 minutes and dilute to 10.0 ml with the same solvent.

Reference solution (a). A solution containing 0.2 per cent w/v of *ramipril IPRS* and 1.0 per cent w/v of *hydrochlorothiazide IPRS* in mobile phase A.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 50.0 ml with mobile phase A. Dilute 5.0 ml of the solution to 100.0 ml with the same solvent.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: A. a mixture of 60 volumes of buffer solution prepared by dissolving 4 g of *sodium perchlorate* in 600 ml of *water*, add 1.0 ml of *triethylamine*, adjusted to pH 2.6 with *orthophosphoric acid* and 40 volumes of *acetonitrile*,
  B. a mixture of 85 volumes of buffer solution and 15 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	0	100
13	0	100
17	50	50
20	100	0
80	100	0
82	0	100
87	0	100

The relative retention time with reference to ramipril for ramipril impurity A is about 0.9, for ramipril impurity B is about 1.2, for ramipril impurity C is about 1.5 and for ramipril impurity D is about 1.7.

Inject reference solution (b). The test is not valid unless the relative standard deviation for replicate injections for each peak is not more than 5.0 per cent.

Inject reference solution (b) and the test solution. The retention time of ramipril is about 30 minutes and of hydrochlorothiazide is about 10 minutes. In the chromatogram obtained with the test solution the area of each peak due to ramipril impurity A, B and C is not be more than the area of the principal peak due to ramipril in the chromatogram obtained

with reference solution (b) (1.0 per cent); the area of peak due to ramipril impurity D is not more than 7 times the area of the principal peak due to ramipril in the chromatogram obtained with reference solution (b) (7.0 per cent); the area of any other secondary peak is not more than the area of the principal peak due to ramipril in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore the peak due to hydrochlorothiazide.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay.

Test solution. Disperse 1 intact tablet in *water* and dilute to 25.0 ml with the mobile phase. Dilute 3.0 ml of the solution to 25.0 ml with the mobile phase and mix.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of powder containing about 25 mg of Ramipril, disperse in *water* and dilute to 250.0 ml with the mobile phase, filter. Dilute 3.0 ml of the solution to 25.0 ml with the mobile phase and mix.

Reference solution (a). A 0.024 per cent w/v solution of *ramipril IPRS* in the mobile phase.

Reference solution (b). A 0.06 per cent w/v solution of *hydrochlorothiazide IPRS* in the mobile phase.

Reference solution (c). Dilute reference solution (a) and (b) with the mobile phase to obtain a solution having a known concentration similar to the test solution.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Thermo quest Hypersil),
- mobile phase: a mixture of 55 volumes of *water*, 45 volumes of *acetonitrile* and 0.1 volume of *triethylamine*, adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

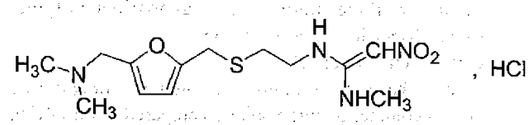
Inject reference solution (c). The test is not valid unless the relative standard deviation for replicate injections for each peak is not more than 2.0 per cent.

Inject reference solution (c) and the test solution.

Calculate the content of $C_{23}H_{32}N_2O_5$ and $C_7H_8ClN_3O_4S_2$ in the tablet.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Ranitidine Hydrochloride



$C_{13}H_{22}N_4O_3S,HCl$ Mol. Wt. 350.9

Ranitidine Hydrochloride is *N*-[2-[[[5-[(dimethylamino) methyl]furan-2-yl]methyl]thio]ethyl]-*N*-methyl-2-nitroethene-1,1-diamine hydrochloride.

Ranitidine Hydrochloride contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{13}H_{22}N_4O_3S,HCl$, calculated on the dried basis.

Category. Antiulcer (Histamine H_2-receptor antagonist).

Description. A white to pale yellow, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ranitidine hydrochloride IPRS* or with the reference spectrum of ranitidine hydrochloride.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

C. A 5 per cent w/v solution gives the reactions of chlorides (2.3.1).

Tests

Appearance of solution. A 1.0 per cent w/v solution is clear (2.4.1), and not more intensely coloured than reference solution BYS5 (2.4.1).

pH (2.4.24). 4.5 to 6.0, determined in a 1.0 per cent w/v solution in *carbon dioxide-free water*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 13 mg of the substance under examination in mobile phase A and dilute to 100.0 ml with mobile phase A.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A.

Reference solution (b). A solution containing 0.013 per cent w/v solution of *ranitidine hydrochloride IPRS* and 0.00002 per cent w/v of *ranitidine impurity J IPRS (1,1'-N-[methylene bis(sulphanediyl-ethylene)] bis(N'-methyl-2-nitroethene-1,1- diamine IPRS)* in mobile phase A.

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Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane amorphous organosilica polymer (3.5 µm) (Such as Xterra MS C18),
- column temperature: 35°,
- mobile phase: A. a mixture of 2 volumes of *acetonitrile* and 98 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 950 ml of *water*, adjusted to pH 7.1 with *sodium hydroxide solution* and dilute to 1000 ml with *water*,

  B. a mixture of 22 volumes of *acetonitrile* and 78 volumes of buffer solution,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
10	0	100
15	0	100
16	100	0
20	100	0

Name	Relative retention time	Correction factor
Ranitidine impurity H[1]	0.1	—
Ranitidine impurity G[2]	0.2	—
Ranitidine impurity F[3]	0.4	—
Ranitidine impurity B[4]	0.5	—
Ranitidine impurity C[5]	0.6	—
Ranitidine impurity E[6]	0.7	—
Ranitidine impurity D[7]	0.8	—
Ranitidine impurity J[8]	0.9	2.0
Ranitidine	1.0	—
Ranitidine impurity I[9]	1.3	—
Ranitidine impurity A[10]	1.7	—

[1] *N*-methyl-2-nitroacetamide,

[2] 3-(methylamino)-5,6-dihydro-2*H*-1,4-thiazin-2-one-oxime,

[3] [5-[(dimethylamino)methyl]furan-2-yl]methanol,

[4] 2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulfanyl] ethanamine,

[5] *N*-[2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulfinyl]ethyl]-*N'*-methyl-2-nitroethene-1,1-diamine,

[6] *N*-[2-[[[5-[(dimethyloxidoamino)methyl]furan-2-yl]methyl]sulfanyl] ethyl]-*N'*- methyl-2-nitroethene-1,1-diamine,

[7] *N*-[2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulfanyl] ethyl]-2-nitroacetamide,

[8] 1,1'-*N*-[methylenebis(sulfanediylethylene)]bis(*N'*-methyl-2-nitroethene-1,1- diamine),

[9] 2,2'-methylenebis[*N*-[2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulfanyl]ethyl]-*N'*-methyl-2-nitroethene-1,1-diamine],

[10] *N*,*N'*-bis[2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulfanyl] ethyl]-2- nitroethene-1,1-diamine.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to ranitidine impurity J and the principal peak is not less than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to ranitidine impurity A is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). The area of any other identified peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). The area of any other secondary peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the area of all the secondary peaks other than ranitidine impurity A is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than 0.05 times the principal peak in the chromatogram obtained with the reference solution (a) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.75 per cent, determined on 1.0 g by drying in an oven at 60° at a pressure not exceeding 2.75 kPa for 3 hours.

Assay. Determine by liquid chromatography (2.4.14)

Test solution. A 0.0112 per cent w/v of the substance under examination in the mobile phase.

Reference solution. A 0.0112 per cent w/v of *ranitidine hydrochloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 85 volumes of *methanol* and 15 volumes of *0.1 M ammonium acetate*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 322 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{13}H_{22}N_4O_3S,HCl$.

Storage. Store protected from light and moisture.

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Ranitidine Injection

Ranitidine Hydrochloride Injection

Ranitidine Injection is a sterile solution of Ranitidine Hydrochloride in Water for Injections and may be suitably buffered.

Ranitidine Hydrochloride Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ranitidine, $C_{13}H_{22}N_4O_3S$.

Usual strength. The equivalent of 50 mg of ranitidine in 2 ml (1.12 g of ranitidine hydrochloride is approximately equivalent to 1 g of ranitidine).

Identification

A. To a volume of the injection containing 25 mg of ranitidine add 20 ml of *methanol*, mix and evaporate to dryness. Add 1 ml of *light petroleum (60° to 80°)* to the resulting residue, scratch the side of the vessel with a glass rod to induce crystallisation, evaporate to dryness and dry the residue at 60° for 10 minutes. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ranitidine hydrochloride IPRS* or with the reference spectrum of ranitidine hydrochloride.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 6.7 to 7.3, if the preparation is buffered; 4.5 to 7.0, if the preparation is unbuffered.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of the injection with *water* to obtain 0.1 per cent w/v of Ranitidine.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with *water*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *water*.

Reference solution (c). Dilute 1.0 ml of the test solution to 200.0 ml with *water*.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 5.0 ml with *water*.

Reference solution (e). A solution containing 0.002 per cent w/v of *ranitidine impurity J IPRS (1,1 '-N-[methylenebis (sulphanediyl-ethylene)] bis(N'-methyl-2-nitroethene-1,1-diamine IPRS)* in mobile phase A. Transfer 1.0 ml of the solution to a 100-ml volumetric flask containing 13 mg of *ranitidine hydrochloride IPRS*, add 40 ml of *water*, shake to dissolve and dilute to volume with *water*.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane amorphous organosilica polymer (3.5 µm) (Such as X-terra MS C18),
- column temperature: 35°,
- mobile phase: A. a mixture of 2 volumes of *acetonitrile* and 98 volumes of *0.05 M potassium dihydrogen orthophosphate*, adjusted to pH 7.1 with *sodium hydroxide solution*,
  B. a mixture of 22 volumes of *acetonitrile* and 78 volumes of *0.05 M potassium dihydrogen orthophosphate*, adjusted to pH 7.1 with *sodium hydroxide solution*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
10	0	100
15	0	100
16	100	0
20	100	0

Inject reference solution (e). The test is not valid unless the resolution between two principal peaks is not less than 1.5.

Inject reference solution (a), (b), (c), (d) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent). The area of not more than one secondary peak is more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), the area of not more than two other secondary peaks is more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent) and the area of not more than two further secondary peaks is more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent).

Bacterial endotoxins (2.2.3). Not more than 7.0 Endotoxin Units per mg of ranitidine.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14)

Test solution. Dilute a volume of the injection containing 10.0 mg of ranitidine to 100.0 ml with the mobile phase.

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Reference solution. A 0.0112 per cent w/v solution of *ranitidine hydrochloride IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.0 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 85 volumes of *methanol* and 15 volumes of *0.1 M ammonium acetate*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 322 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{13}H_{22}N_4O_3S$ in the injection.

Storage. Store protected from light.

Labelling. The label states (1) the strength in terms of the equivalent amount of ranitidine; (2) where appropriate, that the injection is buffered.

Ranitidine Oral Solution

Ranitidine Hydrochloride Oral Solution.

Ranitidine Oral Solution is a solution of ranitidine hydrochloride in a suitable vehicle.

Ranitidine Oral Solution contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of the ranitidine, $C_{13}H_{22}N_4O_3S$.

Usual strength. 75 mg per 5 ml.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 6.7 to 7.5.

Related substances. Determine by liquid chromatography (2.4.14).

Buffer solution. Dissolve 6.804 g of *potassium dihydrogen orthophosphate* in 950 ml of *water*, adjusted to pH 7.1 with *sodium hydroxide solution* and dilute to 1000.0 ml with *water*

Test solution. Prepare a solid phase extraction cartridge containing a C18 sorbent (Such as Sep-Pak C18 cartridges) by passing 10.0 ml of *methanol* followed by 20.0 ml of *0.5 M ammonia* through the cartridge; attach the tip of a suitable syringe to the cartridge. Transfer a weighed quantity of the oral solution containing the equivalent of 15 mg of ranitidine to the barrel of the syringe, add 2.0 ml of *0.5 M ammonia* to the syringe, insert the plunger and slowly force the mixture through the cartridge, discarding the eluant; repeat with two 4.0 ml quantities of *0.5 M ammonia* discarding the eluant. Pass two 5.0 ml quantities of a mixture of 25 volumes of *0.1M hydrochloric acid* and 75 volumes of *methanol* through the cartridge and collect the eluant; add 40.0 ml of *ethanol* to the collected eluant, evaporate the resulting solution to dryness at a temperature not exceeding 30° under reduced pressure and dissolve the residue in 2.0 ml of *water*.

Reference solution (a). Dilute 1 volume of the test solution to 50 volumes with *water*.

Reference solution (b). Dilute 1 volume of the test solution to 100 volumes with *water*.

Reference solution (c). Dilute 1 volume of the test solution to 200 volumes with *water*.

Reference solution (d). Dilute 1 volume of reference solution (b) to 5 volumes with *water*.

Reference solution (e). A solution containing 0.002 per cent w/v of *ranitidine impurity J IPRS (1,1'-N-[methylenebis (sulphanediyl-ethylene)] bis(N'-methyl-2-nitroethene-1,1-diamine IPRS)* in mobile phase A. Transfer 1.0 ml of the solution to a 100-ml volumetric flask containing 13 mg of *ranitidine hydrochloride IPRS*, add 40 ml of *water*, shake to dissolve and dilute to volume with *water*.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane amorphous organosilica polymer (3.5µm) (Such as XTerra MS C_{18}),
- column temperature: 35°,
- mobile phase: A. a mixture of 2 volumes of *acetonitrile* and 98 volumes of buffer solution,
  B. a mixture of 22 volumes of *acetonitrile* and 78 volumes of buffer solution,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
10	0	100
15	0	100
16	100	0
20	100	0

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Inject reference solution (e). The test is not valid unless the resolution between two peaks corresponding to ranitidine impurity J and ranitidine hydrochloride is not less than 1.5.

Inject reference solution (a), (b), (c), (d) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent), the area of not more than one secondary peak is more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), the area of not more than two other secondary peaks is more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent), the area of not more than two further secondary peaks is more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 2.5 times the areas of the principal peak in the chromatogram obtained with reference solution (a) (5.0 per cent). Ignore any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Determine by liquid chromatography (2.4.14)

Test solution. Dilute a volume of oral solution with the mobile phase to obtain a solution containing 0.0025 per cent w/v of ranitidine.

Reference solution. A 0.0028 per cent w/v solution of *ranitidine hydrochloride IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 85 volumes of *methanol* and 15 volumes of buffer solution prepared by dissolving 7.71 gm of *ammonium acetate* in 200 ml of *water*, add 1 ml of *acetic acid* and dilute to 1000 ml with *water*.
- flow rate: 1 ml per minute,
- spectrophotometer set at 322 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent

Inject the reference solution and the test solution.

Calculate the content of $C_{13}H_{22}N_4O_3S$.

Storage. Store protected from light, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of ranitidine.

Ranitidine Tablets

Ranitidine Hydrochloride Tablets

Ranitidine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of the ranitidine, $C_{13}H_{22}N_4O_3S$. The tablets are coated.

Usual strengths. The equivalent of 150 mg, 300 mg of ranitidine (1.12 g of ranitidine hydrochloride is approximately equivalent to 1 g of ranitidine).

Identification

A. Shake a quantity of the powdered tablets containing 25 mg of ranitidine with 5 ml of *methanol* for 5 minutes, filter and evaporate the filtrate to dryness. Add 1 ml of *light petroleum (60° to 80°)* to the resulting residue, scratch the side of the vessel with a glass rod to induce crystallisation, evaporate to dryness and dry the residue at 60° for 10 minutes. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ranitidine hydrochloride IPRS* or with the reference spectrum of ranitidine hydrochloride.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium: 900 ml of *water*,
Speed and time. 50 rpm for 45 minutes.

Withdraw a suitable volume of the medium and filter, rejecting the first few ml of filtrate. Dilute a suitable volume of the filtrate with the medium, if necessary. Measure the absorbance of the resulting solution at the maximum at about 314 nm (2.4.7). Calculate the content of ranitidine, $C_{13}H_{22}N_4O_3S$ in the medium from the absorbance obtained from a solution of known concentration of *Ranitidine hydrochloride IPRS* in the dissolution medium.

Q. Not less than 80 per cent of the stated amount of $C_{13}H_{22}N_4O_3S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing about 0.13 g of Ranitidine in 100.0 ml of *water*, filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with mobile phase A.

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Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with mobile phase A. Dilute 3.0 ml of the solution to 50.0 ml with mobile phase A.

Reference solution (c). A solution containing 0.002 per cent w/v of *ranitidine impurity J IPRS (1,1'-N-[methylenebis (sulphanediyl-ethylene)] bis(N'-methyl-2-nitroethene-1,1-diamine IPRS)* in mobile phase A. Transfer 1.0 ml of the solution to a 100-ml volumetric flask containing 13 mg of *ranitidine hydrochloride IPRS,* add 40 ml of *water,* shake to dissolve and dilute to volume with *water.*

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane amorphous organosilica polymer (3.5 µm) (Such as Xterra MS C18),
- column temperature: 35°,
- mobile phase: A. a mixture of 2 volumes of *acetonitrile* and 98 volumes of *0.05 M potassium dihydrogen orthophosphate,* adjusted to pH 7.1 with *sodium hydroxide solution,*

  B. a mixture of 22 volumes of *acetonitrile* and 78 volumes of *0.05 M potassium dihydrogen orthophosphate,* adjusted to pH 7.1 with *sodium hydroxide solution,*
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
10	0	100
15	0	100
16	100	0
20	100	0

Inject reference solution (c). The test is not valid unless the resolution between two principal peaks is not less than 1.5.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). The area of not more than one secondary peak is more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent) and the area of not more than three other secondary peaks is more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14)

Test solution: Weigh and powder 20 tablets. Disperse a quantity of the powder containing 300 mg of Ranitidine with 80 ml of the mobile phase for 10 minutes and dilute to 100 ml with mobile phase, filter and dilute the filtrate with the mobile phase to obtain a solution containing the equivalent of 0.01 per cent w/v of ranitidine.

Reference solution. A 0.0112 per cent w/v solution of *ranitidine hydrochloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 85 volumes of *methanol* and 15 volumes of *0.1 M ammonium acetate,*
- flow rate: 2 ml per minute,
- spectrophotometer set at 322 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{13}H_{22}N_4O_3S$ in the tablets.

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of the equivalent amount of ranitidine.

Purified Rayon

Viscose Fibre; Absorbent Viscose

Category. Regenerated cellulose used in surgical dressings.

Description. White or very slightly yellow, purified rayon is a fibrous form of bleached regenerated cellulose, which can be produced with a lustrous or matt appearance, and is soft to the touch. The fibres can be produced with average staple length between 32 mm to 80 mm.

Identification

A. When examined under a microscope in the dry state, or when mounted in *ethanol (95 per cent)* and *water,* the following characteristics are observed. They are usually of more or less uniform width. Many longitudinal parallel lines are distributed unequally over the width in the case of standard viscose fibres, but such lines are absent or very few in fibres produced through the zinc-free process. The ends are cut more or less straight. Matt fibres contain numerous granular particles of approximately 1µ average diameter.

B. Treat with *iodinated zinc chloride solution;* the fibres become violet.

C. To 0.1 g add 10 ml of *zinc chloride-formic acid solution,* heat to 40° and allow to stand for 2 hours 30 minutes, shaking occasionally. The fibres dissolve completely except for the matt variety where titanium dioxide particles remain.

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D. Dissolve the residue obtained in the test for Sulphated ash in 5 ml of *sulphuric acid* with slight warming, allow to cool, and carefully add 0.2 ml of *hydrogen peroxide solution (10 volumes)*. The solution does not undergo colour change in case of lustrous variety of fibre, but for matt variety an orange-yellow colour is obtained, the intensity of which depends on the quantity of titanium dioxide present.

Tests

Colour of extract. Take 15 g of material under examination in a suitable vessel, add 150 ml of *water*, close the vessel and allow to macerate for 2 hours. Decant the solution, squeeze the residual liquid carefully from the sample with a glass rod, mix and filter. The filtered extract is colourless. Compare the colour of the extract with water using identical tubes of colourless, transparent, neutral glass 12 mm in diameter measuring 2 ml. Compare the colours in diffused daylight, viewing horizontally against a white background.

Acidity or alkalinity. To 25 ml of filtered extract obtained, add 0.1 ml of *dilute phenolphthalein solution*; to another 25 ml add 0.05 ml of *methyl orange solution*. Neither solution shows a pink colour.

Foreign fibres. When examined under a microscope, it is seen to consist exclusively of viscose fibres, except that occasionally a few isolated foreign fibres may be present.

Fluorescence. Examine a layer about 5 mm in thickness under ultraviolet light at 365 nm. It displays only a slight, brownish-violet fluorescence and a few yellow particles. Not more than a few isolated fibres show an intense blue fluorescence.

Absorbancy

A. Sinking time. Not more than 10 seconds, determined by the following method.

Apparatus

A dry, cylindrical copper wire basket, 80 mm high and 50 mm in diameter, fabricated from wire of diameter 0.4 mm and having a mesh aperture of 15 to 20 mm; the basket weighs 2.4 to 3.0 g.

Method

Weigh the basket to the nearest 10 mg. Take five samples, each of approximately 1 g, from different places in the material under examination, place loosely in the basket and weigh the packed basket to the nearest 10 mg. Hold the basket with its long axis in the horizontal position and drop it from a height of about 10 mm into *water* at 25° contained in a beaker at least 12 cm in diameter and filled to a depth of 10 cm. Measure with a stopwatch the time taken by the basket to sink below the surface of the water. Repeat the procedure on two further samples and calculate the average value.

B. Water-holding capacity. Not less than 18.0 g per g, determined by the following method.

After the sinking time has been recorded in test A, remove the basket from the *water*, allow it to drain for 30 seconds with its long axis in the horizontal position over the beaker, transfer it to a tared beaker and weigh to the nearest 10 mg. Calculate the weight of *water* retained by the sample. Repeat the procedure on two further samples and calculate the average value.

Colouring matter. Slowly extract 10 g in a narrow percolator with *ethanol (95 per cent)* until 50 ml of extract is obtained. The extract is not more intensely coloured than reference solution YS5 *or* GYS6, (2.4.1) or a solution prepared in the following manner. To 3.0 ml of CSS add 7.0 ml of a solution of *hydrochloric acid* containing 1 per cent w/v of *hydrochloric acid* and dilute 0.5 ml of the resulting solution to 10 ml with the same solution of *hydrochloric acid*.

Ether-soluble substances. Not more than 0.5 per cent, determined by the following method. Extract 5 g with *ether* in a continuous extraction apparatus such as a Soxhlet apparatus, for 4 hours in such a way that the rate is at least four extractions per hour. Evaporate the *ether* and dry the residue to constant weight at 105°.

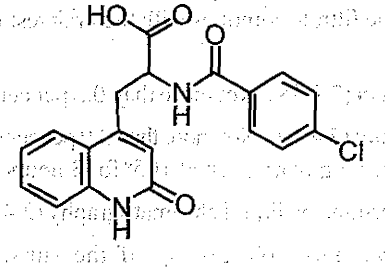
Water-soluble substances. Not more than 0.7 per cent, determined by the following method. Boil 5 g with 500 ml of *water* for 30 minutes, stirring frequently and replacing the *water* lost by evaporation. Decant the liquid into a beaker, squeeze the residual liquid from the material carefully with a glass rod, mix the liquids and filter the extract whilst hot. Evaporate 400 ml of the filtrate and dry the residue to constant weight at 105°.

Hydrogen sulphide. To 10 ml of the filtered extract obtained in the Colour of extract, add 1.9 ml of *water*, 0.15 ml of *dilute acetic acid* and 1 ml of *lead acetate solution*. After 2 minutes, the solution is not more intensely coloured than a reference solution prepared at the same time using 0.15 ml of *dilute acetic acid*, 1.2 ml of *thioacetamide reagent*, 1.7 ml of *lead standard solution (10 ppm Pb)* and 10 ml of filtered extract.

Sulphated ash (2.3.18). Not more than 1.5 per cent.

Loss on drying (2.4.19). Not more than 13.0 per cent, determined on 5 g by drying in an oven at 105°.

Rebamipide



$C_{19}H_{15}ClN_2O_4$ Mol. Wt. 370.8

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Rebamipide is (*RS*)-2-(4-Chlorobenzoylamino)-3-(2-oxo-1,2-dihydroquinolin-4-yl)propionic acid.

Rebamipide contains not less than 98.5 per cent and not more than 102.5 per cent of $C_{19}H_{15}ClN_2O_4$, calculated on the dried basis.

Category. Antiulcer.

Description. A white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rebamipide IPRS* or with the reference spectrum of rebamipide.

B. Dissolve 20 mg of the substance under examination in 5 ml of *1 M sodium hydroxide* and dilute to 50.0 ml with *water*. Dilute 1.0 ml of the solution to 25.0 ml with *water*. When examined in the range of 200 nm to 380 nm (2.4.7), the solution shows an absorption maximum at about 228 nm and 326 nm and absorption minimum at 295 nm.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 10 ml of *dimethylformamide* and dilute to 100.0 ml with *methanol*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 49 volumes of *methanol* and 51 volumes of a 1.0 per cent w/v solution of *sodium acetate* and 2 volumes of *glacial acetic acid*,
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 327 nm,
- injection volume: 20 µl.

Inject the test solution. The sum of the areas of all the secondary peaks is not more than 1.5 per cent, calculated by area normalisation.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Chloride (2.3.12). Shake 1.0 g with 40 ml of *water* for 5 minutes and filter. The filtrate complies with the limit test for chlorides (250 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 2.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 10 ml of *dimethylformamide* and dilute to 100.0 ml with *methanol*. Dilute 2.0 ml of the solution to 25.0 ml of mobile phase.

Reference solution. Dissolve 50 mg of *rebapimide IPRS* in 10 ml of *dimethylformamide* and dilute to 100.0 ml with *methanol*. Dilute 2.0 ml of the solution to 25.0 ml of mobile phase.

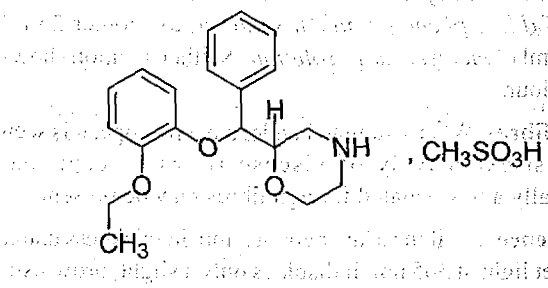
Use chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{15}ClN_2O_4$.

Reboxetine Methanesulphonate



$C_{20}H_{27}NO_6S$ Mol. Wt. 409.5

Reboxetine Methanesulphonate is (±)-2-[(2-Ethoxyphenoxy) benzyl]morpholine methane sulphonic acid.

Reboxetine Methanesulphonate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{20}H_{27}NO_6S$, calculated on the dried basis.

Category. Antidepressant.

Description. A white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *reboxetine methanesulphonate IPRS* or with the reference spectrum of reboxetine methanesulphonate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 2.5 to 5.5, determined in 1 per cent w/v solution in *water*.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

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Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution. A 0.05 per cent w/v solution of *reboxetine methanesulphonate IPRS* in the mobile phase.

Chromatographic system

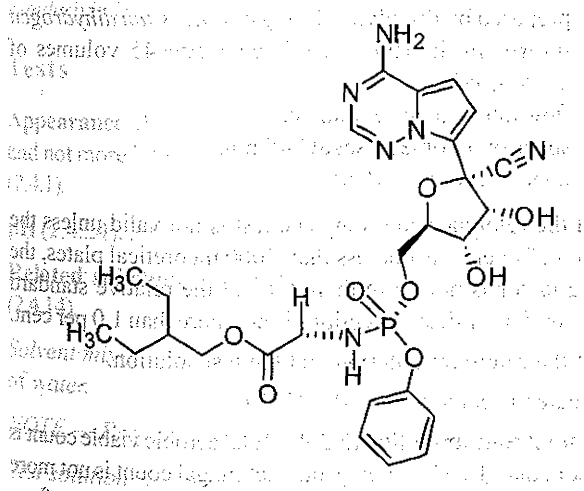
- a stainless steel column 25 cm x 4.0 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 32 volumes of *acetonitrile* and 68 volumes of buffer solution prepared by dissolving 2.0 g of *disodium hydrogen orthophosphate* and 4.0 g of *sodium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 2.7 with *orthophosphoric acid*.
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{27}NO_6S$.

Remdesivir



$C_{27}H_{35}N_6O_8P$ Mol. Wt. 602.6

Remdesivir is (2*S*)-2-{(2*R*,3*S*,4*R*,5*R*)-[5-(4-Aminopyrrolo[2,1-*f*][1,2,4]triazin-7-yl)-5-cyano -3,4-dihydroxy-tetrahydro-furan-2-ylmethoxy]phenoxy-(*S*)-phosphorylamino}propionic acid 2-ethyl-butyl ester.

Remdesivir contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{27}H_{35}N_6O_8P$, calculated on the anhydrous basis.

Category. Antiviral.

Description. A white to off white to yellow powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *remdesivir IPRS* or with the reference spectrum of remdesivir.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Appearance of solution. A 2.0 per cent w/v solution in *methanol* is clear (2.4.1) and colourless (2.4.1).

Specific optical rotation (2.4.22). –23.0° to –17.0°, determined in a 1.0 per cent w/v solution in *methanol*.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 40 volumes of *acetonitrile* and 60 volumes of *water*.

NOTE — Prepare the solutions immediately before use.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). A 0.005 per cent w/v solution of *remdesivir IPRS* in the solvent mixture.

Reference solution (b). Dissolve 12.5 mg of *remdesivir impurity D IPRS (fragment-B)* in 5 ml *acetonitrile* with the aid of ultrasound and dilute to 25.0 ml with the solvent mixture.

Reference solution (c). Dilute 10.0 ml of reference solution (a) and 1.0 ml of reference solution (b) to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane with the extra selectivity of a penta fluoro phenyl phase bonded to porous silica (5 µm) (Such as ACE PFP C18),
- column temperature: 40°,
- sample temperature: 10°,
- mobile phase: A. a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in 1000 ml of *water*,

  B. a mixture of 90 volumes of *acetonitrile* and 10 volumes of *water*,

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– a gradient programme using the conditions given below,
– flow rate: 1.5 ml per minute,
– spectrophotometer set at 245 nm and 275 nm,
– injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
25	40	60
35	40	60
44	20	80
48	20	80
49	85	15
55	85	15

Name	Relative retention time	Correction factor
Remdesivir impurity A[1]	0.17	0.48
Remdesivir impurity B[2]	0.57	0.53
Remdesivir (Retention time: about 21 minutes)	1.0	—
Remdesivir impurity C[3]	1.32	1.03
Remdesivir impurity D[4]	1.74	—

[1](2*R*,3*R*,4*S*,5*R*)-2-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-carbonitrile (Fragment A),

[2](3a*R*,4*R*,6*R*,6a*R*)-4-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-6-(hydroxymethyl)-2,2-dimethyltetrahydrofuran[3,4-d][1,3]dioxole-4-carbonitrile (Acetonide),

[3]2-ethylbutyl((*S*)-(((3aR,4R,6R,6aR)-6-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-6-cyano-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methoxy)(phenoxy)phosphoryl)amino)-*L*-alaninate (Condensed),

[4]*N*-[(*S*)-(4-nitrophenoxy) phenoxyphosphinyl]-l-Alaine 2- ethylbutyl ester (Fragment B).

Inject reference solution (c). The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 10.0 per cent for remdesivir at 245 nm.

Inject reference solution (c) and the test solution at 245 nm. In the chromatogram obtained with the test solution, the area of any peak corresponding to remdesivir impurity A, B and C, each of, is not more than the area of the peak due to remdesivir in the chromatogram obtained with reference solution (c) (0.5 per cent) and the area of any other secondary peak is not more than 0.6 times the area of the peak due to remdesivir in the chromatogram obtained with reference solution (c) (0.3 per cent). Ignore any peak corresponding to remdesivir impurity D.

Inject reference solution (c) and the test solution at 275 nm. In the chromatogram obtained with the test solution, the area of any peak corresponding to remdesivir impurity D is not more than the area of the peak due to remdesivir impurity D in the chromatogram obtained with reference solution (c) (0.5 per cent). Ignore all the peaks other than remdesivir impurity D.

The sum of all secondary peaks at 245 nm and remdesivir impurity D is not more than 1.0 per cent.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Water (2.3.43). Not more than 1.0 per cent, determined on 0.5 g.

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per mg of remdesivir.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 40 volumes of *acetonitrile* and 60 volumes of *water*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *remdesivir IPRS* in the solvent mixture.

Chromatographic system
– a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane with the extra selectivity of a penta fluoro phenyl phase bonded to porous silica (5 µm) (Such as ACE PFP C18),
– column temperature: 40°,
– sample temperature: 10°,
– mobile phase: a mixture of 55 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in 1000 ml of *water* and 45 volumes of *acetonitrile*,
– flow rate: 1.5 ml per minute,
– spectrophotometer set at 245 nm,
– injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{35}N_6O_8P$.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 10^2 CFU per g and total fungal count is not more than 10^2 CFU per g, determined by plate count. 1 g is free from *Escherichia coli*, *Salmonella* and *Shigella*.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

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Remdesivir Injection

Remdesivir for Injection

Remdesivir Injection is a sterile material consisting of Remdesivir with or without buffering agents and other excipients. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Remdesivir Injection contains not less than 90.0 per cent and not more than 105.0 per cent of the stated amount of remdesivir, $C_{27}H_{35}N_6O_8P$.

The contents of the sealed containers comply with the requirements stated under Parenteral Preparations (powder for Injections) and with the following requirements.

Usual strength. 100 mg per vial.

Description. A white to off white to yellow lyophilized solid or powder.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. When examined in the range from 200 nm to 400 nm (2.4.7), a 0.0012 per cent w/v solution in 50 per cent v/v solution of *methanol* shows an absorption maximum as obtained with *remdesivir IPRS* of the same concentration.

Tests

Appearance of solution. A reconstituted solution is clear (2.4.1) and not more intensely coloured than reference solution BYS5 (2.4.1).

pH (2.4.24). 3.0 to 4.0, determined on constituted solution.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 25 volumes of *acetonitrile* and 75 volumes of *water*.

NOTE — Prepare the solutions immediately before use.

Test solution. Reconstitute 5 vials with 20 ml each, with the solvent mixture and pool the contents in 200-ml volumetric flask, dilute to volume with the solvent mixture. Dilute a suitable volume with the solvent mixture to obtain a solution containing 0.1 per cent w/v of Remdesivir.

Reference solution. Dissolve 10 mg of *remdesivir IPRS* in 5 ml of *acetonitrile* and dilute to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane with extra selectivity of a penta fluoro phenyl bonded to porous silica (5 µm) (Such as ACE PFP C18),
- column temperature: 40°,
- sample temperature: 5°,
- mobile phase: A. a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in 1000 ml of *water*,
  B. a mixture of 90 volumes of *acetonitrile* and 10 volumes of *water*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
25	40	60
35	40	60
44	20	80
48	20	80
49	85	15
55	85	15

Name	Relative retention time	Correction factor
Remdesivir impurity A[1]	0.17	0.45
Remdesivir impurity B[2*]	0.57	—
Remdesivir (Retention time: about 21 minutes)	1.0	—
Remdesivir impurity C[3*]	1.32	—
Remdesivir impurity D[4*]	1.8	—

*Process impurity include for identification only and not included in the calculation of total degradation products,

[1](2*R*,3*R*,4*S*,5*R*)-2-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-carbonitrile (Fragment A),

[2](3a*R*,4*R*,6*R*,6a*R*)-4-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-6-(hydroxymethyl)-2,2-dimethyltetrahydrofuran[3,4-d][1,3]dioxole-4-carbonitrile (Acetonide),

[3]2-ethylbutyl((*S*)-(((3a*R*,4*R*,6*R*,6a*R*)-6-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-6-cyano-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methoxy)(phenoxy)phosphoryl)amino)-*L*-alaninate (Condensed),

[4]*N*-[(S)-(4-nitrophenoxy) phenoxyphosphinyl]-l-Alaine 2- ethylbutyl ester (Fragment B).

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Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is more than 2.0 and the relative standard deviation for replicate injections is not more than 10.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to remdesivir impurity A is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent), the area any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent) and the sum of the areas of all the secondary peaks other than remdesivir impurity A is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Water (2.3.43). Not more than 3.0 per cent, determined on 0.1 g.

Bacterial endotoxins (2.2.3).Not more than 0.6 Endotoxin Unit per mg of remdesivir.

Sterility (2.2.11). Complies with the test for sterility.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 40 volumes of *acetonitrile* and 60 volumes of *water*.

Test solution. Reconstitute 5 vials with 20 ml each, with the solvent mixture and pool the contents in 200-ml volumetric flask, dilute to volume with the solvent mixture. Dilute 2.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *remdesivir IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane with extra selectivity of a penta fluoro phenyl bonded to porous silica (5 µm) (Such as ACE PFP C18),
- column temperature: 40°,
- sample temperature: 10°,
- mobile phase: a mixture of 50 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in 1000 ml of *water* and 50 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 10 µl.

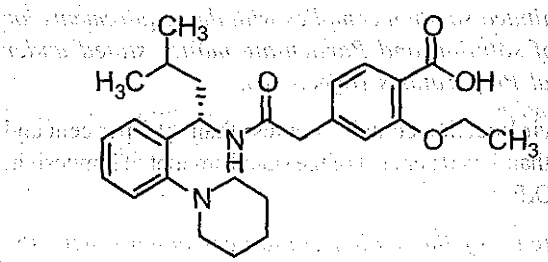
Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{35}N_6O_8P$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Repaglinide



$C_{27}H_{36}N_2O_4$ Mol. Wt. 452.6

Repaglinide is (*S*)-2-Ethoxy-4-[2-[3-methyl-1-(2-(piperidin-1-yl)phenyl)butylamino]-2-oxoethyl]benzoic acid.

Repaglinide contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{27}H_{36}N_2O_4$, calculated on the dried basis.

Category. Antidiabetic.

Description. A white or almost white powder. It shows polymorphism (2.5.11).

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *repaglinide IPRS* or with the reference spectrum of repaglinide.

B. Specific optical rotation (2.4.22). +6.3° to +7.7°, determined in a 5.0 per cent w/v solution in *methanol*.

Tests

Enantiomeric purity. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions in amber flasks and vials.

Test solution. Dissolve 10 mg of the substance under examination in *methanol* and dilute to 10.0 ml with *methanol*.

Reference solution (a). A 0.01 per cent w/v solution of *repaglinide impurity E IPRS (2-ethoxy-4-[2-[[(1R)-3-methyl-1-[2-(piperidin-1-yl)phenyl]butyl]amino]-2-oxoethyl] benzoic acid IPRS)* in *methanol*.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 100.0 ml with *methanol*.

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Reference solution (c). Mix 1.0 ml of the test solution and 10 ml of reference solution (a) and dilute to 50.0 ml with *methanol.*

Chromatographic system
- a stainless steel column 10 cm x 4 mm, packed with silica gel AGP for chiral chromatography (5 µm),
- mobile phase: A. a 0.1 per cent w/v solution of *potassium dihydrogen phosphate*, adjusted to pH 4.7 with *dilute sodium hydroxide solution*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
4	60	40
6	60	40
8	80	20

The retention time of repaglinide is about 3.3 minutes and of repaglinide impurity E is about 5.0 minutes.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to repaglinide and repaglinide impurity E is not less than 1.5.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to repaglinide impurity E is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 30 mg of the substance under examination in *acetonitrile* and dilute to 10.0 ml with *acetonitrile.*

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with *acetonitrile.*

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 100.0 ml with *acetonitrile.*

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with silica alkyl-bonded for use with highly aqueous mobile phases (5 µm),
- column temperature: 45°,
- mobile phase: A. a 0.4 per cent w/v solution of *potassium dihydrogen phosphate*, adjusted to pH 3.2 with *dilute orthophosphoric acid*,
  B. a mixture of 30 volumes of mobile phase A and 70 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	50	50
20	7	93
30	7	93
40	50	50

Name	Relative retention time	Correction factor
Repaglinide impurity A[1]	0.2	0.6
Repaglinide impurity B[2]	0.3	0.7
Repaglinide impurity C[3]	0.4	3.1
Repaglinide (Retention time: about 10 minutes)	1.0	—
Repaglinide impurity D[4]	1.5	—

[1]4-(carboxymethyl)-2-ethoxybenzoic acid,

[2][3-ethoxy-4-(ethoxycarbonyl)phenyl]acetic acid,

[3](1*S*)-3-methyl-1-[2-(piperidin-1-yl)phenyl]butan-1-amine,

[4]ethyl 2-ethoxy-4-[2-[[(1*S*)-3-methyl-1-[2-(piperidin-1-yl)phenyl]butyl]amino]-2-oxoethyl]benzoate.

Inject reference solution (a). The test is not valid unless the column efficiency is not less 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent). The sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.32 g in 10 ml *methanol* and add 60 ml of *anhydrous acetic acid.* Titrate with *0.1M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.04526 g of $C_{27}H_{36}N_2O_4$.

Storage. Store protected from light and moisture.

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Repaglinide Tablets

Repaglinide Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of repaglinide, $C_{27}H_{36}N_2O_4$.

Usual strengths. 0.5 mg; 1 mg; 2 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to peak in the chromatogram obtained with the reference solution (b).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of buffer solution pH 5.0, prepared by dissolving 10.2 g of *citric acid monohydrate* and 18.2 g of *dibasic sodium phosphate dihydrate* with 1000 ml of *water*,

Speed and time. 75 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate and dilute further with dissolution medium, if necessary.

Reference solution. A 0.001 per cent w/v solution of *repaglinide IPRS* in *methanol*. Dilute 5.0 ml of the solution to 100.0 ml with the dissolution medium.

Chromatographic system

- a stainless steel column 12.5 cm x 4 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 49 volumes of *acetonitrile*, 40 volumes of 0.15 per cent w/v of *monobasic potassium phosphate solution*, adjusted to pH 2.3 with *orthophosphoric acid* and 11 volumes of *methanol*,
- flow rate: 1 ml per minute,
- fluorometric detector: excitation wavelength of 244 nm and emission wavelength of 348 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{36}N_2O_4$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{27}H_{36}N_2O_4$.

Related substances. Determine by liquid chromatography (2.4.14) as described under Assay with the following modifications.

Reference solution (d). Dilute 2.5 ml of reference solution (b) to 1000 ml with the solvent mixture.

- spectrophotometer set at 210 nm,

Inject reference solution (c) and (d). The test is not valid unless the resolution between the peaks due to repaglinide and repaglinide impurity A is not less than 7.0 and the tailing factor is not more than 2.0 in the chromatogram obtained with reference solution (c) and the relative standard deviation for replicate injections is not more than 10.0 per cent in the chromatogram obtained with reference solution (d).

Inject the test solution. The sum of areas of all the secondary peaks is not more than 0.5 per cent, calculated by area normalisation.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 70 volumes of *methanol* and 30 volumes of 0.2 per cent w/v of *monobasic ammonium phosphate solution*, adjusted to pH 4.0 with *orthophosphoric acid*.

Test solution. Disperse 8 intact tablets in the solvent mixture and dilute to obtain a solution containing 0.008 per cent w/v of Repaglinide in the solvent mixture, stir for 20 minutes and filter.

Reference solution (a). A 0.08 per cent w/v solution of *repaglinide IPRS* in *methanol*.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 50.0 ml with the solvent mixture.

Reference solution (c). A 0.008 per cent w/v solution of *repaglinide impurity A IPRS (4-(carboxymethyl)-2-ethoxybenzoic acid IPRS)* in *methanol*. To 1.0 ml of the solution, add 5.0 ml of reference solution (a) and dilute to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 6.0 cm x 4 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 70 volumes of *methanol* and 30 volumes of 0.2 per cent w/v solution of *monobasic ammonium phosphate*, adjusted to pH 2.5 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 20 µl.

Inject reference solution (b) and (c). The test is not valid unless the resolution between the peaks due to repaglinide and repaglinide impurity A is not less than 7.0, the tailing factor is not more than 2.0 in the chromatogram obtained with reference solution (c) and the relative standard deviation for replicate injections is not more than 2.0 in the chromatogram obtained with reference solution (b).

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Inject reference solution (b) and the test solution.

Calculate the content of $C_{27}H_{36}N_2O_4$ in the tablets.

Storage. Store protected from light and moisture.

Repaglinide and Metformin Tablets

Repaglinide and Metformin Hydrochloride Tablets

Repaglinide and Metformin Hydrochloride Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of repaglinide, $C_{27}H_{36}N_2O_4$ and metformin hydrochloride, $C_4H_{11}N_5,HCl$.

Usual strengths. Repaglinide, 1 mg and Metformin Hydrochloride 500 mg; Repaglinide, 2 mg and Metformin Hydrochloride 500 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with reference solution (b).

Tests

Dissolution (2.5.2):

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1M hydrochloric acid,*

Speed and time. 75 rpm and 30 minutes.

Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

- injection volume: 50 µl.

Test solution (a). Withdraw a suitable volume of the medium and filter. Dilute further, if necessary, with the dissolution medium for repaglinide.

Test solution (b). Dilute 5.0 ml of the filtrate to 100.0 ml with the dissolution medium for metformin hydrochloride.

Reference solution (a). Weigh accurately about 22 mg of *repaglinide IPRS* and transfer to a 200-ml volumetric flask. Add about 20.0 ml of *methanol* and sonicate to dissolve and make up the volume with the dissolution medium. Dilute 5.0 ml of the solution to 100.0 ml with dissolution medium.

Reference solution (b). Weigh accurately about 28 mg of *metformin hydrochloride IPRS* and transfer to a 200-ml volumetric flask. Add about 150.0 ml of dissolution medium and sonicate to dissolve and make up the volume with the dissolution medium.

Reference solution (c). Dilute reference solution (a) and (b) with dissolution medium to obtain a solution having a known concentration similar to the test solution (a) and (b).

Inject reference solution (c). The test is not valid unless the column efficiency for both repaglinide and metformin hydrochloride peaks is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (c), test solution (a) and (b).

Calculate the content of repaglinide, $C_{27}H_{36}N_2O_4$ and metformin hydrochloride, $C_4H_{11}N_5,HCl$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{27}H_{36}N_2O_4$ and $C_4H_{11}N_5,HCl$.

Related substances.

For Metformin Hydrochloride — Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of powder containing 250 mg of Metformin Hydrochloride to 250-ml volumetric flask. Add about 150.0 ml of *water* and sonicate for 30 minutes. Allow the solution cool to room temperature and dilute to volume with *water* and mix.

Reference solution. 0.0005 per cent solution of *metformin hydrochloride IPRS* in *water*.

Chromatographic system

- a stainless steel column, 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (Such as Inertsil, ODS) (5µm),
- mobile phase: 90 volumes of a buffer solution prepared by dissolving 0.5 g each of *heptane sulphonic acid* and *sodium chloride* in 1000 ml of *water*, adjusted to pH 3.85 with *orthophosphoric acid* and 10 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 218 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for six replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution the area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 1.2 times the area of the principal peak in the chromatogram obtained with the reference solution (0.6 per cent).

For Repaglinide — Determine by liquid chromatography (2.4.14).

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Solvent mixture. A mixture of 30 volumes of *water* and 70 volumes of *methanol*.

Test solution. Weigh and powder 20 tablets. Weigh a quantity of powder containing 10 mg of Repaglinide to a 200-ml volumetric flask and add about 10.0 ml of *water* and sonicate to disperse. Further add 150.0 ml of solvent mixture and sonicate for 30 minutes. Allow the solution cool to room temperature and make up the volume with solvent mixture and filter.

Reference solution. 0.000025 per cent solution of *repaglinide IPRS* in solvent mixture.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm packed with base deactivated, end-capped octadecylsilyl silica gel (3µm) (Such as Hypersil BDS),
- column temperature: 40°,
- mobile phase: 50 volumes of a buffer solution prepared by dissolving 2.0 g *ammonium dihydrogen orthophosphate*, adjusted to pH 2.5 with *orthophosphoric acid* and 50 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

NOTE — Disregard the peak due to blank and placebo up to relative retention time 0.2.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than four times the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent).

Uniformity of content. Complies with the tests stated under Tablets.

For Repaglinide — Determine by liquid chromatography (2.4.14) as described under Assay with the following modifications.

Test solution. Transfer one intact tablet in a 100-ml volumetric flask, add sufficent mobile phase and sonicate for 60 minutes with intermittent shaking. Allow the solution cool to room temperature and dilute to volume with mobile phase, mix and filter.

Reference solution. 0.001 per cent solution of *Repaglinide IPRS* in mobile phase.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{36}N_2O_4$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh accurately a quantity of powder containing about 1.5 mg of Repaglinide in 500-ml volumetric flask. Add 350.0 ml of mobile phase and sonicate for 60 minutes with intermittent shaking. Allow the solution cool to room temperature and make up the volume with mobile phase and mix. Filter the solution and use filtrate for repaglinide content. Further dilute the filtrate to produce a solution containing 0.003 per cent w/v of metformin for metformin content.

Reference solution (a). A 0.03 per cent w/v solution of *repaglinide IPRS* in the mobile phase.

Reference solution (b). Weigh accurately about 30 mg of *metformin hydrochloride IPRS* and transfer to a 100-ml volumetric flask. Add about 70.0 ml of mobile phase and sonicate to dissolve. Add 10.0 ml of reference solution (a), and make up the volume with mobile phase. Further dilute 5.0 ml of the solution to 50.0 ml with mobile phase and mix.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with strong cation exchange packing-aromatic sulphonic acid group bonded to porous silica (Such as Zorbax 300 SCX) (5µm),
- column temperature: 35°,
- mobile phase: a mixture of 72 volumes of buffer solution prepared by dissolving 17.0 g *ammonium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 28 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the column efficiency for both repaglinide and metformin hydrochloride is not less than 2000 theoretical plates, the tailing factor is not be more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (b) and the test solution.

Calculate the content of $C_{27}H_{36}N_2O_4$ and $C_4H_{11}N_5,HCl$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

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Repaglinide and Voglibose Tablets

Repaglinide and Voglibose Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of repaglinide, $C_{27}H_{36}N_2O_4$ and voglibose, $C_{10}H_{21}NO_7$.

Usual strengths. Repaglinide, 0.5 mg and Voglibose 0.2 mg; Repaglinide, 0.5 mg and Voglibose 0.3 mg; Repaglinide, 1.0 mg and Voglibose, 0.2 mg; Repaglinide, 1.0 mg and Voglibose, 0.3 mg.

Identification

In the Assay of repaglinide, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution and in the Assay of voglibose, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

For Repaglinide —

Apparatus No. 2 (Paddle),

Medium. 900 ml of a buffer solution prepared by dissolving 10.3 g of *citric acid monohydrate* and 18.1 g of *disodium hydrogen phosphate dihydrate* in 1000 ml of *water*, adjusted to pH 5.0,

Speed and time. 75 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.0011 per cent w/v solution of *repaglinide IPRS* in *methanol*. To 5.0 ml of the solution, add 6 ml *methanol* and dilute to 100.0 ml with the dissolution medium.

Chromatographic system

- a stainless steel column 5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 30 volumes of a buffer solution prepared by dissolving 2 g of *ammonium dihydrogen phosphate* in 950 ml of *water*, adjusted to pH 2.5 with *orthophosphoric acid*, dilute to 1000 ml with *water* and 70 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{36}N_2O_4$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{27}H_{36}N_2O_4$.

Uniformity of content. Determine by liquid chromatography (2.4.14),

For Repaglinide —

Solvent mixture. 30 volumes of a buffer solution prepared by dissolving 2 g of *ammonium dihydrogen phosphate* in 950 ml of *water*, adjusted to pH 4.0 with *orthophosphoric acid*, dilute to 1000 ml with *water* and 70 volumes of *methanol*.

Test solution. Disperse 1 intact tablet in the solvent mixture, with the aid of ultrasound with intermittent shaking and dilute with the solvent mixture to obtain a solution containing 0.005 per cent w/v of Repaglinide and filter.

Reference solution. A 0.05 per cent w/v solution of *repaglinide IPRS* in *methanol*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Use the chromatographic system as described under dissolution with the following modification.

- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{36}N_2O_4$ in the tablet.

For Voglibose —

Determine by liquid chromatography (2.4.14).

Test solution. Disperse 1 intact tablet in the mobile phase, with the aid of ultrasound with intermittent shaking and dilute to 25.0 ml with the mobile phase, filter.

Reference solution. Dissolve an accurately weighed quantity of *voglibose IPRS* in the mobile phase to obtain a solution of the same concentration as that of the test solution.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with aminopropylsilane bonded to porous silica (5 µm) (Such as Hypersil APS-2),
- column temperature: 20°,
- mobile phase: a mixture of 30 volumes of a buffer solution prepared by dissolving 0.6 g of *potassium dihydrogen phosphate* and 0.28 g of *dipotassium hydrogen*

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phosphate in 1000 ml of *water* and 70 volumes of *acetonitrile*,

- flow rate: 0.8 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 100 µl.

(*NOTE — Equilibrate the column with the mobile phase for at least 4 hours.*)

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{21}NO_7$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

For Repaglinide —

Solvent mixture. 30 volumes of a buffer solution prepared by dissolving 2 g of *ammonium dihydrogen phosphate* in 950 ml of *water*, adjusted to pH 4.0 with *orthophosphoric acid*, dilute to 1000 ml with *water* and 70 volumes of *methanol*.

Test solution. Disperse a sufficient quantity of intact tablets containing 8 mg of Repaglinide in the solvent mixture, with the aid of ultrasound with intermittent shaking and dilute with the solvent mixture to obtain a solution containing 0.008 per cent w/v of Repaglinide.

Reference solution. A 0.08 per cent w/v solution of *repaglinide IPRS* in *methanol*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Use the chromatographic system as described under dissolution with the following modification.

- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{36}N_2O_4$ in the tablets.

For Voglibose —

Test solution. Disperse a sufficient quantity of intact tablets containing 2.4 mg of Voglibose in the mobile phase, with the aid of ultrasound with intermittent shaking and dilute with the mobile phase to obtain a solution containing 0.0012 per cent w/v of Voglibose.

Reference solution. A 0.0012 per cent w/v solution of *voglibose IPRS* in the mobile phase.

Use the chromatographic system as described under Uniformity of content.

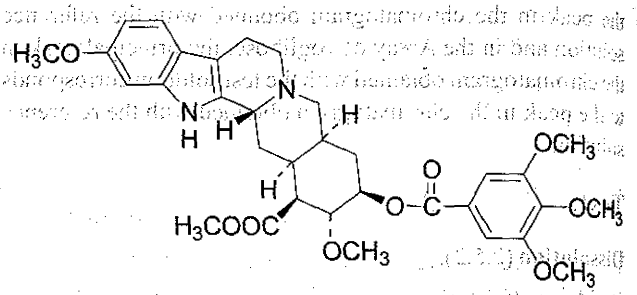
Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{21}NO_7$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Reserpine



$C_{33}H_{40}N_2O_9$ Mol. Wt. 608.7

Reserpine is methyl 11,17α-dimethoxy-18β-[(3,4,5-trimethoxybenzoyl)oxy]-3β,20α-yohimbane-16β-carboxylate.

Reserpine contains not less than 99.0 per cent and not more than 101.0 per cent of total alkaloids and not less than 98.0 per cent and not more than 102.0 per cent of reserpine, $C_{33}H_{40}N_2O_9$, both calculated on the dried basis.

Category. Antihypertensive.

Description. White to slightly yellow small crystals or a crystalline powder which darkens slowly on exposure to light.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests B, C, D and E may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *reserpine IPRS* or with the reference spectrum of reserpine.

B. Dilute 1 ml of a 0.2 per cent w/v solution in *chloroform* to 100.0 ml with *ethanol (95 per cent)*. When examined immediately after preparation, in the range 230 nm to 360 nm (2.4.7), the resulting solution shows an absorption maximum at about 268 nm; absorbance at about 268 nm, about 0.55. Over the range 288 nm to 295 nm, the spectrum exhibits a slight minimum and then a shoulder or a slight maximum; absorbance over this range, about 0.34.

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C. To about 1 mg add 0.1 ml of a 0.1 per cent w/v solution of *sodium molybdate* in *sulphuric acid*; a yellow colour is produced which changes to blue within 2 minutes.

D. To 1 mg add 0.2 ml of a freshly prepared 1 per cent w/v solution of *vanillin* in *hydrochloric acid*; a pink colour develops within 2 minutes.

E. Mix about 0.5 mg with 5 mg of *4-dimethylaminobenzaldehyde* and 0.2 ml of *glacial acetic acid* and add 0.2 ml of *sulphuric acid*; a green colour is produced. Add 1 ml of *glacial acetic acid*; the colour changes to red.

Tests

Specific optical rotation (2.4.22). −128° to −116°, determined in a solution prepared immediately before use by dissolving 0.25 g in sufficient *chloroform* to produce 25 ml.

Oxidation products. Absorbance of a 0.02 per cent w/v solution in *glacial acetic acid* at about 388 nm, measured immediately after preparation, not more than 0.10 (2.4.7).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 0.5 g by drying in an oven over *phosphorus pentoxide* at 60° at a pressure not exceeding 0.7 kPa for 3 hours.

Assay. *For total alkaloids* — Weigh 0.5 g and dissolve in a mixture of 40 ml of *anhydrous glacial acetic acid* and 6 ml of *acetic anhydride*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.06087 g of total alkaloids.

For reserpine, $C_{33}H_{40}N_2O_9$ — Carry out the following procedure protected from light. Weigh 25.0 mg, moisten with 2 ml of *ethanol (95 per cent)*, add 2 ml of *0.25 M sulphuric acid* and 10 ml of *ethanol (95 per cent)* and warm gently to dissolve. Cool, dilute to 100.0 ml with *ethanol (95 per cent)* and dilute 5.0 ml to 50.0 ml with the same solvent (solution A). Transfer 10.0 ml to a boiling tube, add 2 ml of *0.25 M sulphuric acid* and 2 ml of a freshly prepared 0.3 per cent w/v solution of *sodium nitrite*, mix and heat in a water-bath at 55° for 35 minutes. Cool, add 1 ml of a freshly prepared 5 per cent w/v solution of *sulphamic acid* and dilute to 25.0 ml with *ethanol (95 per cent)*. Measure the absorbance of the resulting solution at the maximum at about 388 nm (2.4.7), using as the blank a solution prepared by treating a further 10.0 ml of solution A in the same manner and at the same time but omitting the sodium nitrite solution.

Calculate the content of $C_{33}H_{40}N_2O_9$ from the absorbance obtained by repeating the operation using *reserpine IPRS* in place of the substance under examination.

Storage. Store protected from light.

Reserpine Injection

Reserpine Injection is a sterile solution of Reserpine in Water for Injections prepared with the aid of a suitable acid. It may contain suitable antioxidants.

Reserpine Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of reserpine, $C_{33}H_{40}N_2O_9$.

Usual strengths. 1 mg per ml; 2.5 mg per ml.

Identification

Extract a suitable volume of the injection containing 10 mg of Reserpine with 10 ml of *chloroform* and evaporate the chloroform layer to dryness. The residue complies with the following tests.

Test A may be omitted if tests B, C, D and E are carried out. Tests B, C, D and E may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *reserpine IPRS* or with the reference spectrum of reserpine.

B. Dilute 1 ml of a 0.2 per cent w/v solution in *chloroform* to 100 ml with *ethanol (95 per cent)*. When examined immediately after preparation, in the range 230 nm to 360 nm (2.4.7), the resulting solution shows an absorption maximum at about 268 nm; absorbance at about 268 nm, about 0.55. Over the range 288 nm to 295 nm, the spectrum exhibits a slight minimum and then a shoulder or a slight maximum; absorbance over this range, about 0.34.

C. To about 1 mg add 0.1 ml of a 0.1 per cent w/v solution of *sodium molybdate* in *sulphuric acid*; a yellow colour is produced which changes to blue within 2 minutes.

D. To 1 mg add 0.2 ml of a freshly prepared 1 per cent w/v solution of *vanillin* in *hydrochloric acid*; a pink colour develops within 2 minutes.

E. Mix about 0.5 mg with 5 mg of *4-dimethylaminobenzaldehyde* and 0.2 ml of *glacial acetic acid* and add 0.2 ml of *sulphuric acid*; a green colour is produced. Add 1 ml of *glacial acetic acid*; the colour changes to red.

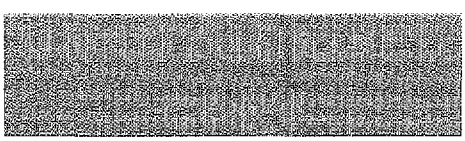
Tests

pH (2.4.24). 3.0 to 4.0.

Bacterial endotoxins (2.2.3). Not more than 71.5 Endotoxin Units per mg of reserpine.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *Protect the solutions from light throughout the assay.*



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Measure a volume of the injection containing about 10 mg of Reserpine and dilute with a 2 per cent w/v solution of *citric acid* to 100.0 ml. Extract 10.0 ml of the solution for 2 minutes with three quantities, each of 15 ml, of *chloroform*. Wash the combined extracts with 10 ml of a 1 per cent w/v solution of *sodium bicarbonate*, add sufficient *chloroform* to produce 50.0 ml, mix and evaporate 10.0 ml to dryness on a water-bath. Dissolve the residue in 10 ml of *ethanol (95 per cent)*, add 2 ml of *0.25 M sulphuric acid* and 10 ml of *ethanol (95 per cent)* and warm gently to dissolve. Cool, dilute to 100.0 ml with *ethanol (95 per cent)* and dilute 5.0 ml to 50.0 ml with the same solvent (solution A). Transfer 10.0 ml to a boiling tube, add 2 ml of *0.25 M sulphuric acid* and 2 ml of a freshly prepared 0.3 per cent w/v solution of *sodium nitrite*, mix and heat in a water-bath at 55° for 35 minutes. Cool, add 1 ml of a freshly prepared 5 per cent w/v solution of *sulphamic acid* and dilute to 25.0 ml with *ethanol (95 per cent)*. Measure the absorbance of the resulting solution at the maximum at about 388 nm (2.4.7), using as the blank a solution prepared by treating a further 10.0 ml of solution A in the same manner and at the same time but omitting the sodium nitrite solution.

Calculate the content of $C_{33}H_{40}N_2O_9$ from the absorbance obtained by repeating the operation using *reserpine IPRS* in place of the substance under examination.

Storage. Store protected from light in single dose (or if stabilising agents are present, in multiple dose) containers.

Reserpine Tablets

Reserpine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of reserpine, $C_{33}H_{40}N_2O_9$.

Usual strengths. 100 µg; 250 µg; 500 µg; 1 mg.

Identification

A. Powder a few tablets and extract with *chloroform*. Evaporate the extract to dryness, add 0.1 ml of a 0.1 per cent w/v solution of *sodium molybdate* in *sulphuric acid*; a yellow colour is produced which changes to blue within 2 minutes.

B. Powder a few tablets and extract with *chloroform*. Evaporate the extract to dryness, add 0.2 ml of a freshly prepared 1 per cent w/v solution of vanillin in *hydrochloric acid*; a pink colour develops within 2 minutes.

Tests

Uniformity of content. *Protect the solutions from light throughout the test.*

Powder one tablet, disperse in 10 ml of a 2 per cent w/v solution of *citric acid* and extract for 2 minutes with three quantities, each of 5 ml, of *chloroform*; filter the extracts through a plug of cotton moistened with *chloroform*. Wash the chloroform extracts with 10 ml of a 1 per cent w/v solution of *sodium bicarbonate* and evaporate the chloroform extracts to dryness on a water-bath. For tablets containing upto 250 µg of Reserpine per tablet, dissolve the residue in 10.0 ml of *ethanol (95 per cent)*. For tablets containing more than 250 µg of Reserpine per tablet, dissolve the residue in a suitable volume of *ethanol (95 per cent)* to give a concentration of 250 µg of Reserpine per 10.0 ml; add 2 ml of *0.25 M sulphuric acid* and 2 ml of a freshly prepared 0.3 per cent w/v solution of *sodium nitrite*, mix and heat in a water-bath at 55° for 35 minutes. Cool, add 1 ml of a freshly prepared 5 per cent w/v solution of *sulphamic acid* and dilute to 25.0 ml with *ethanol (95 per cent)*. Measure the absorbance of the resulting solution at the maximum at about 388 nm (2.4.7), using as the blank a solution prepared by treating a further 10.0 ml of solution A in the same manner and at the same time but omitting the sodium nitrite solution.

Calculate the content of $C_{33}H_{40}N_2O_9$ in the tablet from the absorbance obtained by repeating the operation using *reserpine IPRS* in place of the substance under examination.

Other tests. Comply with the tests stated under Tablets.

Assay. *Protect the solutions from light throughout the assay.*

Weigh a quantity of the powdered tablets containing about 1 mg of Reserpine, add 10 ml of a 2 per cent w/v solution of *citric acid* and extract for 2 minutes with three quantities, each of 15 ml, of *chloroform*. Wash the combined extracts with 10 ml of a 1 per cent w/v solution of *sodium bicarbonate*, add sufficient *chloroform* to produce 50.0 ml and evaporate 10.0 ml to dryness on a water-bath. Dissolve the residue in 10 ml of *ethanol (95 per cent)* and add 2 ml of *0.25 M sulphuric acid* and 10 ml of *ethanol (95 per cent)* and warm gently to dissolve. Cool, dilute to 100.0 ml with *ethanol (95 per cent)* and dilute 5.0 ml to 50.0 ml with the same solvent (solution A). Transfer 10.0 ml to a boiling tube, add 2 ml of *0.25 M sulphuric acid* and 2 ml of a freshly prepared 0.3 per cent w/v solution of *sodium nitrite*, mix and heat in a water-bath at 55° for 35 minutes. Cool, add 1 ml of a freshly prepared 5 per cent w/v solution of *sulphamic acid* and dilute to 25.0 ml with *ethanol (95 per cent)*. Measure the absorbance of the resulting solution at the maximum at about 388 nm (2.4.7), using as the blank a solution prepared by treating a further 10.0 ml of solution A in the same manner and at the same time but omitting the sodium nitrite solution.

Calculate the content of $C_{33}H_{40}N_2O_9$ from the absorbance obtained by repeating the operation using *reserpine IPRS* in place of the substance under examination.

Storage. Store protected from light.

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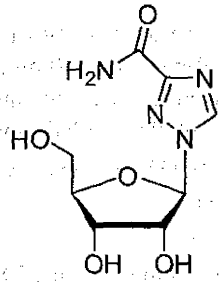
Ribavirin



$C_8H_{12}N_4O_5$ Mol.Wt. 244.2

Ribavirin is 1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide.

Ribavirin contains not less than 98.0 per cent and not more than 102.0 per cent of $C_8H_{12}N_4O_5$, calculated on the dried basis.

Category. Antiviral.

Description. A white or almost white crystalline powder. It shows polymorphism (2.5.11).

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ribavirin IPRS* or with the reference spectrum of ribavirin.

Tests

pH (2.4.24): 4.0 to 6.5, determined in a 2.0 per cent w/v solution in *carbon dioxide-free water*.

Specific optical rotation (2.4.22). −37° to −33°, determined in a 1.0 per cent w/v solution, use freshly prepared solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of substance under examination in 70 ml of *water* and dilute to 100.0 ml with *water*.

Reference solution (a). In order to produce 1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxylic acid (ribavirin impurity A) in situ, mix 5.0 ml of the test solution and 5.0 ml of a 4.2 per cent w/v solution of *sodium hydroxide* and allow to stand for 90 minutes. Neutralise with 5.0 ml of a 10.3 per cent w/v solution of *hydrochloric acid* and mix well.

Reference solution (b) Dilute 1.0 ml of the test solution to 100.0 ml with *water*. Dilute 1.0 ml of the solution to 10.0 ml with *water*.

Reference solution (c) A 0.05 per cent w/v solution of *ribavirin IPRS* in *water*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with end-capped octadecylsilane bonded to porous silica (3 µm),
- mobile phase: A. dissolve 1.0 g of *anhydrous sodium sulphate* in 950 ml of *water*, add 2.0 ml of a 5 per cent v/v solution of *orthophosphoric acid*, adjusted to pH 2.8 with a 5 per cent v/v solution of *orthophosphoric acid* and dilute to 1000 ml with *water*,

  B. 5 volumes of *acetonitrile* and 95 volumes of mobile phase A,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
15	100	0
25	0	100
35	0	100
38	100	0

The relative retention time with reference to ribavirin (retention time is about 6 minutes) for ribavirin impurity A is about 0.8. The correction factor for ribavirin impurity A is 2.3.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to ribavirin and ribavirin impurity A is not less than 4.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak due to ribavirin impurity A is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent). The area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent). The sum of the areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). Dissolve 4.0 g in 20 ml of *water* with heating if necessary and use 10 ml of the solution. The resulting solution complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 5 hour.

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Assay. Determine by liquid chromatography (2.4.14) same as described under Related substances with the following modification.

Inject reference solution (c) and the test solution.

Calculate the content of $C_8H_{12}N_4O_5$.

Storage. Store protected from light.

Ribavirin Capsules

Ribavirin Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ribavirin, $C_8H_{12}N_4O_5$.

Usual strengths. 100 mg; 200 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket).

Medium. 900 ml of *water*.

Speed and time. 100 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.00225 per cent w/v solution of *ribavirin IPRS* in the dissolution medium.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm, packed with a strong cation-exchange resin consisting of sulphonated cross-linked styrene-divinylbenzene copolymer in the hydrogen form (9 µm),
- column temperature: 65°,
- mobile phase: *water*, adjusted to pH 2.5 with *sulphuric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 207 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{12}N_4O_5$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_8H_{12}N_4O_5$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the mixed content containing 50 mg of Ribavirin in 50 ml of the mobile phase, with the aid of ultrasound for 20 minutes with intermittent shaking and dilute to 100.0 ml with the mobile phase, filter.

Reference solution. A 0.0025 per cent w/v solution of *ribavirin IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 7.8 mm, packed with a strong cation-exchange resin consisting of sulphonated cross-linked styrene-divinylbenzene copolymer in the hydrogen form (7 µm),
- column temperature: 65°,
- mobile phase: *water*, adjusted to pH 2.5 with *sulphuric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 207 nm,
- injection volume: 10 µl.

Name	Relative retention time	Correction factor
Ribose triazolole carboxylic acid[1]	0.7	1.43
Ribavirin	1.0	—

[1] 1-β-*D*-Ribofuranosyl-1*H*-1,2,4-triazole-3-carboxylic acid.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to ribavirin impurity A is not more than 0.05 times the area of principal peak in the chromatogram obtained with the reference solution (0.25 per cent), the area of any other secondary peak is not more than 0.02 times the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 0.2 times the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore any peak with an area less than 0.01 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and mix the content of 20 capsules. Disperse a quantity of the mixed content containing 50 mg of Ribavirin in 50 ml of the mobile phase, with the aid of ultrasound for 20 minutes with intermittent shaking and dilute to 100.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution. A 0.0025 per cent w/v solution of *ribavirin IPRS* in the mobile phase.

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Use chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{12}N_4O_5$ in the capsules.

Storage. Store protected from moisture, at a temperature between 15° to 30°.

Ribavirin Inhalation Solution

Ribavirin Solution for Inhalation

Catogery. Antiviral.

Ribavirin Inhalation Solution is a sterile solution of Ribavirin in Water for Injections. It is prepared by dissolving Ribavirin for Inhalation in the requisite amount of Water for Injections.

Ribavirin for Inhalation

Ribavirin for Inhalation is a sterile ribavirin consisting of Ribavirin with or without excipients. It is filled in a sealed container.

The inhalation solution is constituted by dissolving the contents of the sealed container in the requisite amount of sterile water for injection, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and particulate matter stated under Inhalation Preparations.

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Ribavirin Inhalation Solution contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of $C_8H_{12}N_4O_5$.

The content of the sealed container complies with the requirements stated under Inhalation Preparations and with the following requirements.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 20 volumes of *0.1 M ammonium chloride* and 90 volumes of *acetonitrile*.

Test solution. Dissolve ribavirin inhalation solution containing about 0.1 g of Ribavirin in 10 ml of *water*.

Reference solution. A 1.0 per cent w/v solution of *ribavirin IPRS* in *water*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air for 15 minutes, and spray the plate with the mixture of 0.5 ml of *anisaldehyde*, 0.5 ml of *sulphuric acid*, 0.1 ml of *glacial acetic acid* and 9 ml of *ethanol (95 per cent)* and heat at 105° for 40 minutes and allow to cool. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to that in the chromatogram obtained with the reference solution.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. A 0.05 per cent w/v solution of ribavirin in the mobile phase.

Reference solution (a). A 0.000125 per cent w/v solution of ribavirin in the mobile phase.

Reference solution (b). A 0.05 per cent w/v solution of *ribavirin IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 10 cm x 7.8 mm, packed with strong cation-exchange resin of sulphonated, cross-linked *styrene-divinylbenzene co-polymer* in the hydrogen form (7 to 11 µm) (Such as Aminex HPAH),
- column temperature: 40°,
- mobile phase: *water*, adjusted to pH 2.5 with *sulphuric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 207 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the tailing factor for the principal peak is not less than 0.7 and not more than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent) and the sum of the areas of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Sterility (2.2.11). Complies with the test for sterility.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reconstitute the contents of one container as instructed on the label using a measured volume of diluent,

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dilute a suitable volume of the solution to 200 volumes with the mobile phase to produce a solution containing 0.1 per cent w/v of Ribavirin, mix and dilute 5 volumes of the resulting solution to 20 volumes with the mobile phase.

Reference solution. A 0.025 per cent w/v solution of *ribavirin IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 10 cm x 7.8 mm, packed with strong cation-exchange resin of sulphonated, cross-linked *styrene-divinylbenzene co-polymer* in the hydrogen form (7 to 11 µm) (Such as Aminex HPAH),
- column temperature: 40°,
- mobile phase: *water*, adjusted to pH 2.5 with *sulphuric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 207 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not less than 0.7 and not more than 1.5.

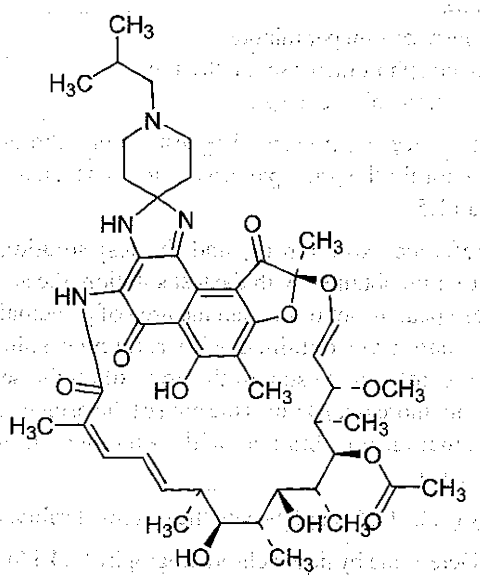
Inject the reference solution and the test solution.

Calculate the content of $C_8H_{12}N_4O_5$ in inhalation solution.

Labelling. The label of the sealed container states (1) the procedure for preparing the solution, (2) the delivery system to be used for the constituted solution (3) the conditions under which the constituted solution should be stored.

Storage. Store procted from light and moisture.

Rifabutin



$C_{46}H_{62}N_4O_{11}$ Mol. Wt. 847.0

Rifabutin is (9*S*,12*E*,14*S*,15*R*,16*S*,17*R*,18*R*,19*R*,20*S*,21*S*,22*E*,24*Z*)-6,16,18,20-Tetrahydroxy-1'isobutyl-14-methoxy-7,9,15,17,19,21,25-heptamethylspirol[9,4-(epoxypentadeca[1,11,13]trienimino)-2*H*-furo[22,32:7,8]naphth[1,2-*d*]imidazole-2,42-piperidine]-5,10,26-(3*H*,9*H*)-trione-16-yl- acetate.

Rifabutin contains not less than 95.0 per cent and not more than 102.0 per cent of $C_{46}H_{62}N_4O_{11}$ calculated on the anhydrous basis.

Category. Antituberculosis.

Description. An amorphous red-violet powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rifabutin IPRS* or with the reference spectrum of rifabutin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

N-isobutylpiperidone. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. Equal volumes of *chloroform* and *methanol*.

Mobile phase. A mixture of 100 volumes of *hexane* and 30 volumes of *acetone*.

Test solution. Dissolve 0.1 g of the substance under examination in 10.0 ml of the solvent mixture.

Reference solution. Prepare 0.0005, 0.001, 0.002, 0.005 and 0.01 per cent w/v solutions of *N-isobutylpiperidone IPRS* in the solvent mixture.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and expose the plate to iodine vapour for about 5 minutes, then spray with *starch solution* and allow to stand for 5 minutes. No spot in the chromatogram of the test solution at R_f value corresponding to *N*-isobutylpiperidone is more intense than that of the principal spot observed in the chromatogram obtained with the reference solution containing 0.005 per cent solution (0.5 per cent).

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay using the following modifications.

Inject the test solution. The area of any impurity detected at a relative retention time of about 0.5, 0.6, 0.8 or 1.4 with respect to rifabutin peak is not more than 1.0 per cent, the area of any other secondary peak is not more than 0.5 per cent and the sum of areas of all the secondary peaks is not more than 3.0 per cent, calculated by area normalisation.

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Water (2.3.43). Not more than 2.5 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in 5 ml of *acetonitrile* and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 25 mg of *rifabutin IPRS* in 5 ml of *acetonitrile* and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of *rifabutin IPRS* and 2 ml of *methanol*, add 1 ml of *2 M sodium hydroxide* and allow to stand for about 4 minutes. Add 1 ml of *2 M hydrochloric acid*, and dilute to 50.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column12.5 cm × 4.6 mm packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of equal volumes of *acetonitrile* and *0.1 M monobasic potassium phosphate*, adjusted pH 6.5 with *2 M sodium hydroxide*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

The relative retention time with reference to rifabutin for a major peak for a degradant, two minor peaks for degradants are about 0.5, 0.6 and 0.8 respectively.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the rifabutin peak and the degradant peak eluting at a relative retention time of about 0.8 is not less than 1.3 in the chromatogram obtained with reference solution (b) and the column efficiency is not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{46}H_{62}N_4O_{11}$.

Storage. Store protected from light and moisture.

Rifabutin Capsules

Rifabutin Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of rifabutin, $C_{46}H_{62}N_4O_{11}$.

Usual strength. 150 mg.

Identification

A. Disperse a quantity of capsule content in *methanol* to obtain a solution containing 0.002 per cent w/v, filter and examine in the range 200 to 400 nm (2.4.7). The spectra obtained with the test solution exhibits the maxima at the same wavelength with that of the reference solution.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.01 M hydrochloric acid*,
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtered solution, suitably diluted with the medium if necessary, at the maximum at about 280 nm (2.4.7). Calculate the content of $C_{46}H_{62}N_4O_{11}$ in the medium from the absorbance obtained by using a solution of known concentration of *rifabutin IPRS*.

Q. Not less than 75 per cent of the stated amount of $C_{46}H_{62}N_4O_{11}$.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay using the following modifications.

Inject the test solution. The area of any secondary peak with a relative retention time of about 0.5, 0.6, 0.8 or 1.4 with respect to rifabutin peak is not more than 1.0 per cent, the area of any other secondary peak is not more than 0.5 per cent and the sum of the areas of all the secondary peaks is not more than 4.5 per cent, calculated by area normalisation.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Mix the contents of 20 capsules. Disperse a quantity of powder containing 25 mg of Rifabutin in 5 ml of *acetonitrile* and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 25 mg of *rifabutin IPRS* in 5 ml of *acetonitrile* and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve about 10.0 mg of *rifabutin IPRS* and 2 ml of *methanol*, add 1 ml of *2 M sodium hydroxide* and allow to stand for about 4 minutes. Add 1 ml of *2 M hydrochloric acid*, and dilute to 50.0 ml with mobile phase.

Chromatographic system
- a stainless steel column12.5 cm × 4.6 mm packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of equal volumes of *acetonitrile* and *0.1 M monobasic potassium phosphate*, adjusted to pH 6.5 with *2 M sodium hydroxide*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

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The relative retention time with reference to rifabutin for a major peak for a degradant, two minor peaks for degradants are about 0.5, 0.6 and 0.8 respectively.

Inject reference solution (a) and reference solution (b).

The test is not valid unless the resolution between the rifabutin peak and the degradant peak eluting at a relative retention time of about 0.8 is not less than 1.3 obtained with reference solution (b) and the column efficiency is not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent obtained with reference solution (a).

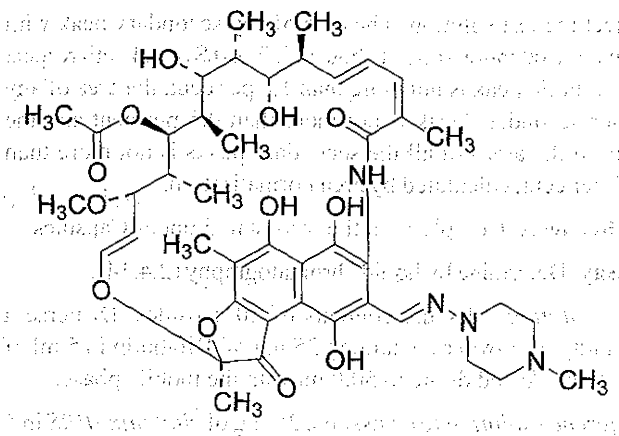
Inject reference solution (a) and the test solution.

Calculate the content of $C_{46}H_{62}N_4O_{11}$.

Storage. Store protected from light and moisture.

Rifampicin

Rifampin



$C_{43}H_{58}N_4O_{12}$ Mol. Wt. 823.0

Rifampicin is (12*Z*,14*E*,24*E*)-(2*S*,16*S*,17*S*,18*R*,19*R*,20*R*,21*S*,22*R*,23*S*)-1,2-dihydro-5,6,9,17,19-pentahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-(4-methylpiperazin-1-yliminomethyl)-1,11-dioxo-2,7-(epoxypentadeca-1,11,13-trienimino)naphtho[2,1-*b*]furan-21-yl acetate.

Rifampicin contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{43}H_{58}N_4O_{12}$, calculated on the dried basis.

Category. Antitubercular.

Description. A brick-red to reddish brown, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rifampicin IPRS* or with the reference spectrum of rifampicin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.5 to 6.5, determined in a 1.0 per cent w/v suspension.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Solvent mixture. A mixture of 10 volumes of a 21.01 per cent w/v solution of *citric acid*, 23 volumes of a 13.61 per cent w/v solution of *potassium dihydrogen phosphate*, 77 volumes of a 17.42 per cent w/v solution of *dipotassium hydrogen phosphate*, 640 volumes of *water* and 250 volumes of *acetonitrile*.

Test solution. Weigh a quantity of powder containing 20 mg of Rifampicin, add 10 ml of *acetonitrile*, shake and filter. Dilute 5 ml of the filtrate to 50 ml with the solvent mixture.

Reference solution. A solution containing 0.02 per cent w/v of *rifampicin quinone IPRS* in *acetonitrile*. To 1 ml of the solution, add 1 ml of the test solution and dilute to 100 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of a solution containing 0.1 per cent v/v of *orthophosphoric acid*, 0.19 per cent w/v of *sodium perchlorate*, 0.59 per cent w/v of *citric acid* and 2.09 per cent w/v of *potassium dihydrogen phosphate* and 35 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the resolution between rifampicin and rifampicin quinone is not less than 4.

In the chromatogram obtained with the test solution the area of any peak due to rifampicin quinone is not more than 1.5 times the area of the peak due to rifampicin quinone in the chromatogram obtained with the reference solution (1.5 per cent); the area of any peak, other than the peak due to rifampicin and rifampicin quinone, is not more than the area of the peak due to rifampicin in the chromatogram obtained with the

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reference solution (1.0 per cent) and the sum of the areas of any such peaks is not more than 3.5 times the area of the peak due to rifampicin in the chromatogram obtained with the reference solution (3.5 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 80° at a pressure not exceeding 0.7 kPa for 4 hours.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Solvent mixture. A mixture of 10 volumes of a 21.01 per cent w/v solution of *citric acid*, 23 volumes of a 13.61 per cent w/v solution of *potassium dihydrogen phosphate*, 77 volumes of a 17.42 per cent w/v solution of *dipotassium hydrogen phosphate*, 640 volumes of *water* and 250 volumes of *acetonitrile*.

Test solution. Dissolve 20.0 mg of the substance under examination in 10.0 ml of *acetonitrile* and filter. Dilute 5.0 ml of the filtrate to 100.0 ml with the solvent mixture.

Reference solution. A solution containing 0.2 per cent w/v of *rifampicin IPRS* in *acetonitrile*. Dilute 5.0 ml of the solution to 100.0 ml with the solvent mixture.

Use the chromatographic system and the system suitability parameters described under the test for Related substances.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{43}H_{58}N_4O_{12}$.

Storage. Store protected from light, in an atmosphere of nitrogen.

Rifampicin Capsules

Rifampin Capsules

Rifampicin Capsules contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of rifampicin, $C_{43}H_{58}N_4O_{12}$.

Usual strengths. 150 mg; 300 mg; 450 mg; 600 mg.

Identification

A. Shake a quantity of the contents of the capsules containing 0.15 g of Rifampicin with 5 ml of *chloroform*, filter and evaporate the filtrate to dryness. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rifampicin IPRS* or with the reference spectrum of rifampicin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium: 900 ml of *0.1 M hydrochloric acid*,
Speed and time: 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter promptly through a membrane filter disc having an average pore diameter not greater than 1.0 µm, rejecting the first 1 ml of the filtrate. Dilute the filtrate, if necessary, with the same solvent. Measure the absorbance (2.4.7) of the resulting solution at the maximum at about 475 nm. Calculate the content of $C_{43}H_{58}N_4O_{12}$ in the medium from the absorbance obtained from a solution of known concentration of *rifampicin IPRS*.

Q. Not less than 75 per cent of the stated amount of $C_{43}H_{58}N_4O_{12}$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Solvent mixture. 10 volumes of a 21.01 per cent w/v solution of *citric acid*, 23 volumes of a 13.61 per cent w/v solution of *potassium dihydrogen phosphate*, 77 volumes of a 17.42 per cent w/v solution of *dipotassium hydrogen phosphate*, 640 volumes of *water* and 250 volumes of *acetonitrile*.

Test solution. Shake a quantity of the contents of the capsules containing 200 mg of Rifampicin, with 100 ml of *acetonitrile* and filter. Dilute 5 ml of the filtrate to 50 ml with the solvent mixture.

Reference solution (a). A solution containing 0.02 per cent w/v of *rifampicin IPRS* in *acetonitrile*. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v each of *rifampicin IPRS* and *rifampicin quinone IPRS* in *acetonitrile*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 10 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 30°,

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- mobile phase: a mixture of 65 volumes of a solution containing 0.1 per cent v/v of *orthophosphoric acid*, 0.19 per cent w/v of *sodium perchlorate*, 0.59 per cent w/v of *citric acid* and 2.09 per cent w/v of *potassium dihydrogen phosphate* and 35 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention times are 1.0, 0.55, 1.25 and 3.56 for rifampicin, rifampicin quinone, rifampicin N-oxide and 3-formylrifamycin SV respectively. The response factors are 1.00, 1.19, 1.03 and 1.25 for rifampicin, rifampicin quinone, rifampicin N-oxide and 3-formylrifamycin SV respectively.

Inject reference solution (b). The test is not valid unless the resolution between rifampicin and rifampicin quinone is not less than 4, the tailing factor is not more than 2.0 and the column efficiency is not less than 2000 theoretical plates.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak due to rifampicin quinone is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent), the area of any peak due to rifampicin N-oxide is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent) and the area of any peak due to 3-formylrifamycin SV is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). In the chromatogram obtained with the test solution the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 10 volumes of a 21.01 per cent w/v solution of *citric acid*, 23 volumes of a 13.61 per cent w/v solution of *potassium dihydrogen phosphate*, 77 volumes of a 17.42 per cent w/v solution of *dipotassium hydrogen phosphate*, 640 volumes of *water* and 250 volumes of *acetonitrile*.

Test solution. Shake a quantity of the contents of the capsules containing about 300.0 mg of Rifampicin, with 200.0 ml of *acetonitrile*, filter. Dilute 10.0 ml of the filtrate to 50.0 ml with *acetonitrile*. Further dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution (a). A 0.15 per cent w/v solution of *rifampicin IPRS* in *acetonitrile*. Dilute 10.0 ml of the solution to 50.0 ml with *acetonitrile*. Further dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v each of *rifampicin IPRS* and *rifampicin quinone IPRS* in *acetonitrile*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Use the chromatographic system and system suitability parameters, as described under the test for Related substances.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{43}H_{58}N_4O_{12}$ in the capsules.

Storage. Store protected from light and moisture.

Rifampicin Oral Suspension

Rifampicin oral suspension contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of rifampicin, $C_{43}H_{58}N_4O_{12}$.

Usual strengths. 10 mg per ml; 20 mg per ml.

Identification

A. To a quantity containing 0.1 g of Rifampicin add 30 ml of *water* and shake with two quantities, each of 50 ml, of *chloroform*. Dry the combined extracts with *anhydrous sodium sulphate*, filter and evaporate the filtrate to dryness at a temperature not exceeding 70°. Wash the residue with 1 ml of *ether* and dry at 70°. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rifampicin IPRS* or with the reference spectrum of rifampicin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution (a).

Tests

pH (2.4.24). 4.2 to 4.8.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Solvent mixture. A mixture of 10 volumes of a 21.01 per cent w/v solution of *citric acid*, 23 volumes of a 13.61 per cent w/v solution of *potassium dihydrogen phosphate*, 77 volumes of a 17.42 per cent w/v solution of *dipotassium hydrogen phosphate*, 640 volumes of *water* and 250 volumes of *acetonitrile*.

Test solution. Add 5 ml of *water* to a quantity of the oral suspension containing 20 mg of Rifampicin and extract with

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four quantities, each of 10 ml, of *dichloromethane*, filter the combined extracts and evaporate to dryness at a temperature not exceeding 40°. Dissolve the residue in 10 ml of *acetonitrile*. Dilute 5.0 ml of the resulting solution to 50 ml with the solvent mixture.

Reference solution (a). A solution containing 0.02 per cent w/v of *rifampicin IPRS* in *acetonitrile*. Dilute 1.0 ml of the solution to a 100.0 ml with the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v each of *rifampicin IPRS* and *rifampicin quinone IPRS* in *acetonitrile*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: a mixture of 65 volumes of a solution containing 0.1 per cent v/v of *orthophosphoric acid*, 0.19 per cent w/v of *sodium perchlorate*, 0.59 per cent w/v of *citric acid* and 2.09 per cent w/v of *potassium dihydrogen phosphate* and 35 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention times are 1.0, 0.55, 1.25 and 2.61 for rifampicin, rifampicin quinone, rifampicin N-oxide and 3-formylrifamycin SV respectively. Multiply the areas of each known impurity by their response factor. The response factors are 1.00, 1.19, 1.03, 1.22 and 1.25 for rifampicin, rifampicin quinone, rifampicin N-oxide, Isonicotinyl hydrazone and 3-formylrifamycin SV respectively.

Inject reference solution (b). The test is not valid unless the resolution between rifampicin and rifampicin quinone is not less than 4; the tailing factor is not more than 2.0 and the column efficiency is not less than 2000 theoretical plates.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any peak due to rifampicin quinone is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent), the area of any peak due to rifampicin N-oxide is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent) and the area of any peak due to 3-formylrifamycin SV is not more than 5.0 times the area of the principal peak in the chromatogram obtained with reference solution (a) (5.0 per cent). The area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a).

Other tests. Comply with the tests stated under Oral Suspensions.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the oral suspension containing 150 mg of rifampicin and dilute to 100.0 ml with *acetonitrile*. Dilute 10.0 ml of the solution to 50.0 ml with *acetonitrile*. Dilute 10.0 ml of the resulting solution to 50.0 ml with the solvent mixture.

Reference solution (a). A 0.15 per cent w/v solution of *rifampicin IPRS* in *acetonitrile*. Dilute 10.0 ml of the solution to 50.0 ml with *acetonitrile*. Dilute 10.0 ml of the resulting solution to 50.0 ml with the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v of each of *rifampicin IPRS* and *rifampicin quinone IPRS* in *acetonitrile*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Use the chromatographic system and the system suitability parameters as described under the test for Related substances.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Determine the weight per ml of the suspension (2.4.29) and calculate the content of $C_{43}H_{58}N_4O_{12}$ weight in volume.

Storage. Store protected from light and moisture.

Rifampicin Tablets

Rifampin Tablets

Rifampicin Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of rifampicin, $C_{43}H_{58}N_4O_{12}$.

Usual strength. 150 mg.

Identification

A. Shake a quantity of the powdered tablets containing 0.15 g of Rifampicin with 5 ml of *chloroform*, filter and evaporate the filtrate to dryness. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rifampicin IPRS* or with the reference spectrum of rifampicin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

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Medium. 900 ml of *0.1 M hydrochloric acid*.

Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter promptly through a membrane filter disc having an average pore diameter not greater than 1.0 µm, rejecting the first 1 ml of the filtrate. Dilute the filtrate, if necessary, with the dissolution medium. Measure the absorbance (2.4.7) of the resulting solution at the maximum at about 475 nm. Calculate the content of $C_{43}H_{58}N_4O_{12}$ in the medium from the absorbance obtained from a solution of known concentration of *rifampicin IPRS*.

Q. Not less than 75 per cent of the stated amount of $C_{43}H_{58}N_4O_{12}$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Solvent mixture. 10 volumes of a 21.01 per cent w/v solution of *citric acid*, 23 volumes of a 13.61 per cent solution w/v of *potassium dihydrogen phosphate*, 77 volumes of a 17.42 per cent w/v solution of *dipotassium hydrogen phosphate*, 640 volumes of *water* and 250 volumes of *acetonitrile*.

Test solution. Weigh a quantity of the powdered tablets containing 200 mg of Rifampicin, dissolve in 100 ml of *acetonitrile*, filter. Dilute 5.0 ml of the filtrate to 50.0 ml with the solvent mixture.

Reference solution (a). A solution containing 0.02 per cent w/v of *rifampicin IPRS* in *acetonitrile*. Dilute 1 ml of the solution to a 100 ml with the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v of each of *rifampicin IPRS* and *rifampicin quinone IPRS* in *acetonitrile*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 10 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column. temperature. 30°,
- mobile phase: a mixture of 65 volumes of a solution containing 0.1 per cent v/v of *orthophosphoric acid*, 0.19 per cent w/v of *sodium perchlorate*, 0.59 per cent w/v of *citric acid* and 2.09 per cent w/v of *potassium dihydrogen phosphate* and 35 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention times are 1.0, 0.55, 1.25 and 2.61 for rifampicin, rifampicin quinone, rifampicin N-oxide, and 3-formylrifamycin SV respectively. The response factors are 1.00, 1.19, 1.03 and 1.25 for rifampicin, rifampicin quinone, rifampicin N-oxide and 3-formylrifamycin SV respectively.

Inject reference solution (b). The test is not valid unless the resolution between rifampicin and rifampicin quinone is not less than 4, the tailing factor is not more than 2.0 and the column efficiency is not less than 2000 theoretical plates.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any peak due to rifampicin quinone is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent), the area of any peak due to rifampicin N-oxide is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent) and the area of any peak due to 3-formyl rifamycin SV is not more than the area of principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). The area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powder containing 300.0 mg of Rifampicin, dissolve in 200.0 ml of *acetonitrile*, filter. Dilute 10.0 ml of the filtrate to 50.0 ml with *acetonitrile*. Further dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution (a). A 0.15 per cent w/v solution of *rifampicin IPRS* in *acetonitrile*. Dilute 10.0 ml of the solution to 50.0 ml with *acetonitrile*. Further dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v each of *rifampicin IPRS* and *rifampicin quinone IPRS* in *acetonitrile*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Use the chromatographic system and system suitability parameters described under the test for Related substances.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{43}H_{58}N_4O_{12}$ in the tablets.

Storage. Store protected from light and moisture.

Rifampicin and Isoniazid Tablets

Rifampin and Isonicotinylhydrazid Tablets

Rifampicin and Isoniazid Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of rifampicin, $C_{43}H_{58}N_4O_{12}$ and isoniazid, $C_6H_7N_3O$.

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Usual strength. Rifampicin 50 mg and Isoniazid 150 mg.

Identification

A. In the Assay, the chromatogram obtained with the test solution shows peaks that correspond to the peaks due to *rifampicin IPRS* and *isoniazid IPRS* in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter promptly through a membrane filter disc with an average pore diameter not greater than 0.8 µm. Reject the first few ml of the filtrate and dilute a suitable volume of the filtrate with the medium.

Reference stock solution. A solution containing 0.0165 per cent of *rifampicin IPRS* and 0.00825 per cent of *isoniazid IPRS* in the dissolution medium.

For Rifampicin — Measure the absorbance of the sample solution and the reference stock solution suitably diluted with the dissolution medium at 475 nm (2.4.7). Calculate the content of $C_{43}H_{58}N_4O_{12}$ in the medium from the absorbance obtained from the reference stock solution.

For Isoniazid — Determine by liquid chromatography (2.4.14).

Use the reference stock solution and sample solution suitably diluted with a solution of *0.05 M potassium dihydrogen orthophosphate*, adjust the pH to 6.2 with *0.1 M sodium hydroxide* to obtain the reference solution and the test solution, respectively.

Chromatographic system
- a stainless steel column 30 cm × 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 99 volumes of *0.05 M potassium dihydrogen phosphate* and 1 volume of *acetonitrile* with the pH adjusted to 4.0 ± 0.05 with 2 per cent w/v solution of *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency in not less than 1500 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_6H_7N_3O$ in the dissolution medium.

Q. Not less than 75 per cent of the stated amounts of $C_{43}H_{58}N_4O_{12}$ and $C_6H_7N_3O$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Solvent mixture. A mixture of 10 volumes of a 21.01 per cent w/v solution of *citric acid*, 23 volumes of a 13.61 per cent w/v solution of *potassium dihydrogen phosphate*, 77 volumes of a 17.42 per cent w/v solution of *dipotassium hydrogen phosphate*, 640 volumes of *water* and 250 volumes of *acetonitrile*.

Test solution. Weigh a quantity of the powdered tablets containing 200 mg of Rifampicin in 100 ml of *acetonitrile* and filter. Dilute 5 ml of the filtrate to 50 ml with the solvent mixture.

Reference solution (a). Dissolve *rifampicin IPRS* in *acetonitrile* to obtain a solution containing 0.2 mg per ml. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v each of *rifampicin IPRS* and *rifampicin quinone IPRS* in *acetonitrile*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 10 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature 30°,
- mobile phase: a mixture of 65 volumes of a solution containing 0.1 per cent v/v of *orthophosphoric acid*, 0.19 per cent w/v of *sodium perchlorate*, 0.59 per cent w/v of *citric acid* and 2.09 per cent w/v of *potassium dihydrogen phosphate* and 35 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention times are 1.0, 0.55, 1.25, 1.51 and 2.61 for rifampicin, rifampicin quinone, rifampicin N-oxide, 3-formylrifamycin SV isonicotinyl hydrazone and 3-formylrifamycin SV respectively. Multiply the area of each known impurity by its response factor. The response factors are 1.00, 1.19, 1.03, 1.22 and 1.25 for rifampicin, rifampicin quinone, rifampicin N-oxide, isonicotinyl hydrazone and 3-formylrifamycin SV respectively.

Inject reference solution (b). The test is not valid unless the resolution between rifampicin and rifampicin quinone is not less than 4, the tailing factor is not more than 2.0 and the column efficiency is not less than 2000 theoretical plates.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any peak due to rifampicin quinone is not more than 4 times the area of the principal peak in the chromatogram obtained with

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reference solution (a) (4.0 per cent), the area of any peak due to rifampicin N-oxide is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent), the area of any peaks due to 3-formylrifamycin SV and isonicotinyl hydrazone is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (5.0 per cent) and the area of any peak due to 3-formylrifamycin SV is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). The area of any other secondary peak is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 1.4 g of *disodium hydrogen orthophosphate anhydrous* in 1000 ml of *water* and adjust the pH to 6.8 with *dilute phosphoric acid*.

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 40 mg of Isoniazid, dissolve in 100.0 ml of *methanol* and dilute to 500.0 ml with the solvent mixture.

Reference solution. A solution containing 0.08 per cent w/v of *rifampicin IPRS* and 0.04 per cent w/v of *isoniazid IPRS* in *methanol*. Dilute 10.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column. temperature 30°,
- mobile phase: A. a mixture of 96 volumes of buffer solution pH 6.8 prepared by dissolving 1.4 g of *disodium hydrogen orthophosphate anhydrous* in 1000 ml of *water* and adjusting the pH to 6.8 ± 0.05 with *dilute orthophosphoric acid*, and 4 volumes of *acetronitrile*.
  B. a mixture of 45 volumes of the buffer solution and 55 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	100	0
6	0	100
15	0	100
16	100	0
20	100	0

NOTE — Saturate the column with mobile phase B for about 1 hour before injection.

The retention times are about 1.0 for rifampicin and about 0.3 for isoniazid.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 for rifampicin and isoniazid; the column efficiency for the isoniazid peak is not less than 3000 and for rifampicin not less than 25000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the contents of $C_{43}H_{58}N_4O_{12}$ and $C_6H_7N_3O$ in the tablets.

Storage. Store protected from moisture.

Rifampicin, Isoniazid and Ethambutol Tablets

Rifampin, Isonicotinylhydrazid and Ethambutol Hydrochloride Tablets

Rifampicin, Isoniazid and Ethambutol Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of rifampicin, $C_{43}H_{58}N_4O_{12}$, isoniazid, $C_6H_7N_3O$ and ethambutol hydrochloride $C_{10}H_{24}N_2O_2.2HCl$.

Usual strength. Rifampicin 300 mg, Isoniazid 200 mg and Ethambutol 200 mg.

Identification

A. In the Assay for rifampicin and isoniazid, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

B. In the Assay for ethambutol hydrochloride the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter promptly through a membrane filter disc with an average pore diameter not greater than 0.8 µm. Reject the first few ml of the filtrate.

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Reference stock solution. A solution containing 0.0165 per cent w/v of *rifampicin IPRS*, 0.00825 per cent w/v of *isoniazid IPRS* and 0.031 per cent w/v of *ethambutol hydrochloride IPRS* in the dissolution medium. Keep this reference stock solution in the dissolution bath during the test run.

For Rifampicin — Dilute the filtrate, if necessary, with the same solvent. Measure the absorbance (2.4.7) of the resulting solution at the maximum at about 475 nm. Calculate the content of $C_{43}H_{58}N_4O_{12}$ in the medium from the absorbance obtained from suitably diluted reference stock solution.

For Isoniazid — Determine by liquid chromatography (2.4.14).

Test solution. Suitably dilute the filtered medium with *0.05 M potassium dihydrogen orthophosphate* with the pH adjusted to 6.2 with *0.1 M sodium hydroxide*.

Reference solution. Suitably dilute the reference stock solution with *0.05 M potassium dihydrogen orthophosphate* with the pH adjusted to 6.2 with *0.1 M sodium hydroxide*.

Chromatographic system
- a stainless steel column 30 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 99 volumes of *0.05 M potassium dihydrogen phosphate* and 1 volume of *acetonitrile* previously adjusted to pH 4.0 with a 2 per cent w/v solution of *phosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency in not less than 1500 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_6H_7N_3O$ in the dissolution medium.

For Ethambutol Hydrochloride — Determine by liquid chromatography (2.4.14).

Test solution. Suitably dilute the filtered medium with *0.05 M potassium dihydrogen orthophosphate* adjusted to pH 6.2 with *0.1 M sodium hydroxide*.

Reference solution. Suitably dilute the reference stock solution with *0.05 M potassium dihydrogen orthophosphate* with the pH adjusted to 6.2 with *0.1 M sodium hydroxide*.

Use the chromatographic system described under the Assay of ethambutol hydrochloride.

Calculate the content of $C_{10}H_{24}N_2O_2.2HCl$ in the medium.

Q. Not less than 75 per cent of the stated amounts of $C_{43}H_{58}N_4O_{12}$, $C_6H_7N_3O$ and $C_{10}H_{24}N_2O_2.2HCl$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Solvent mixture. A mixture of 10 volumes of a 21.01 per cent w/v solution of *citric acid*, 23 volumes of a 13.61 per cent w/v solution of *potassium dihydrogen phosphate*, 77 volumes of a 17.42 per cent w/v solution of *dipotassium hydrogen phosphate*, 640 volumes of *water* and 250 volumes of *acetonitrile*.

Test solution. Weigh a quantity of the powdered tablets containing 200 mg of Rifampicin, dissolve in 100 ml of *acetonitrile* and filter. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution (a). A solution containing 0.02 per cent w/v of *rifampicin IPRS* in *acetonitrile*. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v each of *rifampicin IPRS* and *rifampicin quinone IPRS* in *acetonitrile*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: a mixture of 65 volumes of a solution containing 0.1 per cent v/v of *orthophosphoric acid*, 0.19 per cent w/v of *sodium perchlorate*, 0.59 per cent w/v of *citric acid* and 2.09 per cent w/v of *potassium dihydrogen phosphate* and 35 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between rifampicin and rifampicin quinone is not less than 4, the tailing factor is not more than 2.0 and the column efficiency is not less than 2000 theoretical plates.

Inject reference solution (a) and the test solution. The relative retention times are 1.0, 0.55, 1.25, 1.51 and 2.61 for rifampicin, rifampicin quinone, rifampicin N-oxide, 3-formylrifamycin SV Isonicotinyl hydrazone and 3-formylrifamycin SV respectively. Multiply the areas of each known impurity by its response factor. The response factors are 1.00, 1.19, 1.03, 1.22 and 1.25 for rifampicin, rifampicin quinone, rifampicin N-oxide, Isonicotinyl hydrazone and 3-formylrifamycin SV respectively.

In the chromatogram obtained with the test solution the area of any peak due to rifampicin quinone should not be more than 4 times the area of the principal peak in the chromatogram obtained with the reference solution (4 per cent), the area of any peak due to rifampicin N-oxide should not be more than

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1.5 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent), the area of any peak due to 3-formylrifamycin SV Isonicotinyl hydrazone should not be more than 5 times the area of the principal peak in the chromatogram obtained with the reference solution (5 per cent) and the area of any peak due to 3-formylrifamycin SV should not be more than the area of the principal peak in the chromatogram obtained with the reference solution (1 per cent). In the chromatogram obtained with the test solution the area of any unknown peak should not be more than 1.5 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. *For Rifampicin and Isoniazid* — Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powdered tablets containing about 40 mg of Isoniazid dissolve in 100.0 ml of *methanol*, dilute to 500.0 ml with the diluent and mix.

Reference solution. A solution containing 0.08 per cent w/v of *rifampicin IPRS* and *0.04* per cent w/v of *isoniazid IPRS* in *methanol*. Dilute 10.0 ml of the solution to 50.0 ml with the diluent.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: A. a mixture of 96 volumes of buffer solution pH 6.8 (diluent) prepared by dissolving 1.4 g of *disodium hydrogen orthophosphate anhydrous* in 1000 ml of *water*, and adjusting the pH to 6.8 ± 0.05 with *dilute phosphoroic acid*, and 4 volumes of *acetonitrile*,
  B: a mixture of 45 volumes of the buffer solution and 55 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	100	0
6	0	100
15	0	100
16	100	0
22	100	0

NOTE — Saturate the column with mobile phase B for about 1 hour.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 for rifampicin and isoniazid, the column efficiency determined from isoniazid peak is not less than 3000 and that from rifampicin is not less than 25000 theoretical plates respectively, and the relative standard deviation for replicate injections is not more than 2.0 per cent. The relative retention times are about 1.0 for rifampicin and about 0.3 for isoniazid.

Calculate the contents of $C_{43}H_{58}N_4O_{12}$ and $C_6H_7N_3O$ in the tablets.

For Ethambutol Hydrochloride — Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the powdered tablets containing about 60 mg of Ethambutol Hydrochloride and dissolve in 100.0 ml of the diluent.

Reference solution. A 0.06 per cent w/v solution of *ethambutol hydrochloride IPRS* in the diluent.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with nitrile groups chemically bonded to porous silica particles 5 (µm) (Such as Zorbax SB CN),
- mobile phase: a mixture of 50 volumes of *acetonitrile*, and 50 volumes of a buffer solution pH 7.0 prepared by dissolving 1 ml of *triethylamine* in 1000 ml of *water* and adjusting the pH to 7.0 with *dilute phosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 200 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent, the tailing factor is not more than 3.0 and the column efficiency determined from Ethambutol hydrochloride peak is not less than 1500 theoretical plates.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{24}N_2O_2.2HCl$ in the tablets.

Storage. Store protected from moisture.

Rifampicin, Isoniazid and Pyrazinamide Tablets

Rifampin, Isonicotinylhydrazid and Pyrazinamide Tablets

Rifampicin, Isoniazid and Pyrazinamide Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of rifampicin, $C_{43}H_{58}N_4O_{12}$, isoniazid, $C_6H_7N_3O$ and pyrazinamide $C_5H_5N_3O$.

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Identification

In the Assay of rifampicin, isoniazid and pyrazinamide, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium: 900 ml of a solution containing 2 g of *sodium chloride* and 7.0 ml of *hydrochloric acid* in 1000 ml of *water*, with a pH of about 1.2,

Speed and time: 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter through a membrane filter disc with an average pore diameter not greater than 0.8 µm. Reject the first few ml of the filtrate.

Reference stock solution. A solution containing 0.0165 per cent w/v of *rifampicin IPRS*, 0.00825 per cent w/v of *isoniazid IPRS* and 0.04375 per cent w/v of *pyrazinamide IPRS* in the dissolution medium and filtered. Keep this reference stock solution in the dissolution bath during the test run.

For Rifampicin — Dilute the filtrate, if necessary, with the same solvent. Measure the absorbance (2.4.7) of the resulting solution at the maximum at about 475 nm. Calculate the content of $C_{43}H_{58}N_4O_{12}$ in the medium from the absorbance obtained from suitably diluted reference stock solution.

For Isoniazid and Pyrazinamide — Determine by liquid chromatography (2.4.14).

NOTE — Use this solution within one hour from preparation.

Test solution. To 15.0 ml of the filtered medium, add 15 ml of *1 M dibasic potassium phosphate*, dilute to 100.0 ml with the mobile phase and mix.

Reference solution (a). To 15.0 ml of the reference stock solution, add 15 ml of *1 M dibasic potassium phosphate*, dilute to 100.0 ml with the mobile phase and mix.

Reference solution (b). To 10.0 ml of a 0.0125 per cent w/v solution of *isonicotinic acid* in the dissolution medium, add 4.0 ml of the reference stock solution and 15 ml of *1 M dibasic potassium phosphate*, dilute to 100.0 ml with the mobile phase and mix.

Chromatographic system

- a stainless steel column 30 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 860 volumes of *water*, 100 volumes of *1 M monobasic potassium phosphate* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 50 µl.

Inject reference solution (a), (b) and the test solution. The relative retention times are about 0.7 for isonicotinic acid, 1.0 for pyrazinamide and 1.8 for isoniazid.

The test is not valid unless in the chromatogram obtained with reference solution (b) the resolution between isonicotinic acid and pyrazinamide is not less than 2.5 and between pyrazinamide and isoniazid is not less than 4.0.

Calculate the contents of $C_6H_7N_3O$ and $C_5H_5N_3O$ in the medium.

Q: Not less than 75 per cent of the stated amounts of $C_{43}H_{58}N_4O_{12}$, $C_6H_7N_3O$ and $C_5H_5N_3O$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Solvent mixture. A mixture of 10 volumes of a 21.01 per cent w/v solution of *citric acid*, 23 volumes of a 13.61 per cent w/v solution of *potassium dihydrogen phosphate*, 77 volumes of 17.42 per cent w/v solution of *dipotassium hydrogen phosphate*, 640 volumes of *water* and 250 volumes of *acetonitrile*.

Test solution. Weigh a quantity of the powdered tablets containing about 200 mg of Rifampicin, dissolve in 100 ml of *acetonitrile* and filter. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution (a). A solution containing 0.02 per cent w/v of *rifampicin IPRS* in *acetonitrile*. Dilute 1.0 ml of the solution to a 100.0 ml with the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v each of *rifampicin IPRS* and *rifampicin quinone IPRS* in *acetonitrile*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: a mixture of 65 volumes of a solution containing 0.1 per cent v/v of *orthophosphoric acid*, 0.19 per cent w/v of *sodium perchlorate*, 0.59 per cent w/v of *citric acid* and 2.09 per cent w/v of *potassium dihydrogen phosphate*, 35 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between rifampicin and rifampicin quinone is not less than 4, the tailing factor is not more than 2.0 and the column efficiency is not less than 2000 theoretical plates.

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Inject reference solution (a) and the test solution. The relative retention times are 1.0, 0.55, 1.25, 1.51 and 2.61 for rifampicin, rifampicin quinone, rifampicin N-oxide, 3-formylrifamycin SV Isonicotinyl hydrazone and 3-formylrifamycin SV respectively. Multiply the area of each known impurity by its response factor. The response factors are 1.00, 1.19, 1.03, 1.22 and 1.25 for rifampicin, rifampicin quinone, rifampicin N-oxide, Isonicotinyl hydrazone and 3-formylrifamycin SV respectively.

In the chromatogram obtained with the test solution the area of any peak due to rifampicin quinone should not be more than 4 times the area of the principal peak in the chromatogram obtained with the reference solution (4 per cent), the area of any peak due to rifampicin N-oxide should not be more than 1.5 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent), the area of any peak due to 3-formylrifamycin SV Isonicotinyl hydrazone should not be more than 5 times the area of the principal peak in the chromatogram obtained with the reference solution (5 per cent) and the area of any peak due to 3-formylrifamycin SV should not be more than the area of the principal peak in the chromatogram obtained with the reference solution (1 per cent). In the chromatogram obtained with the test solution the area of any unknown peak should not be more than 1.5 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. *For Rifampicin, Isoniazid and Pyrazinamide* — Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powdered tablets containing about 40 mg of Isoniazid, dissolve in 100.0 ml of *methanol*, dilute to 500 ml with the buffer solution pH 6.8 and mix.

Reference solution. A solution containing 0.08 per cent w/v of *rifampicin IPRS*, 0.04 per cent w/v of *isoniazid IPRS* and 0.2 per cent w/v of *pyrazinamide IPRS* in *methanol*. Dilute 10.0 ml of the solution to 50.0 ml with the buffer solution pH 6.8.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: A. a mixture of 96 volumes of buffer solution pH 6.8 (diluent) prepared by dissolving 1.4 g of *disodium hydrogen orthophosphate anhydrous* in 1000 ml of *water*, adjusted to pH 6.8 ± 0.05 with *dilute orthophosphoroic acid* and 4 volumes of *acetonitrile*,
  B. a mixture of 45 volumes of the buffer solution and 55 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	100	0
6	0	100
15	0	100
16	100	0
22	100	0

NOTE — Saturate the column with mobile phase (B) for about 1 hour.

Inject the reference solution and the test solution. The test is not valid unless the tailing factor is not less than 2.0 for rifampicin, isoniazid and pyrazinamide, the column efficiencies determined from Isoniazid, pyrazinamide and that from rifampicin are not less than 3000, 5000 and 25000 theoretical plates respectively, and the relative standard deviation for replicate injections is not more than 2.0 per cent. The relative retention times are about 1.8 for rifampicin, about 0.7 for isoniazid and about 1.0 for pyrazinamide.

Inject the reference solution and the test solution.

Calculate the contents of $C_{43}H_{58}N_4O_{12}$, $C_6H_7N_3O$ and $C_5H_5N_3O$ in the tablets.

Storage. Store protected from moisture.

Rifampicin, Isoniazid, Pyrazinamide and Ethambutol Tablets

Rifampin, Isonicotinylhydrazid, Pyrazinamide and Ethambutol Hydrochloride Tablets

Rifampicin, Isoniazid, Pyrazinamide and Ethambutol Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of rifampicin, $C_{43}H_{58}N_4O_{12}$, isoniazid, $C_6H_7N_3O$, pyrazinamide, $C_5H_5N_3O$ and ethambutol hydrochloride, $C_{10}H_{24}N_2O_2.2HCl$.

Usual strength. Rifampicin 150 mg, Isoniazid 75 mg, Pyrazinamide 400 mg and Ethambutol 275 mg.

Identification

A. In the Assay for rifampicin, isoniazid and pyrazinamide, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

B. In the Assay for ethambutol hydrochloride, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

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Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *sodium phosphate buffer pH 6.8* prepared by dissolving 7 g of *dibasic sodium phosphate anhydrous* in 5000 ml of *water* and adjusting to pH 6.8 with *orthophosphoric acid*,

Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter through a membrane filter disc with an average pore diameter not greater than 0.8 µm. Reject the first few ml of the filtrate. Determine the amounts of rifampicin, $C_{43}H_{58}N_4O_{12}$, isoniazid, $C_6H_7N_3O$, pyrazinamide, $C_5H_5N_3O$ and ethambutol hydrochloride, $C_{10}H_{24}N_2O_2.2HCl$ by using the methods described under Assay for rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride.

Q. Not less than 75 per cent of the stated amounts of $C_{43}H_{58}N_4O_{12}$, $C_6H_7N_3O$, $C_5H_5N_3O$ and $C_{10}H_{24}N_2O_2$,2HCl.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Solvent mixture. A mixture of 10 volumes of a 21.01 per cent w/v solution of *citric acid*, 23 volumes of a 13.61 per cent w/v solution of *potassium dihydrogen phosphate*, 77 volumes of a 17.42 per cent w/v solution of *dipotassium hydrogen phosphate*, 640 volumes of *water* and 250 volumes of *acetonitrile*.

Test solution. Weigh a quantity of powdered tablets containing 200 mg of Rifampicin, dissolve in 100 ml of *acetonitrile* and filter. Dilute 5 ml of the solution to 50 ml with the solvent mixture.

Reference solution (a). Dissolve 20 mg of *rifampicin IPRS* in 100 ml of *acetonitrile*. Dilute 1.0 ml of the solution to a 100.0 ml with the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v each of *rifampicin IPRS* and *rifampicin quinone IPRS* in *acetonitrile*. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: a mixture of 65 volumes of a solution containing 0.1 per cent v/v of *orthophosphoric acid*, 0.19 per cent w/v of *sodium perchlorate*, 0.59 per cent w/v of *citric acid* and 2.09 per cent w/v of *potassium dihydrogen phosphate*, 35 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between rifampicin and rifampicin quinone is not less than 4, the tailing factor is not more than 2.0 and the column efficiency is not less than 2000 theoretical plates.

Inject reference solution (a) and the test solution. The relative retention times are 1.0, 0.55, 1.25, 1.51 and 2.61 for rifampicin, rifampicin quinone, rifampicin N-oxide, 3-formylrifamycin SV Isonicotinyl hydrazone and 3-formylrifamycin SV respectively. Multiply the area of each known impurity by its response factor. The response factors are 1.00, 1.19, 1.03, 1.22 and 1.25 for rifampicin, rifampicin quinone, rifampicin N-oxide, Isonicotinyl hydrazone and 3-formylrifamycin SV respectively.

In the chromatogram obtained with the test solution the area of any peak due to rifampicin quinone should not be more than 4 times the area of the principal peak in the chromatogram obtained with the reference solution (4 per cent), the area of any peak due to rifampicin N-oxide should not be more than 1.5 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent), the area of any peak due to 3-formylrifamycin SV Isonicotinyl hydrazone should not be more than 5 times the area of the principal peak in the chromatogram obtained with the reference solution (5 per cent) and the area of any peak due to 3-formylrifamycin SV should not be more than the area of the principal peak in the chromatogram obtained with the reference solution (1 per cent). In the chromatogram obtained with the test solution the area of any unknown peak should not be more than 1.5 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. *For Rifampicin, Isoniazid and Pyrazinamide* — Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powdered tablets containing about 40 mg of Isoniazid, dissolve in 100 ml of *methanol*, dilute to 500 ml with the buffer solution pH 6.8, mix and filter.

Reference solution. A solution containing 0.08 per cent w/v of *rifampicin IPRS*, 0.04 per cent w/v of *isoniazid IPRS* and 0.2 per cent w/v of pyrazinamide *IPRS* in *methanol*. Dilute 10.0 ml of the solution to 50.0 ml with the buffer solution pH 6.8.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column. temperature 30°,
- mobile phase: A. a mixture of 96 volumes of buffer solution pH 6.8 prepared by dissolving 1.4 g of *disodium hydrogen orthophosphate anhydrous* in 1000 ml of

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water, adjusting to pH 6.8 ± 0.05 with *dilute phosphoric acid* and 4 volumes of *acetonitrile*,

B. a mixture of 45 volumes of the buffer solution and 55 volumes of *acetonitrile*,

– a gradient programme using the conditions given below,
– flow rate: 1.5 ml per minute,
– spectrophotometer set at 238 nm,
– injection volume: 20 µl.

Time (in min.)	Solution A (per cent v/v)	Solution B (per cent v/v)
0	100	0
5	100	0
6	0	100
15	0	100
16	100	0
22	100	0

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent, the tailing factor is not more than 2.0 for rifampicin, isoniazid and pyrazinamide and the column efficiencies for isoniazid, pyrazinamide and rifampicin are not less than 3000, 5000 and 25000 theoretical plates respectively.

Inject the reference solution and the test solution. The relative retention time for rifampicin is 1.8, for isoniazid, 0.7 and for pyrazinamide, 1.0.

Calculate the contents of $C_{43}H_{58}N_4O_{12}$, $C_6H_7N_3O$ and $C_5H_5N_3O$ in the tablets.

For Ethambutol Hydrochloride — Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the powdered tablets containing about 60 mg of Ethambutol Hydrochloride and dissolve in 100 ml of the solvent mixture.

Reference solution. A 0.06 per cent w/v solution of *ethambutol hydrochloride IPRS* in the solvent mixture.

Chromatographic system

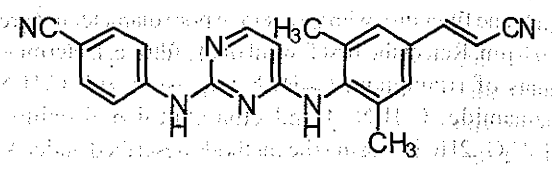
– a stainless steel column 15 cm × 4.6 mm, packed with nitrile groups chemically bonded to porous silica particles (5 µm),
– mobile phase: a mixture of 50 volumes of *acetonitrile*, and 50 volumes of buffer solution pH 7.0 (diluent) prepared by dissolving 1 ml of *triethylamine* in 1000 ml of *water* and adjusting the pH to 7.0 with *dilute phosphoric acid*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 200 nm,
– injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent, the tailing factor is not more than 3.0 and the column efficiency determined from ethambutol hydrochloride peak is not less than 1500 theoretical plates.

Calculate the content of $C_{10}H_{24}N_2O_2.2HCl$ in the tablets.

Storage. Store protected from moisture.

Rilpivirine



$C_{22}H_{18}N_6.HCl$ Mol Wt. 402.9

Rilpivirine is 4-{[4-({4-[(*E*)-2-Cyanovinyl]-2,6-dimethylphenyl}amino)pyrimidin-2-yl]amino}benzonitrile.

Rilpivirine contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{22}H_{18}N_6.HCl$, calculated on the anhydrous basis.

Category. Antiretroviral.

Description. A white to pale brown colour powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rilpivirine IPRS* or with the reference spectrum of rilpivirine.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in *methanol* and dilute to 100.0 ml with *methanol*.

Reference solution. A 0.003 per cent w/v solution of *rilpivirine IPRS*, *impurity A IPRS* and *impurity B IPRS* in *methanol*. Dilute 0.5 ml of the solution to 20.0 ml with *methanol*.

Chromatographic system

– a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
– column temperature: 45°,
– mobile phase: A. dissolve 3.08 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 6.0 with *acetic acid*,

B. *methanol*,

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– a gradient programme using the conditions given below,
– flow rate: 0.6 ml per minute,
– spectrophotometer set at 295nm,
– injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	40	60
12	20	80
15	20	80
18	40	60
20	40	60

Inject reference solution. The test is not valid unless the the resolution between rilpivirine impurity A (2E)-3-(4-amino-3,5dimethyl phenyl) acrylonitrile hydrochlorid and rilpivirine impurity B (4-(4-chloropyrimidin -2 yl-amino)- benzonitrile) is not less than 5.0.

Inject reference solution and the test solution. In the chromatogram obtained with the test solution, the area of peak due to rilpivirine impurity A is not more than area of reference solution (0.3 per cent), the area of peak due to rilpivirine mpurity B is not more than area of reference solution (0.3 per cent) , area of any other secondary peak is not more than area of rilpivirine peak in the chromatograpm obtained with the reference solution (0.3 per cent) and the sum of area of all the secondary peaks is not more than 3.3 times the area of rilpivirine peak in the chromatograpm obtained with the reference solution (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 0.3 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in *methanol* and dilute to 100.0 ml with *methanol*. Dilute 5.0 ml of the solution to 50.0 ml with *methanol*.

Reference solution. A 0.0025 per cent w/v solution of *rilpivirine IPRS* in *methanol*.

Chromatographic system
– a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
– column temperature: 45°,
– mobile phase: A. dissolve 3.08 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 6.0 with *acetic acid*,
B. a mixture of 50 volumes of *acetonitrile* and 50 volumes of *methanol*,
– a gradient programme using the conditions given below,
– flow rate: 0.6 ml per minute,
– spectrophotometer set at 295nm,
– injection volume: 10 µl.

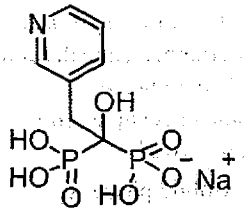
Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	40	60
12	20	80
15	20	80
18	40	60
20	40	60

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates , the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution and the test solution.

Calculate the content of $C_{22}H_{18}N_6.HCl$.

Risedronate Sodium



$C_7H_{10}NNaO_7P_2$ Mol. Wt. 305.1

$C_7H_{10}NNaO_7P_2,H_2O$(Monohydrate) Mol. Wt. 323.1

$C_7H_{10}NNaO_7P_2,2.5H_2O$ (Hemi-pentahydrate) Mol. Wt. 350.1

Risedronate Sodium is 1-Hydroxy-2-(3-pyridyl)ethane-1,1-diylbis(phosphonic acid) monosodium salt.

Risedronate Sodium contains not less than 98.0 per cent and not more than 102.0 per cent of $C_7H_{10}NNaO_7P_2$, calculated on the anhydrous basis.

Category. Calcium regulator.

Description. A white to off-white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6), compare the spectrum with that obtained with *risedronate sodium IPRS* or with the reference spectrum of risedronate sodium.

B. A 1.0 per cent w/v solution gives the reactions of sodium salt (2.3.1).

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Tests

Related substances. A. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 10 mg of the substance under examination in mobile phase A and dilute to 10.0 ml with mobile phase A.

Reference solution (a). A 0.0001 per cent w/v solution of *risedronate sodium IPRS* in mobile phase A.

Reference solution (b). A solution containing 0.1 per cent w/v of *risedronate sodium IPRS* and 0.0001 per cent w/v of risedronate impurity A in mobile phase A.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (3.5 µm),
- mobile phase: A. a mixture of 900 volumes of buffer solution prepared by dissolving 1.42 g of *disodium hydrogen orthophosphate*, 1.7 g of *tetrabutyl ammonium phosphate* and 0.38 g of *ethylenediamine tetraacetate* in 950 ml of *water*, adjusting to pH 7.5 with *ammonium hydroxide solution* and 100 volumes of *acetonitrile*,

  B. a mixture of 800 volumes of buffer solution and 200 volumes of *acetonitrile*,
- a gradient programme using the condition given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 263 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
10	100	0
40	0	100
55	0	100
67	100	0
75	100	0

Name	Relative retention time	Relative response factor
3-Pyridyl acetic acid	0.3	1.32
Risedronate impurity A[1]	0.51	1.76
Risedronate sodium	1.0	—

[1] [1-hydroxy-2-(2-pyridinyl)ethylidene] bisphosphonic acid.

Equilibrate the column with the mobile phase for at least 2 hours.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to risedronate and risedronate impurity A is not less than 5.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

B. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 0.46 g of *disodium edetate* in 500 ml of *water*, adjusted to pH 7.5 with *1 M sodium hydroxide*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 25.0 ml with the solvent mixture.

Reference solution. A 0.0005 per cent w/v solution of *risedronate sodium impurity B IPRS (3,6-bis[(3-pyridinyl)-methyl]-2,5-dihydroxy-2,5-dioxido-1,4,2,5 dioxadiphosphorinane-3,6-diyl]bis[phosphonic acid] disodium tetrahydrate IPRS*) in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 16.15 g of *dibasic potassium phosphate* and 0.46 g of *disodium edetate* in 400 ml of *water*, add 5 ml of *tetrabutylammonium dihydrogen phosphate* and 1 ml of *hydrochloric acid*, adjusted to pH 7.5 with *1 M sodium hydroxide* or *1 M hydrochloric acid* and dilute to 480 ml with *water*, add 20 ml of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 263 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5.

Inject the test solution. The area of any peak corresponding to risedronate impurity B is not more than 0.1 per cent and the area of any other secondary peak is not more than 0.1 per cent. The sum of areas of all the secondary peaks of tests A and B is not more than 0.5 per cent, calculated by area normalization. Ignore any peak eluting before risedronate impurity B.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). 5.5 to 7.5 per cent for monohydrate; 11.9 to 13.9 per cent for hemi-pentahydrate, determined on about 0.25 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 10 mg of the substance under examination in mobile phase A and dilute to 10.0 ml with mobile phase A.

Reference solution. A 0.1 per cent w/v solution of *risedronate sodium IPRS* in mobile phase A.

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Use chromatographic system as described under Related substances, test A.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_7H_{10}NNaO_7P_2$.

Storage. Store protected from moisture.

Labelling. Label to indicate whether it is monohydrate or hemipentahydrate.

Risedronate Sodium Tablets

Risedronate Sodium Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of risedronate sodium, $C_7H_{10}NNaO_7P_2$.

Usual strengths. 35 mg; 75 mg; 150 mg.

Identification

A. Disperse a quantity of the powdered tablets containing 50 mg of risedronate sodium in 10 ml of *water,* shake and filter. Add 10 ml of *0.2M cupric chloride solution*, mix well and allow the solution to stand for about 10 minutes. Add 2 ml of *dehydrated alcohol*, mix well and allow the solution to stand for a minimum of 1 hour, to form a blue precipitate of the copper complex. Collect the precipitate using a nylon filter of 0.45-µm pore size, wash it with 10 ml of *dehydrated alcohol*, and allow it to dry on the filter paper, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *risedronate sodium IPRS,* treated in the same manner or with the reference spectrum of risedronate sodium.

NOTE — Dry the precipitate under ambient conditions, do not heat the precipitate. A modest change of colour (from blue to green) may be observed upon drying.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution .

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Dilute the filtrate, if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *risedronate sodium IPRS* in the dissolution medium to obtain a solution of known concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 5 cm x 4.0 mm, packed with a strong anion-exchange microbeads consisting of sulphonated cross-linked polystyrene (10 µm),
- mobile phase: a buffer solution prepared by dissolving 1.8 g of *disodium edetate* in 1000 ml of *water,* adjusted to pH 9.5 with dilute *sodium hydroxide*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 263 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution.

Q. Not less than 75 per cent of the stated amount of $C_7H_{10}NNaO_7P_2$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 25 mg of Risedronate Sodium in 50 ml of mobile phase, shake for 10 minutes and sonicate for 5 minutes, dilute to 100.0 ml with the mobile phase, filter.

Reference solution. A 0.025 per cent w/v solution of *risedronate sodium IPRS* and 0.0004 per cent w/v solution of *risedronate related compound A IPRS [(2- pyridinil isomer [1- hydroxyl-2-(2- pyridinyl) ethyl- idene] bis (phosphonic acid) monohydrate IPRS]* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.0 mm, packed with a strong anion-exchange microbeads consisting of sulphonated cross-linked polystyrene (10 µm),
- mobile phase: a buffer solution prepared by dissolving 1.8 g of *disodium edetate* in 1000 ml of *water,* adjusted to pH 9.5 with dilute *sodium hydroxide*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 263 nm,
- injection volume: 50 µl.

Inject the reference solution . The test is not valid unless the resolution between risedronate related compound A peak and risedronate peak is not less than 2.0, the tailing factor for risedronate peak is not more than 1.5 and the relative standard deviation of risedronate peaks for replicate injections is not more than 1.5 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_7H_{10}NNaO_7P_2$ in the tablets.

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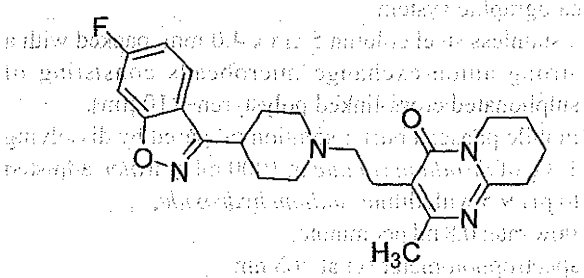
Storage. Store protected from moisture, at a temperature not exceeding 30°.

Risperidone



$C_{23}H_{27}FN_4O_2$ Mol. Wt. 410.5

Risperidone is 3-[2-[4-(6-Fluoro-1,2-benzisoxazol-3-yl)piperidino]ethyl]-6,7,8,9-tetrahydro-2-methyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one.

Risperidone contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{23}H_{27}FN_4O_2$, calculated on dried basis.

Category. Antipsychotic.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *risperidone IPRS* or with the reference spectrum of risperidone.

B. In the Assay, the principal peak in the chromatogram obtained with test solution (b) corresponds to the peak in the chromatogram obtained with reference solution (b).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in *methanol* and dilute to 10.0 ml with the same solvent.

Reference solution (a). A solution containing 0.002 per cent w/v of *risperidone related compound G IPRS [3-[2-[4-(4-Fluoro-2-hydroxybenzoyl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one hydrochloride IPRS]* and 1.0 per cent w/v of *risperidone IPRS* in *methanol*.

Reference solution (b). A 0.002 per cent w/v solution of *risperidone IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: A. a 0.5 per cent w/v solution of *ammonium acetate*, adjusted to pH 6.0 with *glacial acetic acid* and filter;

  B. a mixture of 40 volumes of *acetonitrile* and 60 volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 2 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
12	65	35
18	65	35
25	35	65
35	30	70
40	30	70
42	70	30
50	70	30

Name	Relative retention time	Correction factor
E-Oxime[1]	0.52	1.0
Z-Oxime[2]	0.64	1.0
9-Hydroxyrisperidone[3]	0.71	1.0
Desfluoro risperidone[4-5]	0.79	1.0
Risperidone difluoroketone[6]	0.90	0.71
5-Fluororisperidone[7]	0.94	1.0
Risperidone	1.0	1.0
Risperidone related compound G[5]	1.08	0.4
6-Methylrisperidone[8]	1.44	1.0
Individual unidentified impurities	—	1.0
Total impurities	—	—

[1]3-(2-[4-[(E)-(2,4-Difluorophenyl)(hydroxyimino)methyl]piperidin-1-yl]ethyl)-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-a]pyrimidin-4-one,

[2]3-[2-[4-[(Z)-(2,4-Difluorophenyl)(hydroxyimino)methyl]piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

[3](9*RS*)-3-[2-[4-(6-Fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-9-hydroxy-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

[4]3-{2-[4-(Benzisoxazol-3-yl)piperidin-1-yl]ethyl}-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

[5]This impurity may not be relevant to all manufacturing processes.

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[a]3-[2-[4-(2,4-Difluorobenzoyl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

[b]3-[2-[4-(5-Fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (also known as 5-fluororisperidone),

[c](6*RS*)-3-[2-[4-(6-Fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-2,6-dimethyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to risperidone and risperidone related compound G is not less than 1.5 in the chromatogram obtained with reference solution (a), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to E-oxime, Z-oxime, 9-hydroxy-risperidone, 5-fluororisperidone and 6-methylrisperidone is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any peak due to desfluoro risperidone, risperidone difluoroketone and risperidone related compound G is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent), the area of any other secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent). Ignore any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g, dry under vacuum at 80° for 4 hours.

Assay. Determine by liquid chromatography (2.4.14).

Solution A. A 0.5 per cent w/v solution of *ammonium acetate*, adjusted to pH 6.0 with *glacial acetic acid* and filter.

Solution B. A mixture of 80 volumes of *acetonitrile* and 20 volumes of *tetrahydrofuran*.

Test solution (a). Dissolve a suitable quantity of the substance under examination in *methanol* containing 20 per cent of the final volume and dilute with the mobile phase to obtain a 0.1 per cent w/v solution.

Test solution (b). Dilute test solution (a) with the mobile phase to obtain a 0.02 per cent w/v solution of risperidone.

Reference solution (a). Dissolve a suitable quantity of *risperidone IPRS* in *methanol* containing 20 per cent of the final volume and dilute with the mobile phase to obtain a 0.1 per cent w/v solution of risperidone.

Reference solution (b). Dilute reference solution (a) with the mobile phase to obtain a 0.02 per cent w/v solution of risperidone.

Reference solution (c). A 0.002 per cent w/v solution of *risperidone related compound G IPRS* in reference solution (b).

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: a mixture of 80 volumes of solution A and 20 volumes of solution B,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 275 nm,
- injection volume: 10 µl.

Run the chromatogram twice the retention time of the principal peak.

The relative retention time with reference to risperidone for risperidone related compound G is about 1.1.

Inject reference solution (b) and (c). The test is not valid unless the resolution between the peaks due to risperidone and risperidone related compound G is not less than 1.5 in the chromatogram obtained with reference solution (c), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (b) for risperidone peak.

Inject reference solution (b) and test solution (b).

Calculate the content of $C_{23}H_{27}FN_4O_2$.

Storage. Store protected from moisture, at temperature not exceeding 30°.

Risperidone Syrup

Risperidone Oral Solution

Risperidone Syrup contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of risperidone, $C_{23}H_{27}FN_4O_2$. It may contain suitable preservative.

Usual strength. 1 mg per ml.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (b).

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Tests

pH (2.4.24). 2.0 to 4.0.

Related substances. Determine by liquid chromatography (2.4.14).

Buffer solution. A 0.5 per cent w/v solution of *ammonium acetate* in *water*.

Test solution. Transfer a suitable quantity of the syrup containing 10 mg of Risperidone to a 50-ml volumetric flask, add 25 ml of the buffer solution, fill with *methanol* almost to volume and mix. Allow to cool to room temperature and dilute with *methanol* to volume.

Reference solution (a). Dissolve 2.5 mg of *risperidone related compounds mixture IPRS* in 2 ml of *methanol*. Add 2 ml of *water* and 5 ml of the buffer solution and allow to cool to room temperature, dilute to 10.0 ml with *methanol*.

Reference solution (b). A 0.0005 per cent w/v solution of *risperidone IPRS* in *methanol*.

Reference solution (c). Transfer 10.0 ml of reference solution (b) to a 50-ml volumetric flask. Add 10 ml of *water* and 25 ml of the buffer solution and allow to cool to room temperature, dilute with *methanol* to volume.

Reference solution (d). Transfer 2.0 ml of reference solution (b) to a 50-ml volumetric flask. Add 10 ml of *water* and 25 ml of the buffer solution and allow to cool to room temperature, dilute with *methanol* to volume.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- mobile phase: a mixture of 78 volumes of the buffer solution and 22 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 275 nm,
- injection volume: 10 µl.

Name	Relative retention time	Correction factor
Risperidone *cis-N*-oxide[1]	0.33	1.03
Bicyclorisperidone[2]	0.43	1.49
Risperidone *Z*-oxime[3]*	0.53	—
Risperidone	1.0	—

*Process impurity included for identification only. Process impurities are controlled in the drug substance, and are not to be reported or included in the total impurities for the drug product.

[1]*cis*-3-{2-[4-(6-Fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]ethyl}-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one *N*-oxide,

[2]3-(4-Fluoro-2-hydroxyphenyl)-1-{2-(6,7,8,9-tetrahydro-2-methyl-4-oxo-4*H*-pyrido-[1,2-*a*]pyrimidin-3-yl)ethyl}-2-aza-1-azonia-bicyclo[2.2.2]oct-2-ene iodide,

[3]3-(2-{4-[(*Z*)-(2,4-Difluorophenyl)(hydroxyimino)methyl]piperidin-1-yl}ethyl)-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one.

Inject reference solution (a), (c) and (d). The test is not valid unless the resolution between the peaks due to bicyclorisperidone and risperidone *Z*-oxime is not less than 1.5 in the chromatogram obtained with reference solution (a), the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (c) and the signal-to-noise ratio for the principal peak is not less than 10.0 in the chromatogram obtained with reference solution (d).

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to risperidone *cis-N*-oxide and bicyclorisperidone, each of, is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent), the area of any other secondary peak is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent). Ignore any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Microbial contamination (2.2.9). Total microbial count not more than 100 CFU per ml, total yeast and mould count not more than 10 CFU per ml. 1 ml is free from *Escherichia coli*.

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modifications.

Reference solution (a). Dissolve 2.5 mg *risperidone related compounds mixture IPRS* in 2 ml of *methanol*. Add 2 ml of *water* and 5 ml of the buffer solution and allow to cool to room temperature, dilute to 10.0 ml with *methanol*.

Reference solution (b). A 0.1 per cent w/v solution of *risperidone IPRS* in *methanol*. Transfer 10.0 ml of the solution to a 50-ml volumetric flask. Add 10 ml of *water* and 25 ml of the buffer solution and allow to cool to room temperature, dilute to volume with *methanol*.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to bicyclorisperidone and risperidone 2-oxime is not less than 1.5 in the chromatogram obtained with reference solution (a) and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution.

Determine the weight per ml of the oral solution (2.4.29) and calculate the content of $C_{23}H_{27}FN_4O_2$.

Storage. Store protected from light, at a temperature not exceeding 30°. Do not freeze.

Risperidone Tablets

Risperidone Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of risperidone, $C_{23}H_{27}FN_4O_2$.

Usual strengths. 1 mg; 2 mg; 3 mg; 4 mg.

Identification

A. Powder an appropriate number of tablets to prepare about 0.055 per cent w/v solution of risperidone in *ethyl acetate*, shake for 30 minutes and centrifuge for 20 minutes. Evaporate 5 ml of the supernatant liquid with the aid of a stream of nitrogen to dryness; the residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *risperidone IPRS* or with the reference spectrum of risperidone.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium, 500 ml of *0.1 M hydrochloric acid*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Dilute the filtrate, if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *risperidone IPRS* in the dissolution medium and dilute with the same solvent to obtain a solution having a known concentration similar to the test solution.

Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of *water*, 35 volumes of *acetonitrile* and 0.1 volume of *trifluoroacetic acid*, adjust the pH to 3.0 with *ammonia solution*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 237 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{27}FN_4O_2$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{23}H_{27}FN_4O_2$.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Dilute sodium hydroxide solution. To 1000 ml of *water* in a beaker, add *0.1 M sodium hydroxide* dropwise to obtain a pH of about 8.5.

Dilute hydrogen peroxide solution. Dilute 1 ml of *hydrogen peroxide* solution (10 volume) with *water* to 500 ml.

Reference solution. Dissolve 10 mg of *risperidone IPRS* in 10 ml of *dilute sodium hydroxide solution* in a 100-ml volumetric flask. Store the flask at 90° for 24 hours. Cool the solution to room temperature. Add 10 ml of dilute hydrogen peroxide solution to the flask, and store at 90° for an additional two hours. Cool the mixture to room temperature, and dilute with *methanol* to volume.

Name	Relative retention time	Correction factor
Bicyclorisperidone[1]	0.68	1.23
Risperidone	1.0	—
Risperidone *trans-N*-oxide[2]	1.65	—
Risperidone *cis-N*-oxide[3]	1.81	1.05
Individual unidentified impurities	—	—
Total impurities	—	—

[1]3-(4-fluoro-2-hydroxyphenyl)-1-[2-(6,7,8,9-tetrahydro-2-methyl-4-oxo-4*H*-pyrido-[1,2-a]pyrimidin-3-yl)ethyl]-2-aza-1-azoniabicyclo [2.2.2]oct-2-ene iodide,

[2]*trans*-3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]ethyl]-6,7,8,9-tetrahydro-2-methyl-4*H*-pyrido[1,2-a]pyrimidin-4-one, *N*-oxide monohydrate,

3*cis*-3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]ethyl]-6,7,8,9-tetrahydro-2-methyl-4*H*-pyrido[1,2-a]pyrimidin-4-one, *N*-oxide monohydrate.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to risperidone *trans-N*-oxide and risperidone *cis-N*-oxide is not less than 1.2.

Inject the test solution. The area of any peak corresponding to bicyclorisperidone, and risperidone *cis-N*-oxide is not more than 0.5 per cent, the area of any other secondary peak is not more than 0.3 per cent and the sum of areas of all the secondary peaks is not more than 1.0 per cent, calculated by area normalisation.

Uniformity of content. Complies with the test stated under Tablets.

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Determine by liquid chromatography (2.4.14), using the chromatographic system as described under Dissolution, except using

– injection volume: 20 µl.

Test solution. Transfer 1 tablet to a 100-ml volumetric flask. Add 50 ml of *0.1 M hydrochloric acid* and shake mechanically for 30 minutes, dilute to the volume with *0.1 M hydrochloric acid*, filter.

Reference solution. Weigh accurately and dissolve the *risperidone IPRS* in *0.1 M hydrochloric acid* to obtain a solution having a known concentration similar to the test solution.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{27}FN_4O_2$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of *methanol* and 20 volumes of *water.*

Test solution. Transfer not less than 10 tablets to a suitable volumetric flask to obtain a 0.01 per cent w/v solution of risperidone. Add an appropriate amount of *water* equivalent to 20 per cent of the total volume of the volumetric flask, and shake mechanically for about 30 minutes. Add a volume of *methanol* equivalent to 60 per cent of the total volume of the volumetric flask, and shake mechanically for about 30 minutes, dilute with *methanol* to volume and filter.

Reference solution. A 0.01 per cent w/v solution of *risperidone IPRS* in the solvent mixture.

Chromatographic system

– a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
– mobile phase: A. a mixture of 80 volumes of *water,* 19.5 volumes of *acetonitrile* and 0.1 volume of *trifluoroacetic acid*, adjust the pH to 3.0 with *ammonium hydroxide*,
B. a mixture of 61 volumes of *water,* 39 volumes of *methanol* and 0.1 volume of *trifluoroacetic acid*, adjust the pH to 3.0 with *ammonium hydroxide*,
– a gradient programme using the conditions given below,
– flow rate: 2.5 ml per minute,
– spectrophotometer set at 275 nm,
– injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
8	100	0
16	0	100
20	0	100
21	100	0
25	100	0

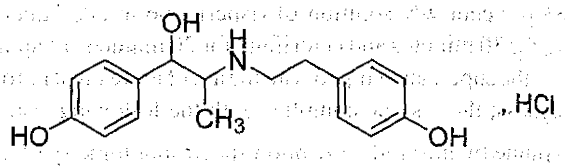
Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{27}FN_4O_2$ in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 25°.

Ritodrine Hydrochloride



$C_{17}H_{21}NO_3,HCl$ Mol. Wt. 323.8

Ritodrine Hydrochloride is (*RS*)-*erythro*-1-(4-hydroxyphenyl)-2-(4-hydroxyphenethylamino)-1-propanol.

Ritodrine Hydrochloride contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{17}H_{21}NO_3,HCl$, calculated on the dried basis.

Category. Tocolytic

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ritodrine hydrochloride IPRS* or with the reference spectrum of ritodrine hydrochloride.

B. A 1.0 per cent w/v solution gives the reactions of chloride (2.3.1).

Tests

pH (2.4.24). 4.5 to 6.0, determined on a 2.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in 100.0 ml of the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 20 mg of the substance under examination in 50 ml of the mobile phase, add 5.6 ml of *sulphuric acid* and dilute to 100.0 ml with the mobile phase,

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heat at a temperature of 85° for 2 hours. Cool to room temperature, mix 10 ml of the cooled solution with 8 ml of a 10 per cent w/v solution of *sodium hydroxide* and allow to cool (generation of *threo*- diastereoisomer).

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a solution containing 6.6 g of *diammonium hydrogen orthophosphate* and 1.1 g of *sodium heptanesulphonate* in 700 ml of *water* and 300 ml of *methanol*, adjusting the pH to 3.0 with 85 per cent v/v solution of *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Name	Relative retention time
Ritodrine impurity A[1]	0.3
Ritodrine impurity C[3]	0.7
Ritodrine impurity B[2]	0.9
Ritodrine hydrochloride	1.0
Ritodrine impurity D[4]	1.2
Ritodrine impurity E[5]	2.3

[1]Tyramine,

[2]rac-4-[(1R,2R)-2-{[1-hydroxy-1-(4-hydroxyphenyl)propan-2-yl]amino}ethyl]cyclohexan-1-one (hexehydroxketonel),

[3]rac-4-[(1R,2R)-1-hydroxy-2-{[2-(4-hydroxyphenyl)ethyl]amino}propyl]cyclohexan-1-one (hexehydroketone II),

[4]rac-4-[(1R,2R)-1-hydroxy-2-{[2-(4-hydroxyphenyl)ethyl]amino}propyl]phenol(threo-diastereoisomer),

[5]rac-1-(4-hydroxy)-3-{[2-(4-hydroxyphenyl)ethyl]amino}peopan-1-one (aminoketone).

Inject reference solution (b). The test is not valid unless the resolution between the principal peak and *threo*-diastereoisomer peak is not less than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak due to impurity C, multiplied by correction factor 2.9, is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). The area of any peak due to impurity A and impurity E, each of, is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any peak due to impurity B, multiplied by correction factor 2.0 and impurity D, each of, is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent). The area of any other secondary peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method A (20 ppm).

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). A 0.02 per cent w/v solution of *ritodrine hydrochloride IPRS* in the mobile phase.

Reference solution (b). Dissolve 20 mg of the substance under examination in 50 ml of the mobile phase, add 5.6 ml of *sulphuric acid* and dilute to 100.0 ml with the mobile phase, heat at a temperature of 85° for 2 hours. Cool to room temperature, mix 10 ml of the cooled solution with 8 ml of a 10 per cent w/v solution of *sodium hydroxide* and allow to cool (generation of *threo*- diastereoisomer).

Use chromatographic system as described under Related substances.

Inject reference solution (b). The test is not valid unless the resolution between the principal peak and *threo*- diastereoisomer peak is not less than 1.5.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{17}H_{21}NO_3$,HCl.

Storage. Store protected from moisture.

Ritodrine Injection

Ritodrine Hydrochloride Injection

Ritodrine Injection is a sterile solution of Ritodrine Hydrochloride in Water for Injections.

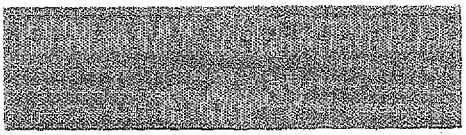
Ritodrine Injection contains not less than 93.0 per cent and not more than 105.0 per cent of the stated amount of ritodrine hydrochloride, $C_{17}H_{21}NO_3$,HCl.

Usual strength. 10 mg per ml.

Identification

A. Dilute a suitable volume of the injection with *methanol (30 per cent)* to obtain a solution containing 0.01 per cent w/v of Ritodrine Hydrochloride. When examined in the range 230 nm to 350 nm (2.4.7) exhibits a maximum at 275 nm and a shoulder at about 280 nm.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal



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peak in the chromatogram obtained with reference solution (a).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of the injection, if necessary with the mobile phase to obtain a solution containing 0.1 per cent w/v of Ritodrine Hydrochloride.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase.

Reference solution (b). Heat a solution containing 0.1 per cent w/v of *ritodrine hydrochloride IPRS* in the mobile phase containing 5.6 per cent v/v of *sulphuric acid* for 2 hours at 85°. Cool and mix 10 volumes of the solution with 8 volumes of a 10 per cent w/v solution of *sodium hydroxide* (generation of *threo*-diastereoisomer).

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (7 µm),
- mobile phase: a mixture of a solution containing 6.6 g of *diammonium hydrogen orthophosphate* and 1.1 g of *sodium heptanesulphonate* in 700 ml of *water* and 300 ml of *methanol*, adjusting to pH 3.0 with *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 275 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the principal peak and the peak correponding to *threo*- diastereoisomer is not less than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent) and not more than one such peak has an area more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent) and not more than one such peak has an area greater than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). The sum of areas of all the secondary peaks is not more than 2.5 times the area of the peak in the chromatogram obtained with reference solution (a) (5.0 per cent).

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per mg of ritodrine hydrochloride.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of the injection with mobile phase to obtain a solution containing 0.02 per cent w/v of Ritodrine Hydrochloride.

Reference solution (a). A 0.02 per cent w/v solution of *ritodrine hydrochloride IPRS* in the mobile phase.

Reference solution (b). Heat a solution containing 0.1 per cent w/v of *ritodrine hydrochloride IPRS* in the mobile phase containing 5.6 per cent v/v of *sulphuric acid* for 2 hours at 85°. Cool and mix 10 volumes with 8 volumes of a 10 per cent w/v solution of *sodium hydroxide* (generation of *threo*-diastereoisomer).

Use chromatographic system as described under Related substances.

Inject reference solution (b). The test is not valid unless the resolution between the principal peak and the peak due to *threo*- diastereoisomer is not less than 1.5.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{17}H_{21}NO_3$,HCl in the injection.

Storage. Store protected from light.

Ritodrine Tablets

Ritodrine Hydrochloride Tablets

Ritodrine Tablets contain not less than 93.0 per cent and not more than 105.0 per cent of ritodrine hydrochloride, $C_{17}H_{21}NO_3$,HCl.

Usual strength. 10 mg.

Identification

A. Shake a quantity of the powdered tablets containing 10 mg of Ritodrine Hydrochloride with 30 ml of *methanol* for 20 minutes, filter. Dilute 15 ml of the filtrate to 50 ml with *water*. The resulting solution, when examined in the range 230 nm to 350 nm (2.4.7) exhibits a maximum at 275 nm and a shoulder at about 280 nm.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets containing 20 mg of Ritodrine Hydrochloride in 50 ml of the mobile phase for 5 minutes and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 20 mg of *ritodrine hydrochloride IPRS* in 50 ml of the mobile phase, add 5.6 ml of *sulphuric acid* and dilute to 100.0 ml with the mobile phase, heat at a temperature of 85° for 2 hours. Cool to room temperature, mix 10 ml of the cooled solution with 8 ml of a 10 per cent w/v solution of *sodium hydroxide* and allow to cool (generation of *threo*-diastereoisomer).

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 300 ml of *methanol* and 6.6 g of *diammonium hydrogen orthophosphate* and 1.1 g of *sodium heptanesulphonate* in 700 ml of *water*, adjusted to pH 3.0 with an 85 per cent v/v solution of *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 275 nm,
- injection volume: 20 µl.

Name	Relative retention time
Tyramine	0.3
Ritodrine Hydrochloride	1.0
threo-diastereoisomer	1.15
Aminoketone	2.3

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to ritodrine hydrochloride and *threo*-diastereoisomer is not less than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak due to tyramine and the *threo*-diastereoisomer is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). The area of any peak due to aminoketone, multiplied with 0.2 is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 20 mg of Ritodrine Hydrochloride in 50 ml of the mobile phase and dilute to 100.0 ml with the mobile phase and filter.

Reference solution (a). A 0.02 per cent w/v solution of *ritodrine hydrochloride IPRS* in the mobile phase.

Reference solution (b). Dissolve 20 mg of *ritodrine hydrochloride IPRS* in 50 ml of the mobile phase, add 5.6 ml of *sulphuric acid* and dilute to 100 ml with the mobile phase, mix and heat at a temperature of 85° for 2 hours. Cool to room temperature, mix 10 ml of the cooled solution with 8 ml of a 10 per cent w/v solution of *sodium hydroxide* and allow to cool (generation of *threo*-diastereoisomer).

Use chromatographic system as described under Related substances.

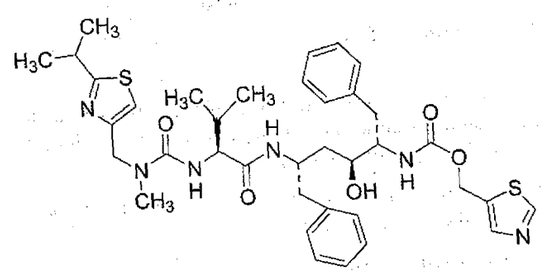
Inject reference solution (b). The test is not valid unless the resolution between the peaks due to ritodrine hydrochloride and the peak due to *threo*-diastereoisomer is not less than 1.5.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{17}H_{21}NO_3$,HCl in the tablets.

Storage. Store protected from light.

Ritonavir



$C_{37}H_{48}N_6O_5S_2$ Mol. Wt. 721.0

Ritonavir is (5*S*,8*S*,10*S*,11*S*)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid 5-thiazolylmethyl ester.

Ritonavir contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{37}H_{48}N_6O_5S_2$, calculated on the anhydrous basis.

Category. Antiretroviral.

Description. A white to off-white powder. It shows polymorphism (2.5.11).

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ritonavir IPRS* or with the reference spectrum of ritonavir.

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B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). +7.0° to +10.5°, determined in a 0.2 per cent w/v solution in *methanol*.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay and calculate the percentage of each impurity peak in the chromatogram obtained with the test solution. Not more than 0.5 per cent of any individual impurity and not more than 1.0 per cent of total impurities is found.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent

Water (2.3.43). Not more than 0.5 per cent, determined on 2.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 45 volumes of *acetonitrile* and 55 volumes of *water*.

Test solution. Dissolve 25.0 mg of the substance under examination in sufficient in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution. A 0.025 per cent w/v solution of *ritonavir IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 45 volumes of *acetonitrile* and 55 volumes of a buffer solution prepared by dissolving 3.4 g of *sodium acetate* and 0.94 g of *sodium hexanesulphonate* in 1000 ml of *water*, adjusted to pH 4.0 with *hydrochloric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 239 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency determined from the ritonavir peak is not less than 1000 theoretical plates, the tailing factor is not more than 2.0, and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{37}H_{48}N_6O_5S_2$.

Storage. Store protected from light.

Ritonavir Capsules

Ritonavir Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ritonavir, $C_{37}H_{48}N_6O_5S_2$.

Usual strength. 100 mg.

Identification

A. In the test for Assay, the principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. When examined in the range 200 nm to 350 nm (2.4.7), a 0.001 per cent w/v solution in *methanol* shows absorption maxima at the same wavelengths as the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid* with 25 mM polyoxyethylene 10 lauryl ether prepared by dissolving 15.65 g of *polyoxyethylene 10 lauryl ether* in 950 ml of *water*. Add 8.5 ml of *hydrochloric acid* and dilute to 1000 ml with *water*,
Speed and time. 50 rpm and 90 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate and if necessary, dilute with the dissolution medium.

Reference solution. A 0.1 per cent w/v solution of *ritonavir IPRS* in *methanol*. Dilute 5 ml of the solution to 50 ml with the dissolution medium.

Use the chromatographic system described under Assay.

Inject the reference solution and the test solution.

Q. Not less than 70 per cent of the stated amount of $C_{37}H_{48}N_6O_5S_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 40 volumes of *0.03M monobasic potassium phosphate buffer* and 60 volumes of *acetonitrile*.

Test solution. Weigh a quantity of the mixed contents of 20 capsules containing about 25 mg of Ritonavir, dissolve in 50 ml of the solvent mixture and filter.

Reference solution (a). A 0.05 per cent w/v solution of *ritonavir IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with butyl silane chemically bonded to porous silica (3 µm) (Such as ACE C4),
- mobile phase: A. a mixture of 69 volumes of *0.03 M mono-basic potassium phosphate buffer* prepared by dissolving 4.1 g of *monobasic potassium phosphate* in 1000 ml of *water*, 18 volumes of acetonitrile, 8 volumes of *tetrahydrofuran* and 5 volumes of *n-butanol*,
  B. a mixture of 40 volumes of *0.03 M mono-basic potassium phosphate buffer*, 47 volumes of acetonitrile, 8 volumes of *tetrahydrofuran* and 5 volumes of *n-butanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
60	100	0
120	0	100
155	100	0

Inject the reference solution (a). The test is not valid unless the tailing factor is not more than 2.0 and the column efficiency in not less than 5000 theoretical plates.
Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 2.5 times the area of the peak in the chromatogram obtained with the reference solution (b) (2.5 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the peak in the chromatogram obtained with the reference solution (b) (5.0 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the mixed contents of 20 capsules containing about 25 mg of Ritonavir, disperse in 100.0 ml of *methanol* and filter.

Reference solution. A 0.025 per cent w/v solution of *ritonavir IPRS* in *methanol*.

Chromatographic system
- a stainless steel column 5 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm),
- column temperature 45°,
- mobile phase: a mixture of 55 volumes of a buffer solution prepared by dissolving 3.4 g of *sodium acetate trihydrate* and 0.94 g of *sodium 1-hexanesulphonate* in 1000 ml of *water*, adjusted to pH 4.0 with *hydrochloric acid* and 45 volumes of *acetonitrile*,
- flow rate: 2.5 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 10 µl.

Inject the reference solution. Run the chromatogram 1.5 times the retention time (about 3 minutes) of the principal peak The test is not valid unless the tailing factor is not more than 2.0, the column efficiency in not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{37}H_{48}N_6O_5S_2$ in the capsules.

Storage. Store protected from light in a refrigerator (2° to 8°).

Ritonavir Tablets

Ritonavir Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ritonavir, $C_{37}H_{48}N_6O_5S_2$.

Usual strengths. 25 mg; 100 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2)

Apparatus No. 2 (Paddle),
Medium. 900 ml of a solution prepared by dissolving 15.7 g of *polyoxyethylene 10-lauryl ether* in 1000 ml of a 0.85 per cent v/v solution of *hydrochloric acid*,
Speed and time. 100 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14)

Test solution. The filtrate obtained as given above. Dilute the filtrate if necessary, with the dissolution medium.

Reference solution. A 0.1 per cent w/v solution of *ritonavir IPRS* in *methanol*. Dilute 5 ml of the solution to 50 ml with the dissolution medium.

Use the chromatographic system described under Assay.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation of replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

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Calculate the content of $C_{37}H_{48}N_6O_5S_2$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{37}H_{48}N_6O_5S_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture: 40 volumes of *0.03 M monobasic potassium phosphate* and 60 volumes of *acetonitrile*.

Test solution: Shake a quantity of the powdered tablets containing 25 mg of Ritonavir with 50 ml of the solvent mixture.

Reference solution (a). A 0.05 per cent w/v solution of *ritonavir IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with butyl silane chemically bonded to porous silica (3 µm) (Such as ACE C4),
- mobile phase: A. a mixture of 69 volumes of *0.03 M mono-basic potassium phosphate solution*, 18 volumes of *acetonitrile*, 8 volumes of *tetrahydrofuran* and 5 volumes of *n- butanol*,

  B. a mixture of 40 volumes of *0.03 M mono-basic potassium phosphate solution*, 47 volumes of *acetonitrile*, 8 volumes of *tetrahydrofuran* and 5 volumes of *n-butanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 100 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
60	100	0
120	0	100
121	100	0
155	100	0

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 2.5 times the area of the peak in the chromatogram obtained with the reference solution (b) (2.5 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the peak in the chromatogram obtained with the reference solution (b) (5.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14)

Test solution. Weigh a quantity of the powdered tablets containing 25 mg of Ritonavir and disperse in 100.0 ml of *methanol*.

Reference solution. A 0.025 per cent w/v solution of *ritonavir IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 5 cm × 4.6 mm, packed with phenyl stationary phase bonded to porous silica (3µm),
- column temperature 45°,
- mobile phase: a mixture of 55 volumes of a buffer solution prepared by dissolving 3.4 g of *sodium acetate trihydrate* and 0.94 g of *sodium 1-hexanesulphonate* to 1000 ml with *water*, adjusted to pH 4.0 with *dilute hydrochloric acid*, and 45 volumes of *acetonitrile*.
- flow rate: 2.5 ml per minute,
- spectrophotometer set at 239 nm,
- injection volume: 10 µl.

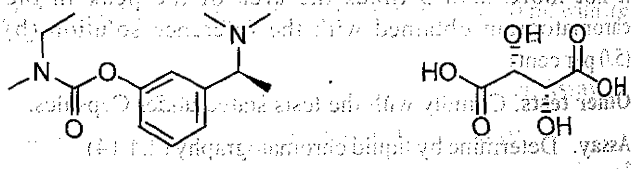
Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{37}H_{48}N_6O_5S_2$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Rivastigmine Tartrate



$C_{14}H_{22}N_2O_2,C_4H_6O_6$ Mol. Wt. 400.4

Rivastigmine Tartrate is ethylmethylcarbamic acid,3-[(S)-1-(dimethylamino)ethyl]phenyl ester, (2R,3R)-2,3-dihydroxybutanedioate.

Rivastigmine Tartrate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{14}H_{22}N_2O_2,C_4H_6O_6$, calculated on the anhydrous basis.

Category. Anti-Dementia Drug.

Description. A white to off white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rivastigmine*

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tartrate IPRS or with the reference spectrum of rivastigmine tartrate.

B. In the test for Enantiomeric purity, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with reference solution (c).

Tests

Enantiomeric purity. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 10 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). A 0.00001 per cent w/v solution of *rivastigmine tartrate R-Isomer IPRS* in the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (c). A solution containing 0.01 per cent w/v of *rivastigmine tartrate IPRS* and 0.00001 per cent w/v of *rivastigmine tartrate R-Isomer IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 10 cm × 4.0 mm, packed with immobilized α1-acid glycoprotein on spherical silica particles (5 µm) (Such as Chiralpak AGP),
- mobile phase. a mixture of 98 volumes of a buffer solution prepared by dissolving 1.78 g of *dibasic sodium phosphate dihydrate* and 1.38 g of *monobasic sodium phosphate* in 1000 ml of *water*, adjusted to pH 6.0 with *dilute orthophosphoric acid*, 2 volumes of *acetonitrile* and 0.02 volume of *N,N-dimethyloctylamine*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 200 nm,
- injection volume: 20 µl.

Inject reference solution (a), (b) and (c). The test is not valid unless the resolution between the peaks due to R-enantiomer and S-enantiomer (rivastigmine) is not less than 0.8 in the chromatogram obtained with reference solution (c), the signal-to-noise ratio is not less 10 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 10 per cent in the chromatogram obtained with reference solution (a).

NOTE —The elution order is the R-enantiomer, followed by S-enantiomer (rivastigmine).

Inject reference solution (a) and the test solution. The area of the peak corresponding to R-enantiomer is not more than three times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). A 0.0001 per cent w/v solution of *rivastigmine tartrate IPRS* in the mobile phase.

Reference solution (b). A solution containing 0.005 per cent w/v each of *rivastigmine related compound A IPRS* and *rivastigmine related compound B IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase. a mixture of 70 volumes of a buffer solution prepared by dissolving 8.6 g of *monobasic ammonium phosphate* in 900 ml of *water*, adjusted to pH 7.0 with *ammonia solution* and dilute to 1000 ml with *water*, 15 volumes of *methanol* and 15 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Tartaric acid	0.18	—
Phenol impurity[1]	0.28	0.63
DPTTA[2]	0.46	1.20
Nor impurity[3]	0.57	0.83
Rivastigmine	1.0	—
Carbamate impurity[4]	4.1	0.77
Ether impurity[5]	6.5	0.71

[1](S)-3-[1-(Dimethylamino)ethyl]phenol,

[2](+)-Di-(*P*-toluoyl)-D- tartric acid (rivastigmine related compound A),

[3](S)-3-[1-(Dimethylamino)ethyl]phenyldimethylcarbamate (racemic mixture is rivastigmine related compound B),

[4]3-Nitrophenyl ethyl(methyl)carbamate,

[5](*S*)-*N*,*N*-Dimethyl-1-[3-(4-nitrophenoxy)phenyl]ethanamine.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to rivastigmine related compound A and rivastigmine related compound B is not less than 1.5 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 10 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. Run the chromatogram 8 times the retention time of the principal peak. The area of any peak corresponding to phenol impurity is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). The area of any peak corresponding to DPTTA, nor impurity, carbamate impurity and ether impurity, each of , is

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not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent). The area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore the peak due to tartaric acid.

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.5 per cent.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modification.

Test solution. Dissolve 20 mg of the substance under examination in 70 ml of the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). A 0.02 per cent w/v solution of *rivastigmine tartrate IPRS* in the mobile phase.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to rivastigmine related compound A and rivastigmine related compound B is not less than 1.5 in the chromatogram obtained with reference solution (b), the column efficiency is not less than 5000 theoretical plates, the tailing factor is not more than 3.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{14}H_{22}N_2O_2$, $C_4H_6O_6$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Rivastigmine Capsules

Rivastigmine Tartrate Capsules

Rivastigmine Capsules contain Rivastigmine Tartrate equivalent to not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of rivastigmine, $C_{14}H_{22}N_2O_2$.

Usual strengths. 1.5 mg; 3 mg; 4.5 mg; 6 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 500 ml of *water*,

Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14) as described under Assay with the following modifications.

Test solution. Use the filtrate, dilute if necessary with the dissolution medium.

Reference solution. A 0.0192 per cent w/v solution of *rivastigmine tartrate IPRS* in the mobile phase and dilute with the dissolution medium to obtain a solution having a known concentration similar to the expected concentration of the test solution.

– injection volume: 100 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_{14}H_{22}N_2O_2$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{14}H_{22}N_2O_2$.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Disperse a quantity of the mixed content containing 25 mg of Rivastigmine in 10 ml of the mobile phase, with the aid of ultrasound and dilute to 25.0 ml with the mobile phase, centrifuge and use the clear supernatant liquid.

Reference solution (a). A 0.00016 per cent w/v solution of *rivastigmine tartrate IPRS* in the mobile phase.

Name	Relative retention time	Correction factor
Phenol impurity[1]	0.28	0.63
Rivastigmine	1.0	—

[1](S)-3-[1-(Dimethylamino)ethyl]phenol.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to rivastigmine related compound A and rivastigmine related compound B is not less than 1.5 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 10 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area any peak corresponding to phenol impurity is not more than

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6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent), the area of any other secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore the peak due to tartaric acid.

Uniformity of content. Complies with the test stated under Capsules.

Determine by liquid chromatography (2.4.14), as described under Assay with the following modification.

Test solution. Disperse the content of one capsule in 2.5 ml of *methanol* and 5 ml of the mobile phase, with the aid of ultrasound for 15 minutes with intermittent shaking and dilute to 25.0 ml with the mobile phase, centrifuge and use the clear supernatant liquid. Dilute the supernatant, if necessary, with the mobile phase to obtain a solution having the similar concentration to the reference solution.

Reference solution. Dissolve 34 mg of *rivastigmine tartrate IPRS* in 2 ml of *methanol* and 25 ml of the mobile phase, with the aid of ultrasound and dilute to 100.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Inject the reference solution and the test solution.

Calculate the content of $C_{14}H_{22}N_2O_2$ in the capsules.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and mix the content of 20 capsules or more. Disperse a quantity of the mixed content containing 20 mg of rivastigmine in 10 ml of *methanol* and 25 ml of the mobile phase with the aid of ultrasound for 15 minutes with intermittent shaking and dilute to 100.0 ml with the mobile phase, centrifuge. Dilute 5.0 ml of supernatant liquid to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 64 mg of *rivastigmine tartrate IPRS* in 10 ml of *methanol* and 25 ml of the mobile phase with the aid of ultrasound and dilute to 100.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.001 per cent w/v each of *rivastigmine related compound A IPRS* and *rivastigmine related compound B IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 70 volumes of a buffer solution prepared by dissolving 8.6 g of *monobasic ammonium phosphate* in 1000 ml of *water*, adjusted to pH 7.0 with *ammonia solution*, 15 volumes of *methanol* and 15 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

The relative retention time with reference to rivastigmine for rivastigmine related compound A is about 0.46 and rivastigmine related compound B is about 0.57.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to rivastigmine related compound A and rivastigmine related compound B is not less than 1.5 in the chromatogram obtained with reference solution (b), the column efficiency is not less than 5000 theoretical plates, the tailing factor is not more than 2.3 and the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

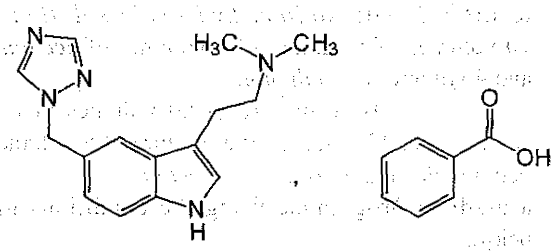
Calculate the content of $C_{14}H_{22}N_2O_2$ in the capsules.

1 mg of $C_{14}H_{22}N_2O_2.C_4H_6O_6$ is equivalent to 0.6252 mg of $C_{14}H_{22}N_2O_2$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of rivastigmine.

Rizatriptan Benzoate



$C_{15}H_{19}N_5,C_7H_6O_2$ Mol. Wt. 391.5

Rizatriptan Benzoate is *N,N*-Dimethyl-2-[5-(1,2,4-triazol-1-ylmethyl)-1*H*-indol-3-yl] ethyl amine benzoate.

Rizatriptan Benzoate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{15}H_{19}N_5,C_7H_6O_2$, calculated on the dried basis.

Category. Antimigraine.

Description. A white to off white crystalline powder.

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Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rizatriptan benzoate IPRS* or with the reference spectrum of rizatriptan benzoate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of mobile phase A and mobile phase B.

Test solution. Dissolve 25 mg of the substance under examination in 25.0 ml of the solvent mixture.

Reference solution (a). A 0.0001 per cent w/v solution of *rizatriptan benzoate IPRS* in the solvent mixture.

Reference solution (b). A 0.0001 per cent w/v solution of *dimethyl-[2-(5-[1,2,4]-triazol-1-ylmethyl-1H-indol-3-yl)-ethyl]-amine-N-oxide IPRS (rizatriptan impurity C IPRS)* in reference solution (a).

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 90 volumes of buffer solution prepared by dissolving 2.6 g of *sodium dihydrogen phosphate*, 1.0 g of *dibutyl amine* and 0.8 g of *orthophosphoric acid* in 1000 ml of *water*, adjusted to pH 6.5 with *sodium hydroxide solution* and 10 volumes of a mixture of 6 volumes of *acetonitrile* and 4 volumes of *methanol*,

  B. a mixture of 60 volumes of buffer solution and 40 volumes of a mixture of 6 volumes of *acetonitrile* and 4 volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
35	45	55
40	100	0
45	100	0

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to rizatriptan impurity C and rizatriptan benzoate is not less than 2.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore the peak due to benzoic acid.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *rizatriptan benzoate IPRS* in the solvent mixture.

Use chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{19}N_5.C_7H_6O_2$.

Storage. Store protected from light.

Rizatriptan Tablets

Rizatriptan Benzoate Tablets

Rizatriptan Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of rizatriptan, $C_{15}H_{19}N_5$.

Usual strengths. 5 mg; 10 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

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Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *water*,

Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Reject the first few ml of the filtrate and dilute a suitable volume of the filtrate with dissolution medium. Measure the absorbance of the resulting solution at the maximum at about 280 nm (2.4.7).Calculate the content of $C_{15}H_{19}N_5$ in the medium from the absorbance obtained from a solution of known concentration of *rizatriptan benzoate IPRS*.

Q. Not less than 80 per cent of the stated amount of $C_{15}H_{19}N_5$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture: Equal volumes of mobile phase A and mobile phase B.

Test solution. Weigh a quantity of the powdered tablets containing about 50 mg of rizatriptan, dissolve in 30 ml of the solvent mixture and dilute to 50.0 ml with the solvent mixture, filter.

Reference solution (a). A solution of *rizatriptan benzoate IPRS* equivalent to 0.0001 per cent w/v of rizatriptan in the solvent mixture.

Reference solution (b). A 0.0001 per cent w/v solution of *dimethyl-[2-(5-[1,2,4]-triazol-1-ylmethyl-1H-indol-3-yl)-ethyl]-amine-N-oxide IPRS (rizatriptan impurity C IPRS)* in reference solution (a).

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS-3V),
- column temperature: 40°,
- mobile phase: A. a mixture of 90 volumes of buffer solution prepared by dissolving 2.6 g of *sodium dihydrogen phosphate*, 1.3 ml of *dibutylamine* and 0.5 ml of *orthophosphoric acid* in 1000 ml of *water*, adjusted to pH 6.5 with *sodium hydroxide solution* and 10 volumes of a mixture of 6 volumes of *acetonitrile* and 4 volumes of *methanol*,

  B. a mixture of 60 volumes of buffer solution and 40 volumes of a mixture of 6 volumes of *acetonitrile* and 4 volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
35	45	55
40	100	0
55	100	0

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to rizatriptan impurity C and rizatriptan is not less than 3.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14).

Test solution. Disperse one tablet in the mobile phase and dilute suitably to obtain a concentration of 0.005 per cent w/v of Rizatriptan Benzoate equivalent to Rizatriptan.

Reference solution. A solution of *rizatriptan benzoate IPRS* equivalent to 0.005 per cent w/v of rizatriptan in the mobile phase.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and transfer a quantity of powdered tablets containing about 20 mg of rizatriptan, dissolve in 50 ml of mobile phase and dilute to 100 ml with the mobile phase, filter. Dilute 5.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution. A solution of *rizatriptan benzoate IPRS* equivalent to 0.005 per cent w/v of rizatriptan in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 87 volumes of buffer solution prepared by dissolving 2.6 g of *sodium dihydrogen phosphate*, 1.3 ml of *dibutylamine* and 0.5 ml of *orthophosphoric acid* in 1000 ml *water*, adjusted to pH 6.5 with *sodium hydroxide solution*, 7.8 volumes of *acetonitrile* and 5.2 volumes of *methanol*,

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– flow rate: 1 ml per minute,
– spectrophotometer set at 225 nm,
– injection volume: 20 µl.

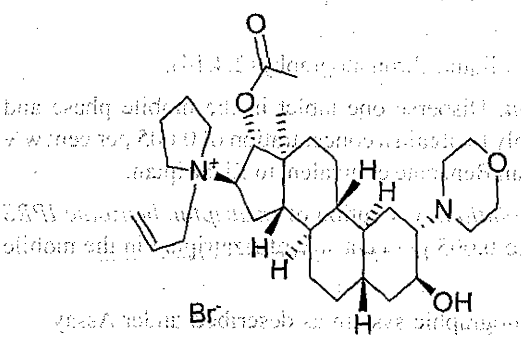
Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent. Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{19}N_5$ in the tablet.

Storage. Store protected from light.

Labelling. The label states the strength in terms of the equivalent amount of Rizatriptan.

Rocuronium Bromide



$C_{32}H_{53}BrN_2O_4$ Mol. Wt. 609.7

Rocuronium Bromide is 1-[17β-Acetoxy-3α-hydroxy-2β-(morpholin-4-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium bromide.

Rocuronium Bromide contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{32}H_{53}BrN_2O_4$, calculated on the anhydrous basis.

Category. Non-depolarizing neuromuscular blocker.

Description. Almost white or pale yellow, slightly hygroscopic powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rocuronium bromide IPRS* or with the reference spectrum of rocuronium bromide.

B. A 1 per cent w/v solution in *carbon dioxide-free water* (solution A) gives reaction (a) of bromides (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1) and not more intensely coloured than reference solution BYS5 (2.4.1).

pH (2.4.24). 8.9 to 9.5, determined in solution A.

Specific optical rotation (2.4.22). +28.5° to +32.0°, determined in a 1 per cent w/v solution in *0.05 M hydrochloric acid*.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 10 volumes of *water* and 90 volumes of *acetonitrile*.

Test solution. Dissolve 100 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). A 0.001 per cent w/v solution of *rocuronium bromide IPRS* in the solvent mixture.

Reference solution (b). A 0.1 per cent w/v solution of *rocuronium for peak identification IPRS (containing impurities A, B, C, F, G and H)* in the solvent mixture.

Chromatographic system
– a stainless steel column 25 cm x 4.6 mm, packed with porous silica (5 µm),
– mobile phase: a mixture of 10 volumes of a buffer solution prepared by dissolving 4.53 g of *tetramethylammonium hydroxide* in 1000 ml of *water*, adjusted to pH 7.4 with *orthophosphoric acid* and 90 volumes of *acetonitrile*,
– flow rate: 2 ml per minute,
– spectrophotometer set at 210 nm,
– injection volume: 5 µl.

Name	Relative retention time	Correction factor
Rocuronium impurity A[1]	0.2	0.5
Rocuronium impurity G[2]	0.4	0.4
Rocuronium impurity F[3]	0.75	1.3
Rocuronium impurity B[4]	0.8	–
Rocuronium impurity H[5]	0.95	0.4
Rocuronium (retention time is about 9 minutes)	1.0	–
Rocuronium impurity C[6]	1.2	–

[1]3α-hydroxy-2β-(morpholin-4-yl)-16β-(pyrrolidin-1-yl)-5α-androstan-17β-yl acetate,

[2]2β-(morpholin-4-yl)-16β-(pyrrolidin-1-yl)-5α-androstane-3α,17β-diol,

[3]1-[3α,17β-acetoxy-2β-(pyrrolidin-1-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium,

[4]1-[3α,17β-diacetoxy-2β-(morpholin-4-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium,

[5]1-[17β-acetoxy-2-(morpholin-4-yl)-3-oxo-5α-androst-1-en-16β-yl]-1-(prop-2-enyl)pyrrolidinium,

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1-[3α,17β-dihydroxy-2β-(morpholin-4-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium.

NOTE — Equilibrate the column at least for 4 hours.

Inject reference solution (b). The test is not valid unless the peak-to-valley ratio is not less than 3.0, where Hp is the height above the baseline of the peak due to impurity H and Hv is the height above the baseline of the lowest point of the curve separating this peak from the peak due to rocuronium.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to rocuronium impurity A, B, C, each of, is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any peak corresponding to rocuronium impurity F, G, H, each of, is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak eluting before impurity A and with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Chlorides. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in *water* and dilute to 20.0 ml with *water*.

Reference solution (a). A solution containing 0.0644 per cent w/v of *sodium bromide* and 0.0824 per cent w/v of *sodium chloride* in *water*. Dilute 1.0 ml of the solution to 50.0 ml with *water*.

Reference solution (b). A 0.00824 per cent w/v solution of *sodium chloride* in *water*. Dilute 1.0 ml of the solution to 25.0 ml with *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with strong anion exchange resin (13 µm),
- mobile phase: a solution containing 0.0063 per cent w/v of *sodium hydrogen carbonate* and 0.0212 per cent w/v of *anhydrous sodium carbonate* in *water*,
- flow rate: 2 ml per minute,
- conductivity detector set at 100 µs/v and maintained at 30°, use a self-regenerating anion suppressor at 100 mA,
- injection volume: 25 µl.

The retention time of chloride and bromide are about 1.7 minutes and about 2.8 minutes, respectively.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to chloride and bromide is not less than 2.5.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to chloride is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),

2-Propanol. Not more than 1.0 per cent, determined by method described under Residual solvents (5.4).

Heavy metals (2.3.13). 1 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 4.5 per cent, determined on 0.4 g.

Assay. Dissolve 0.4 g in 40 ml of *glacial acetic acid*. Titrate with *0.1M perchloric acid*, determining the end- point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.06097 g of $C_{32}H_{53}BrN_2O_4$.

Storage. Store protected from light and moisture, and a temperature below - 15°.

Rocuronium Injection

Rocuronium Bromide Injection

Rocuronium Injection is a sterile solution of Rocuronium Bromide in Water for Injections.

Rocuronium Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of rocuronium bromide, $C_{32}H_{53}BrN_2O_4$.

Usual strength. 10 mg per ml.

Description. A clear colourless solution.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 3.0 to 4.5.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 90 volumes of *acetonitrile* and 10 volumes of *water*.

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Test solution. Dilute a suitable volume of the injection with the solvent mixture to obtain a solution containing 0.1 per cent w/v of Rocuronium Bromide.

Reference solution (a). A 0.001 per cent w/v solution of *rocuronium bromide IPRS* in the solvent mixture.

Reference solution (b). A 0.1 per cent w/v solution of *rocuronium for peak identification IPRS* (containing impurities A, B, C, F, G and H) in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with porous silica (5 µm),
- mobile phase: a mixture of 10 volumes of a buffer solution prepared by dissolving 4.53 g of *tetramethyl ammonium hydroxide* in 1000 ml of *water*, adjusted to pH 7.4 with *orthophosphoric acid* and 90 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Name	Relative retention time	Correction factor
Rocuronium impurity A[1]	0.2	0.5
Rocuronium impurity G[2]	0.4	0.4
Rocuronium impurity F[3]	0.75	1.3
Rocuronium impurity B[4]	0.8	—
Rocuronium impurity H[5]	0.95	0.4
Rocuronium (retention time is about 9 minutes)	1.0	—
Rocuronium impurity C[6]	1.2	—

[1]3α-hydroxy-2β-(morpholin-4-yl)-16β-(pyrrolidin-1-yl)-5α-androstan-17β-yl acetate,

[2]2β-(morpholin-4-yl)-16β-(pyrrolidin-1-yl)-5α-androstane-3α,17β-diol,

[3]1-[3α,17β-acetoxy-2β-(pyrrolidin-1-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium,

[4]1-[3α,17β-diacetoxy-2β-(morpholin-4-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium,

[5]1-[17β-acetoxy-2-(morpholin-4-yl)-3-oxo-5α-androst-1-en-16β-yl]-1-(prop-2-enyl)pyrrolidinium,

[6]1-[3α,17β-dihydroxy-2β-(morpholin-4-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium.

NOTE — Equilibrate the column at least for 4 hours.

Inject reference solution (a) and (b). The test is not valid unless the peak-to-valley ratio is not less than 3.0, where Hp is the height above the baseline of the peak due to impurity H and Hv is the height above the baseline of the lowest point of the curve separating this peak from the peak due to rocuroniumin the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to rocuronium impurity A, B, C, F, G, H, each of, is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent), the area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent). Ignore any peak eluting before impurity A and with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Other tests. Comply with the tests stated under Parenteral preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modifications.

Reference solution. A 0.1 per cent w/v solution of *rocuronium bromide IPRS* in the solvent mixture.

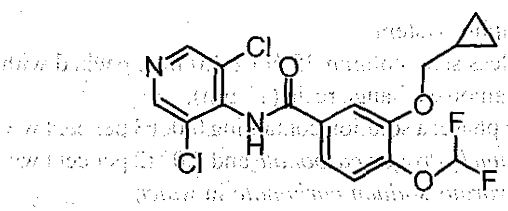
Inject the reference solution. The test is not valid unless the column efficiency is not less than 5000 theoretical plates, the tailing factor is more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solutionand the test solution.

Calculate the content of $C_{32}H_{53}BrN_2O_4$ in the injection.

Storage. Store at a temperature between 2° to 8°.

Roflumilast



$C_{17}H_{14}Cl_2F_2N_2O_3$ Mol Wt. 403.2

Roflumilast is *N*-(3,5-Dichloropyridin-4-yl)-3-(cyclopropylmethoxy)-4-(difluoromethoxy)benzamide.

Roflumilast contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{17}H_{14}Cl_2F_2N_2O_3$, calculated on the dried basis.

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Category. Anti-asthmatic.

Description. A white to off white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *roflumilast IPRS* or with the reference spectrum of roflumilast.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in *methanol* and dilute to 25.0 ml with *methanol.*

Reference solution. Dissolve 1 mg of *4-amino-3,5 dichloropyridine IPRS* and 25 mg of *roflumilast IPRS* in *methanol* and dilute to 25.0 ml with *methanol.*

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. 0.07 per cent w/v solution of *ammonium acetate* in *water*,
  B. a mixture of 80 volumes of *acetonitrile and* 20 volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	60	40
30	25	75
32	60	40
40	60	40

The relative retention time with respect to roflumilast for *4-amino-3,5dichloropyridine* is about 0. 2.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the test solution. The area of any peak due to 4-amino-3,5dichloropyridine is not more than 0.5 per cent, the area of any other secondary peak is not more than 0.5 per cent and the sum of areas of all the secondary peaks is not more than 1.0 per cent, calculated by area normalization.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in *methanol* and dilute to 25.0 ml with *methanol*. Dilute 5.0 ml of the solution to 50.0 ml with *methanol.*

Reference solution. A 0.01 per cent w/v solution of *roflumilast IPRS* in *methanol.*

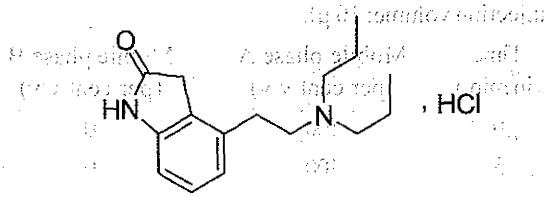
Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 40 volumes of 0.07 per cent w/v solution of *ammonium acetate* in *water*, 60 volumes of a mixture of 80 volumes of *acetonitrile and* 20 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent. Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{14}Cl_2F_2N_2O_3$.

Ropinirole Hydrochloride



$C_{16}H_{24}N_2O,HCl$ Mol. Wt. 296.8

Ropinirole Hydrochloride is 2*H*-Indol-2-one, 4-[2-(dipropylamino)ethyl]-1,3-dihydro-, monohydrochloride; 4-[2-(Dipropylamino)ethyl]-2-indolinone monohydrochloride.

Ropinirole Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{16}H_{24}N_2O,HCl$, calculated on the anhydrous basis.

Category. Anti-Parkinsonian.

Description. A pale cream to yellow powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ropinirole*

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hydrochloride IPRS or with the reference spectrum of ropinirole hydrochloride.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

C. It gives reaction (A) of chlorides (2.3.1).

Tests

Related substances. If methylene ropinirole and propylidene ropinirole are known process impurities, Method B is applicable. If ropinirole indole derivative and ropinirole oxime are known process impurities, Method C is applicable.

A. Determine by liquid chromatography (2.4.14), use buffer solution and solvent mixture as described under Assay.

Test solution. Dissolve 25 mg of the substance under examination in 25.0 ml of the solvent mixture.

Reference solution. A solution containing 0.1 per cent w/v of *ropinirole hydrochloride IPRS*, and 0.00015 per cent w/v of *ropinirole impurity B IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 15 volumes of *acetonitrile* and 85 volumes of buffer solution,

  B. a mixture of 60 volumes of *acetonitrile* and 40 volumes of *water*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	100	0
30	60	40
50	60	40
55	100	0
60	100	0

Name	Relative retention time	Correction factor
Open ring nitro derivative[1]	0.39	1.39
Monopropyl ropinirole[2]	0.58	0.91
N-hydroxyropinirole[3]	0.84	1.53
Ropinirole impurity B[4]	0.97	1.52
Ropinirole	1.00	—

[1]2-{2-[2-(Dipropylamino)ethyl]-6-nitrophenyl}acetic acid,

[2]4-[2-(Propylamino)ethyl]indolin-2-one,

[3]4-[2-(Dipropylamino)ethyl]-1-hydroxyindolin-2-one,

[4]4-[2-(Dipropylamino)ethyl]indoline-2,3-dione hydrochloride.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to ropinirole hydrochloride and ropinirole impurity B is not less than 1.5, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent using ropinirole peak.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of each peak due to impurities open ring nitro derivative, monopropyl ropinirole, N-hydroxyropinirole and impurity B is not more than 0.15 per cent, the area of any other secondary peak is not more than 0.1 per cent and the sum of areas of all secondary peaks is not more than 1.0 per cent. Ignore any peak with an area less than 0.05 per cent, calculated by area normalisation.

B. Determine by liquid chromatography (2.4.14).

Solvent mixture. 84 volumes of mobile phase A and 16 volumes of mobile phase B.

Test solution. Dissolve 25 mg of the substance under examination in 25.0 ml of the solvent mixture.

Reference solution (a). A solution containing 0.005 per cent w/v of each *ropinirole hydrochloride IPRS*, *ropinirole impurity A IPRS* and *ropinirole impurity B IPRS* in the solvent mixture.

Reference solution (b). A 0.0003 per cent w/v solution of *ropinirole impurity B IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. dissolve 3.85 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 2.5 with *ortho phosphoric acid*,

  B. a mixture of 70 volumes of *acetonitrile* and 30 volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 250 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	84	16
16	84	16
36	20	80
37	84	16
40	84	16

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Name	Relative retention time
Monopropyl ropinirole[1]	0.37
Ropinirole impurity A[2]	0.70
Ropinirole impurity B[3]	0.85
Ropinirole	1.0
Methylene ropinirole[4]	1.39
Ropinirole isohexyl analog[5]	1.64
Propylidene ropinirole[6]	1.70

[1] 4-[2-(Propylamino)ethyl]indolin-2-one,

[2] 4-(2-Hydroxyethyl)indolin-2-one,

[3] 4-[2-(Dipropylamino)ethyl]indoline-2,3-dione hydrochloride,

[4] 4-[2-(Dipropylamino)ethyl]-3-methyleneindolin-2-one,

[5] 4-[2-(2-Methylpentylamino)ethyl]indolin-2-one,

[6] (Z)-4-[2-(Dipropylamino)ethyl]-3-propylideneindolin-2-one.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to ropinirole impurity A and ropinirole impurity B is not less than 2.0, the resolution between the peaks due to ropinirole hydrochloride and ropinirole impurity B is not less than 3.0, the tailing factor is not more than 1.5 in the chromatogram obtained with reference solution (a) and the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (b).

Inject the test solution. The area of each peak due to monopropyl ropinirole, methylene ropinirole and propylidene ropinirole is not more than 0.2 per cent, the area of each peak due to ropinirole impurity B and ropinirole isohexyl analog is not more than 0.3 per cent, the area of any other secondary peak is not more than 0.1 per cent and the sum of areas of all the secondary peaks is not more than 1.0 per cent, calculated by area normalisation.

C. Determine by liquid chromatography (2.4.14).

Solvent mixture. 32 volumes of *acetonitrile* and 68 volumes of *water*.

Test solution. Dissolve 30 mg of the substance under examination in 25.0 ml of the solvent mixture.

Reference solution (a). A 0.00012 per cent w/v solution of *ropinirole hydrochloride IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.1 per cent w/v of *ropinirole hydrochloride IPRS* and 0.001 per cent w/v of *ropinirole impurity B IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm × 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. dissolve 3.95 g of *ammonium bicarbonate* in 1000 ml of *water*, adjusted to pH 9.2 to 9.3 with 25 per cent *ammonia solution*,

B. *acetonitrile*,

- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	68	32
12	68	32
28	20	80
34	20	80
34.1	68	32
40	68	32

Name	Relative retention time	Correction factor
Ropinirole oxime[1]	0.69	1.0
Ropinirole	1.0	—
Ropinirole impurity B	1.2	1.0
Ropinirole indole derivative[2]	2.0	0.25

[1] 4-[2-(Dipropylamino)ethyl]-3-(hydroxyimino)indolin-2-one,

[2] *N,N*-Dipropyl-*N*-(2-indol-4-ylethyl)amine.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to ropinirole hydrochloride and ropinirole impurity B is not less than 1.5 in the chromatogram obtained with reference solution (b), the tailing factor is between 0.8 to 1.5 and the relative standard deviation for replicate injections is not more than 5.0 per cent for ropinirole in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of each peak due to ropinirole oxime and ropinirole impurity B is not more than 0.15 per cent, the area of each peak due to ropinirole indole derivative and any other secondary peaks is not more than 0.1 per cent, and the sum of areas of all the secondary peaks is not more than 0.5 per cent, calculated by area normalisation.

Palladium. Not more than 10 ppm.

NOTE —Perform this test if Pd is a known inorganic impurity.

Solution A. Dissolve 2.4 g of *sodium hydroxide* in 20 ml of *water*, and dilute with *glycerol* to 100 ml.

Solution B. A 4.0 per cent w/v solution of *thioacetamide* in *water*.

NOTE —The solution may be filtered if necessary.

Solvent mixture. Carefully add 27 ml of *hydrochloric acid* to 25 g of *ammonium acetate*, and dilute with *water* to 100.0 ml.

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Test solution. Transfer 1 g of the substance under examination to a platinum crucible, and heat until the material is completely carbonized. Moisten the contents with 0.5 ml of *sulphuric acid*, and heat until fumes are no longer emitted. Place the crucible in a muffle furnace at 700° and heat. Cool, add 5 ml of *hydrochloric acid* to the residue, and evaporate to dryness. Add 1 ml of *2 M hydrochloric acid* and, if necessary, dissolve the residue with gentle heating. Cool and transfer the contents to a 50-ml beaker. Wash the crucible twice with 1-ml aliquots of *water*, and add the washings to the beaker. Pass the solution through a membrane filter of 2-µm pore size into a centrifuge tube.

Reference solution. Pipet 1.0 ml of 10 ppm Pd solution into a platinum crucible, and gently evaporate to dryness. Moisten the residue with 0.5 ml of *sulphuric acid*, and heat until fumes are no longer emitted. Place the crucible in a muffle furnace at 700°, and heat for additional 5 minutes, Cool, add 5 ml of *hydrochloric acid* to the residue, and evaporate to dryness. Add 1 ml of *2 M hydrochloric acid* and, if necessary, dissolve the residue with gentle heating. When cool, transfer the contents to a 50-ml beaker. Wash the crucible twice with 1-ml aliquots of *water*, and add the washings to the beaker. Pass the solution through a membrane filter of 2-µm pore size into a centrifuge tube.

To the filtered reference solution and the test solution, add 1 ml of Solution A, 0.2 ml of Solution B, and 2 ml of solvent mixture. Cap the centrifuge tubes and incubate at 37° for 2 hours. Pass the test solution and reference solution through a membrane filter of 2-µm pore size, wash the membrane with 2-ml aliquots of *water*, and allow the membrane to dry.

The intensity of the spot due to the test is not more intense than the spot due to the reference.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.5 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 20 volumes of *acetonitrile* and 80 volumes of *water*.

Test solution. Dissolve 10 mg of the substance under examination in 100.0 ml of the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *ropinirole hydrochloride IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 80 volumes of a buffer solution prepared by dissolving 1.88 g of *sodium hexanesulphonate*, 1 g of *orthophosphoric acid* in 1000 ml of *water* and adjusting the pH to 6.5 with dilute triethylamine (1:10) and 20 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.6 and the relative standard deviation for replicate injections is not more than 1.5 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{24}N_2O$, HCl.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Ropinirole Prolonged-release Tablets

Ropinirole Hydrochloride Prolonged-release Tablets; Ropinirole Hydrochloride Sustained-release Tablets; Ropinirole Hydrochloride Extended-release Tablets; Ropinirole Sustained-release Tablets; Ropinirole Extended-release Tablets

Ropinirole Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Ropinirole Prolonged-release Tablets contain Ropinirole Hydrochloride equivalent to not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ropinirole, $C_{16}H_{24}N_2O$.

Usual strengths. 2 mg; 4 mg; 8 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2). Complies with the test stated under Tablets.

1 mg of $C_{16}H_{24}N_2O$,HCl is equivalent to 0.8771 mg of $C_{16}H_{24}N_2O$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture A. 80 volumes of *acetonitrile* and 20 volumes of mobile phase A.

Solvent mixture B. 20 volumes of solvent mixture A and 80 volumes of mobile phase A.

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Test solution. Weigh a quantity of the powdered tablets containing about 27 mg of ropinirole to a 200-ml volumetric flask, add about 100 ml of solvent mixture A, shake for 30 minutes and mix with the aid of ultrasound and dilute to volume with the same solvent and filter. Dilute 2.0 ml of the solution to 10.0 ml with mobile phase A.

Reference solution (a). A 0.000015 per cent w/v solution of *ropinirole hydrochloride IPRS* in solvent mixture B.

Reference solution (b). A solution containing 0.003 per cent w/v of *ropinirole hydrochloride IPRS* and 0.0001 per cent w/v of *ropinirole impurity B IPRS* in solvent mixture B.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 10.0 ml with solvent mixture B.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A, a 0.05 per cent v/v solution of *trifluoroacetic acid* in *water*;
  B, a mixture of 80 volumes of *acetonitrile* and 20 volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 250 nm,
- injection volume: 100 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	84	16
23	84	16
36	40	60
36.1	84	16
40	84	16

Name	Relative retention time	Correction factor
Monopropyl ropinirole[1]	0.42	—
Ropinirole impurity B[2]	0.89	—
Ropinirole N-hydroxymethyl[3]	0.94	1.41
Ropinirole	1.0	—
Ropinirole N-oxide[4]	1.31	—
Ropinirole methylene dimer[5]	1.82	—
Propylidene ropinirole[6]	1.96	0.5

[1]4-[2-(Propylamino)ethyl]indolin-2-one,

[2]4-[2-(Dipropylamino)ethyl]indoline-2,3-dione hydrochloride,

[3]4-[2-(Dipropylamino)ethyl]-1-hydroxymethyl)indolin-2-one,

[4]N-[2-(2-Oxoindolin-4-yl)ethyl]-N-propylpropan-1-amine oxide,

[5]4-[2-(Dipropylamino)ethyl]-3-({4-[2-(dipropylamino)ethyl]-2-oxo-2,3-dihydro-1H-indol-3-yl}methyl)-2,3-dihydro-1H-indol-2-one,

[6](Z)-4-[2-(Dipropylamino)ethyl]-3-propylideneindolin-2-one; process impurity included for identification only.

Inject reference solution (a), (b) and (c). The test is not valid unless the resolution between the peaks due to ropinirole and ropinirole impurity B is not less than 2.0 in the chromatogram obtained with reference solution (b), the relative standard deviation for replicate injections is not more than 10.0 per cent in the chromatogram obtained with reference solution (a) and the signal to noise ratio is not less than 10 in the chromatogram obtained with reference solution (c).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of each peak corresponding to monopropyl ropinirole, ropinirole impurity B, ropinirole N-hydroxymethyl, ropinirole N-oxide and ropinirole methylene dimer is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent), the area of any other secondary peak is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). The sum of areas of all secondary peaks is not more than 2.9 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Uniformity of content. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Disperse 1 tablet in the solvent mixture with the aid of ultrasound, and dilute if necessary to obtain a solution having 0.01 per cent w/v solution of ropinirole.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{16}H_{24}N_2O$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2. 4.14).

Solvent mixture. 80 volumes of *acetonitrile* and 20 volumes of a solution prepared by diluting 0.7 ml of *orthophosphoric acid* in 1000 ml with *water*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 5 mg of Ropinirole Hydrochloride in 25 ml of the solvent mixture, with the aid of ultrasound for 30 minutes and dilute to 50.0 ml with the same solvent and filter.

Reference solution (a). A 0.011 per cent w/v solution of *ropinirole hydrochloride IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v of *ropinirole hydrochloride IPRS* and 0.0003 per cent w/v of *ropinirole impurity B IPRS* in the solvent mixture.

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Chromatographic system
- a stainless steel column 12.5 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 75 volumes of *methanol* and 25 volumes of a buffer solution prepared by dissolving 4.5 g of *dibasic sodium phosphate dihydrate* in 900 ml of *water*, adjusted to pH 7.0 with *orthophosphoric acid* and diluting to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 250 nm,
- injection volume: 10 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to ropinirole hydrochloride and ropinirole impurity B is not less than 2.0 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.5 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{16}H_{24}N_2O$ in the tablets.

1 mg of $C_{16}H_{24}N_2O$,HCl is equivalent to 0.8771 mg of $C_{16}H_{24}N_2O$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of ropinirole.

Ropinirole Tablets

Ropinirole Hydrochloride Tablets

Ropinirole Tablets contain Ropinirole Hydrochloride equivalent to not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ropinirole, $C_{16}H_{24}N_2O$.

Usual strengths. 0.25 mg; 0.5 mg; 1 mg; 2 mg; 5 mg.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium: 500 ml of a buffer solution pH 4.0, prepared by dissolving 2.9 g of *sodium citrate dihydrate* and 3.3 g of *anhydrous citric acid* in 1000 ml of *water*,

Speed and time: 50 rpm and 15 minutes.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary with the dissolution medium.

Reference solution. Dissolve a quantity of *ropinirole hydrochloride IPRS* in dissolution medium and dilute with dissolution medium to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 7.5 cm × 3.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 1 volume of *acetonitrile* and 4 volumes of medium,
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 250 nm,
- injection volume: 50 µl.

Inject the reference solution and the test solution.

1 mg of $C_{16}H_{24}N_2O$,HCl is equivalent to 0.8771 mg of $C_{16}H_{24}N_2O$.

Q: Not less than 85 per cent of the stated amount of $C_{16}H_{24}N_2O$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of a buffer solution prepared by dissolving 5 g of *sodium lauryl sulphate* in 800 ml of *water*, adjusted to pH 6.8 with *orthophosphoric acid* or *sodium hydroxide* and 20 volumes of *methanol*.

Test solution. Weigh a quantity of the powdered tablets containing about 5 mg of ropinirole hydrochloride to a 50-ml volumetric flask, add about 35 ml of the solvent mixture, shake for 30 minutes and mix with the aid of ultrasound and dilute to volume with the solvent mixture and filter.

Reference solution (a). A 0.002 per cent w/v solution of *ropinirole hydrochloride IPRS* in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the same solvent.

Reference solution (b). A 0.003 per cent w/v solution of *ropinirole hydrochloride IPRS* and 0.005 per cent w/v of *ropinirole impurity B IPRS* in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the same solvent.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: A. a mixture of 20 volumes of *methanol* and 80 volumes of a buffer solution prepared by dissolving 1.8 g of *dibasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 7.4 with *orthophosphoric acid*,

  B. a mixture of 80 volumes of *methanol* and 20 volumes of a buffer solution prepared by dissolving 1.8 g of *dibasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 7.4 with *orthophosphoric acid*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 250 nm,
- injection volume: 100 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
8	85	15
30	30	70
40	10	90
60	10	90
60.1	85	15
65	85	15

Name	Relative retention time	Correction factor
Monopropyl ropinirole[1]	0.24	0.91
Ropinirole N-Oxide[2]	0.27	—
Cyclopentanylindolinone[3]	0.55	—
Hydroxy ropinirole[4]	0.64	3.03
Ropinirole impurity B[5]	0.95	0.71
Ropinirole	1.0	—
Ethyl ropinirole[6]	1.2	—
Propylidene ropinirole[7]	1.35	0.62

[1]4-[2-(Propylamino)ethyl]indolin-2-one,

[2]N-[2-(2-Oxoindolin-4-yl)ethyl]-*N*-propylpropan-1-amine oxide,

[3]1,2a,3,4-Tetrahydro-2*H* -cyclopenta(cd)indol-2-one,

[4]4-[2-(Dipropylamino)ethyl]-1-hydroxy-1,3-dihydro-2*H*-indol-2-one,

[5]4-[2-(Dipropylamino)ethyl]indoline-2,3-dione hydrochloride,

[6]4-[2-(Dipropylamino)ethyl]-1-ethyl-1,3-dihydro-2*H*-indol-2-one; process impurity included for identification only,

[7](Z)-4-[2-(Dipropylamino)ethyl]-3-propylideneindolin-2-one.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to ropinirole hydrochloride and ropinirole impurity B is not less than 2.0 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (a).

Inject the test solution. The area of each peak due to monopropyl ropinirole and ropinirole impurity B is not more than 0.6 per cent, the area of each peak due to ropinirole N-oxide, cyclopentanylindolinone and hydroxy ropinirole is not more than 0.5 per cent, the area of peak due to propylidene ropinirole is not more than 0.4 per cent, the area of any secondary peak is not more than 0.3 per cent and the sum of areas of all the secondary peaks is not more than 2.0 per cent calculated by area normalisation.

Uniformity of content. Determine by liquid chromatography (2.4.14), as described under Assay with the following modification.

Test solution. Disperse 1 tablet in the solvent mixture with the aid of ultrasound, and dilute if necessary to obtain a solution containing 0.01 per cent w/v solution of ropinirole.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{16}H_{24}N_2O$ in the tablets.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2. 4.14).

Solvent mixture. Dissolve 3.85 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 2.5 with *orthophosphoric acid*.

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 5 mg of Ropinirole to a 50-ml volumetric flask, add about 25 ml of the solvent mixture, shake for 30 minutes and mix with the aid of ultrasound and dilute to volume with the solvent mixture and filter.

Reference solution (a). A 0.011 per cent w/v solution of *ropinirole hydrochloride IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v of *ropinirole hydrochloride IPRS* and 0.00005 per cent w/v of *ropinirole impurity B IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 14 volumes of *acetonitrile*, 6 volumes of *methanol* and 80 volumes of a solution prepared by dissolving 3.85 g of *ammonium acetate* in 900 ml of *water*, adjusted to pH 2.5 with *orthophosphoric acid* and diluting to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 250 nm,
- injection volume: 20 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to ropinirole hydrochloride and ropinirole impurity B is not less than 2.0 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{16}H_{24}N_2O$ in the tablets.

1 mg of $C_{16}H_{24}N_2O$,HCl is equivalent to 0.8771 mg of $C_{16}H_{24}N_2O$.

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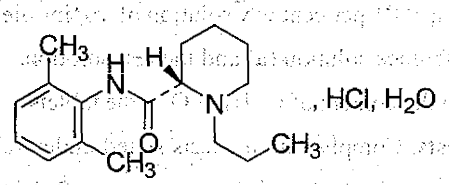
Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of ropinirole.

Ropivacaine Hydrochloride



$C_{17}H_{26}N_2O,HCl,H_2O$ Mol. Wt. 328.9

Ropivacaine Hydrochloride is (*S*)-(–)-1-Propylpiperidine-2-carboxylic acid (2,6-dimethylphenyl)amide hydrochloride monohydrate; (*S*)-(–)-1-Propyl-2',6'-pipecoloxylidine hydrochloride monohydrate.

Ropivacaine Hydrochloride contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{17}H_{26}N_2O,HCl$, calculated on the anhydrous basis.

Category. Local anaesthetic.

Description. A white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ropivacaine hydrochloride IPRS* or with the reference spectrum of ropivacaine hydrochloride.

B. A 1.0 per cent w/v solution gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution (2.4.1). A 2.0 per cent w/v solution in *carbon dioxide free water* is not more intensely coloured than reference solution YS7 (2.4.1) and is not more opalescent than standard OS1 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution (a). Dissolve 30 mg of the substance under examination in 10.0 ml of mobile phase.

Test solution (b). Dilute 1.0 ml of test solution (a) to 100.0 ml with mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with mobile phase.

Reference solution. A solution containing 0.001 per cent w/v each of *ropivacaine hydrochloride IPRS* and *bupivacaine hydrochloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (4 µm),
- mobile phase: a mixture of 50 volumes of *acetonitrile* and 50 volumes of a buffer solution prepared by dissolving 0.18 g of *monobasic sodium hydrogen phosphate* and 2.9 g of *disodium hydrogen phosphate dihydrate* in 1000 ml of *water*, adjusted to pH 8.0,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 20 µl.

The relative retention times are about 1.0 for ropivacaine and 1.6 for bupivacaine respectively.

Inject the reference solution and test solution (b). The test is not valid unless the resolution between the peaks due to ropivacaine and bupivacaine obtained with reference solution is not less than 6.0 and the signal to noise ratio for the principal peak obtained with test solution (b) is not less than 10.0.

Inject the reference solution and the test solution (a). The area of any peak due to bupivacaine is not more than 0.2 per cent. The area of any other secondary peak is not more than 0.1 per cent and the sum of the areas of all the secondary peaks is not more than 0.5 per cent, calculated by area normalisation.

Limit of Ropivacaine related compound A (2,6-Dimethylaniline hydrochloride). Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in 10.0 ml of mobile phase.

Reference solution. A solution containing 0.00001 per cent w/v of *ropivacaine hydrochloride impurity A IPRS* in the mobile phase.

Use chromatographic system as described under Related substances.

Inject the reference solution. In the chromatogram obtained with the reference solution signal- to- noise ratio for the principal peak is not less than 10.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak due to ropivacaine impurity A is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.001 per cent).

Enantiomeric purity. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 75 mg of the substance under examination in *water* and dilute to 100.0 ml with *water*. Dilute 10.0 ml of this resulting solution to 100.0 ml with mobile phase.

Reference solution. A solution containing 0.0075 per cent w/v of *ropivacaine hydrochloride IPRS and* 0.000075 per cent w/v of *ropivacaine impurity B IPRS* in the mobile phase.

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Chromatographic system

- a stainless steel column 10 cm x 4.0 mm, packed with chiralpak AGP (5 µm),
- mobile phase: dilute 35.0 ml of *isopropyl alcohol* into a 500-ml volumetric flask with buffer solution prepared by diluting 7.5 ml of *1 M monobasic sodium phosphate* solution and 28.5 ml of *0.5 M dibasic sodium phosphate dihydrate* solution to a 1000 ml with *water* and mix. Adjusted to pH 7.2, if necessary,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

The relative retention times are about 0.75 for ropivacaine impurity B and 1.0 for ropivacaine.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to ropivacaine impurity B (R enantiomer) and ropivacaine (S enantiomer) is not less than 1.5.

Inject the test solution. The area of any peak corresponding to ropivacaine hydrochloride impurity B is not more than 0.5 per cent, calculated by area normalisation.

pH (2.3.24). 4.5 to 6.0, determined on 1.0 per cent w/v solution.

Water (2.3.43). 5.0 to 6.0 per cent, determined on 0.1 g.

Heavy metals (2.3.13). 2.0 g complies with limit test for heavy metals, method B (10 ppm).

Assay. Dissolve 1.0 g of the substance in 10 ml of *water* and 40 ml of *ethanol*. Add 1 ml of *1 M hydrochloric acid*. Titrate with 1M sodium hydroxide, determining the end point potentiometrically (2.4.25). Read the volumes added between the two points of inflection.

1 ml of *1M sodium hydroxide* is equivalent to 0.3109 g of $C_{17}H_{26}N_2O,HCl$.

Ropivacaine hydrochloride intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 60 Endotoxin Unit per g of ropivacaine hydrochloride.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store in well-closed containers and at a temperature not exceed 30°.

Ropivacaine Injection

Ropivacaine Hydrochloride Injection

Ropivacaine Hydrochloride Injection is a sterile solution of Ropivacaine Hydrochloride in Water for Injections.

Ropivacaine Hydrochloride Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of $C_{17}H_{26}N_2O,HCl$.

Usual strengths. 2.0 mg per ml; 5.0 mg per ml; 7.5 mg per ml; 10 mg per ml.

Description. A clear, colourless solution.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution.

B. In the Enantiomeric purity, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.0 to 6.0.

Limit of Ropivacaine related compound A (2,6-Dimethylaniline hydrochloride). Determine by liquid chromatography (2.4.14) as described under Assay with the following modifications.

Test solution. Dilute a volume of injection to obtain a solution containing 0.2 per cent w/v of ropivacaine hydrochloride in the mobile phase.

Use chromatographic system and reference solution as described under assay.

- flow rate: 1.5 ml per minute,

Inject the reference solution. The test is not valid unless the resolution between the peaks due to ropivacaine hydrochloride and ropivacaine hydrochloride impurity A is not less than 5.0 and signal- to- noise ratio for the ropivacaine hydrochloride impurity A is not less than 10.

Inject the reference solution and the test solution. In the chromatogram obtained with test solution, the area of the peak due to ropivacaine impurity A is not more than 0.1 times of the area of the principal peak in the chromatogram obtained with the reference solution (0.01 per cent).

Enantiomeric purity. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of injection with mobile phase to obtain a solution containing 0.0075 per cent w/v of Ropivacaine Hydrochloride.

Reference solution. A solution containing 0.0075 per cent w/v of *ropivacaine hydrochloride IPRS* and 0.000075 per cent of *ropivacaine hydrochloride impurity B IPRS* in the mobile phase.

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Chromatographic system

- a stainless steel column 10 cm x 4.0 mm packed with immobilised α-1 acid glycoprotein on spherical silica particles (5 µm),
- mobile phase: take 35.0 ml of *isopropyl alcohol* into a 500-ml volumetric flask and make up the volume with buffer solution prepared by diluting 7.5 ml of *1 M monobasic sodium phosphate* solution and 28.5 ml of *0.5 M dibasic sodium phosphate dihydrate* solution to a 1000 ml with *water* and mix. Adjusted to pH 7.2, if necessary,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

The relative retention times are about 0.75 for ropivacaine impurity B and 1.0 for ropivacaine.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to ropivacaine hydrochloride and ropivacaine hydrochloride impurity B is not less than 1.5.

Inject the test solution. The area of any secondary peak corresponding to ropivacaine hydrochloride impurity B is not more than 2.0 per cent, calculated by area normalisation.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Bacterial endotoxins (2.2.3). Not more than 60 Endotoxin Unit per g of Ropivacaine Hydrochloride.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of injection with mobile phase to obtain a solution containing 0.025 per cent w/v of Ropivacaine Hydrochloride.

Reference solution. A solution containing 0.025 per cent w/v of *ropivacaine hydrochloride IPRS* and 0.000025 per cent w/v of *ropivacaine hydrochloride impurity A IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm).
- mobile phase: a mixture of 40 volumes of a buffer solution prepared by dissolving 1.8 g of *monobasic sodium hydrogen phosphate* and 2.9 g of *disodium hydrogen phosphate dihydrate* in 1000 ml of *water* adjusted to pH 8.0, if necessary and 60 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the resolution between the principal peak and the peak due to ropivacaine hydrochloride impurity A RS is not less than 5 and the relative standard deviation for replicate injection is not more than 1.0 per cent obtained with the reference solution.

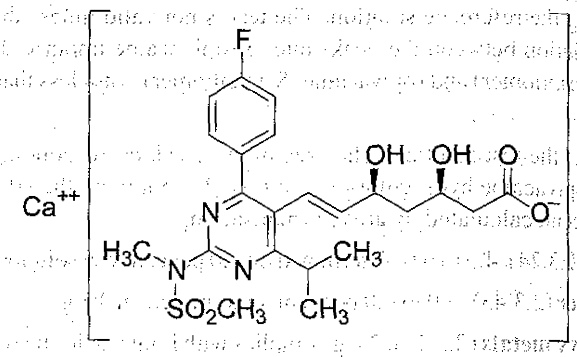
Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{26}N_2O,HCl$ in the Injection.

Storage. Store in single dose or multiple dose containers, preferably of Type I glass or of suitable plastic.

Labelling. The label states the quantity of active ingredient in terms of the equivalent amount of ropivacaine hydrochloride.

Rosuvastatin Calcium



$(C_{22}H_{27}FN_3O_6S)_2.Ca$ Mol. Wt. 1001.1

Rosuvastatin Calcium is (*E*)-(3*R*,5*S*)-7-{4-(4-fluorophenyl)-6-isopropyl-2-{methyl(methylsulphonylamino)}pyrimidin-5-yl}-3,5-dihydroxyhepten-6-oic acid calcium.

Rosuvastatin Calcium contains not less than 98.0 per cent and not more than 102.0 per cent of $(C_{22}H_{27}FN_3O_6S)_2.Ca$, calculated on the anhydrous basis.

Category. Antihyperlipidaemic.

Description. An off- white to creamish white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rosuvastatin calcium IPRS* or with the reference spectrum of rosuvastatin calcium.

B. Dissolve 20 mg in 25 ml of *methanol*, add 2 drops of *methyl red indicator* neutralise with *6 M ammonium hydroxide*. Add *3 M hydrochloric acid* until the solution is acidic to the indicator. Add *ammonium oxalate solution*, a white precipitate is obtained.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

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Test solution. Dissolve 50 mg of the substance under examination in 100 ml of the mobile phase.

Reference solution (a). A 0.05 per cent w/v solution of *rosuvastatin calcium IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of 0.2 per cent w/v *acetic acid* in *water*, 25 volumes of *acetonitrile* and 25 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 248 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the peak in the chromatogram obtained with the reference solution (b) (2.0 per cent).

Water (2.3.43). Not more than 8.0 per cent, determined on 0.3 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 100.0 ml of mobile phase. Dilute 5.0 ml of the solution to 25.0 ml with mobile phase, mix and filter.

Reference solution. A 0.05 per cent w/v solution of *rosuvastatin calcium IPRS* in mobile phase. Dilute 5.0 ml of the solution to 25.0 ml with mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of 0.2 per cent *acetic acid* in *water*, 25 volumes of *acetonitrile* and 25 volumes of *methanol*, filter and degas.
- flow rate: 1 ml per minute,
- spectrophotometer set at 248 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $(C_{22}H_{27}FN_3O_6S)_2Ca$.

Storage. Store protected from light and moisture.

Rosuvastatin Tablets

Rosuvastatin Calcium Tablets

Rosuvastatin Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of rosuvastatin, $C_{22}H_{28}FN_3O_6S$.

Usual strengths. 5 mg; 10 mg; 20 mg.

(1 mg of Rosuvastatin Calcium $(C_{22}H_{27}FN_3O_6S)_2Ca$ is equivalent to 961.9 µg of Rosuvastatin $(C_{22}H_{28}FN_3O_6S)_2$).

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of phosphate buffer pH 6.8,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. The filtrate obtained as given above.

Reference solution. Dissolve a quantity of *rosuvastatin calcium IPRS* in the mobile phase and dilute with dissolution medium to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{28}FN_3O_6S$ in the medium.

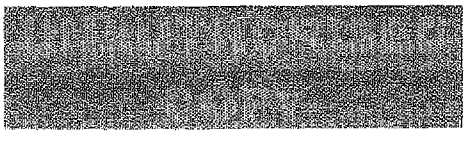
Q. Not less than 70 per cent of the stated amount of $C_{22}H_{28}FN_3O_6S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of powdered tablet containing 25 mg of rosuvastatin, disperse in 50.0 ml of the mobile phase and filter.

Reference solution (a). Dissolve a suitable quantity of *rosuvastatin calcium IPRS* in the mobile phase to obtain a solution containing 0.05 per cent w/v rosuvastatin.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with mobile phase.



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Chromatographic system as described under Assay.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 1.5 times the area of the peak in the chromatogram obtained with reference solution (b) (1.5 per cent) and the sum of areas of all the secondary peaks is not more than 3.0 times the area of the peak in the chromatogram obtained with reference solution (b) (3.0 per cent).

Uniformity of content. Complies with the tests stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay using following modication.

Test solution. Disperse one tablet in 50.0 ml of mobile phase, mix with the aid of ultrasound and dilute to 100.0 ml with the mobile phase, filter. Dilute further, if necessary, with the mobile phase to produce a solution containing 0.005 per cent w/v solution of rosuvastatin.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of powder containing 25 mg of Rosu-vastatin, disperse in 100.0 ml of the mobile phase. Dilute 5.0 ml of the solution to 25.0 ml with mobile phase and filter.

Reference solution. Dissolve a quantity of *rosuvastatin calcium IPRS* in mobile phase to obtain a solution containing 0.025 per cent w/v of rosuvastatin. Dilute 5.0 ml of the solution to 25.0 ml with mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: a mixture of 585 volumes of a buffer solution prepared by dissolving 1.54 g of *ammonium acetate* in 900 ml *water*, adjust pH to 4.0 with *glacial acetic acid* and dilute to 1000 ml with *water*, 360 volumes of *acetonitrile* and 50 volumes of *tetrahydrofurane*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 248 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{28}FN_3O_6S$.

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of the equivalent amount of Rosuvastatin.

Rosuvastatin and Ezetimibe Tablets

Rosuvastatin Calcium and Ezetimibe Tablets

Rosuvastatin and Ezetimibe Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of rosuvastatin, $C_{22}H_{28}FN_3O_6S$ and ezetimibe, $C_{24}H_{21}F_2NO_3$.

Usual strengths. Rosuvastatin 5 mg, Ezetimibe 10 mg; Rosuvastatin 20 mg, Ezetimibe 10 mg; Rosuvastatin 40 mg, Ezetimibe 10 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium, 900 ml of a buffer solution prepared by dissolving 10 g of *sodium lauryl sulphate* in 1000 ml of *water*,
Speed and time. 50 rpm for 45 minutes.
Withdraw a suitable volume of the medium and filter, rejecting the first few ml of the filtrate.

Determine by liquid chromatography (2.4.14).

Test solution. The filtrate obtained as given above, diluted suitably if necessary, with the dissolution medium.

Reference solution. Dissolve an accurately weighed quantity of *rosuvastatin calcium IPRS* and *ezetimibe IPRS* in the solvent mixture and dilute with the dissolution medium to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Use chromatographic system (except the requirement of sample temperature 5°) and solvent mixture as described under Assay.

Inject the reference solution and the test solution.

Calculate the contents of $C_{22}H_{28}FN_3O_6S$ and $C_{24}H_{21}F_2NO_3$, in the tablets.

Q. Not less than 70 per cent of the stated amounts of $C_{22}H_{28}FN_3O_6S$ and $C_{24}H_{21}F_2NO_3$.

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Related substances. Determine by liquid chromatography (2.4.14).

Use chromatographic system, solvent mixture and test solution as described under Assay.

Inject the test solution. The area of any secondary peak is not more than 1.0 per cent and the sum of areas of all the secondary peaks is not more than 2.0 per cent, calculated by area normalisation.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14).

Test solution. Disperse one tablet with 70 ml of solvent mixture ultrasound for 10 minutes, add sufficient solvent mixture to produce 100.0 ml, mix, centrifuge at 2500 rpm for about 20 minutes and filter. Dilute the filtrate, if necessary.

Use chromatographic system, solvent mixture and reference solution described under Assay.

Inject the reference solution and the test solution.

Calculate the contents of $C_{22}H_{28}FN_3O_6S$ and $C_{24}H_{21}F_2NO_3$ in the tablets.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 50 volumes of *dimethyl formamide* and 50 volumes of *methanol*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 5 mg of Rosuvastatin with 70 ml of solvent mixture, ultrasound for 10 minutes, add sufficient solvent mixture to produce 100.0 ml, mix and filter.

Reference solution. A solution containing 0.053 per cent w/v of *rosuvastatin calcium IPRS* and 0.1 per cent w/v solution of *ezetimibe IPRS* in solvent mixture. Dilute to 1.0 ml to 10.0 ml with solvent mixture to obtain a solution having a known concentration similar to the test solution.

Chromatographic system

- a stainless steel column 25 cm ´ 4.6 mm, packed with octadecylsilane chemically bonded to porous silica (5µm) (Such as Hypersil BDS),
- sample temperature: 5°,
- mobile phase: a mixture of 600 volumes of a buffer solution prepared by dissolving 0.77 g of *ammonium acetate* in 1000 ml of *water*, 400 volumes of *acetonitrile* and 5 volumes of *triethylamine* mix and adjusted to pH 4.0 with dilute *orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Inject the reference solution.The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent, and tailing factor is not more than 2.0, the column efficiency is not less than 6000 theoretical plates.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{28}FN_3O_6S$ and $C_{24}H_{21}F_2NO_3$, in the tablets.

Storage. Store protected from moisture.

Labelling. The label states the quantity of rosuvastatin calcium in terms of the equivalent amount of rosuvastatin, and the quantity of ezetimibe.

Rosuvastatin and Fenofibrate Tablets

Rosuvastatin Calcium and Fenofibrate Tablets

Rosuvastatin and Fenofibrate Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of rosuvastatin, $C_{22}H_{28}FN_3O_6S$ and fenofibrate, $C_{20}H_{21}ClO_4$.

Usual strengths. Rosuvastatin, 5 mg and Fenofibrate, 160 mg; Rosuvastatin, 10 mg and Fenofibrate, 80 mg; Rosuvastatin, 10 mg and Fenofibrate, 160 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (c).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium, 900 ml of a solution, prepared by dissolving 10 g of *sodium lauryl sulphate* in 1000 ml of *water*,
Speed and time. 75 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14), using the chromatographic system, solvent mixture and system suitability criteria as described under Assay.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution (a). A 0.052 per cent w/v solution of *rosuvastatin calcium IPRS* in solvent mixture.

Reference solution (b). A 0.18 per cent w/v solution of *fenofibrate IPRS* in solvent mixture.

Reference solution (c). Dilute a suitable volume of reference solution (a) and (b) with dissolution medium, to obtain a solution having concentration similar to the test solution.

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Calculate the content of $C_{22}H_{28}FN_3O_6S$ and $C_{20}H_{21}ClO_4$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{22}H_{28}FN_3O_6S$ and $C_{20}H_{21}ClO_4$.

Related substances. Determine by liquid chromatography (2.4.14).

Use chromatographic system and test solution as described under Assay.

Inject the test solution. The area of any secondary peak is not more than 1.0 per cent and the sum of areas of all the secondary peaks is not more than 2.0 per cent, calculated by area normalisation.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14).

Test solution. Disperse 1 tablet in 10 ml of *water* with the aid of ultrasound. Add about 30 ml of solvent mixture and disperse with aid of ultrasound for 15 minutes. Further add 30 ml of mobile phase and disperse with aid of ultrasound for 25 minutes, cool and dilute to 100.0 ml with mobile phase, mix and centrifuge at 2500 rpm for 10 minutes. Rejecting the first few ml of filtrate, dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.012 per cent w/v solution of *rosuvastatin calcium IPRS* in the solvent mixture and dilute to volume with mobile phase to obtain a solution having similar concentration to the test solution.

Use the solvent mixture and chromatographic system as described under Assay.

Calculate the content of $C_{22}H_{28}FN_3O_6S$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 50 volumes of *methanol* and 50 volumes of *N,N-diamethylformamide.*

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 800 mg of Fenofibrate in 125 ml of the solvent mixture, with the aid of ultrasound for 25 minutes with intermittent shaking and dilute to 250 ml with the mobile phase, mix and centrifuge. Dilute 5.0 ml of clear supernatant to 50.0 ml with the mobile phase.

Reference solution(a). A 0.052 per cent w/v solution of *rosuvastatin calcium IPRS* in the solvent mixture.

Reference solution(b). A 0.064 per cent w/v solution of *fenofibrate IPRS* in the solvent mixture.

Reference solution (c). Dilute a suitable volume of reference solution (a) and (b) with mobile phase, to obtain a solution having concentration similar to the test solution.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 40 volumes of a buffer solution prepared by dissolving 0.77 g of *ammonium acetate*, add 5 ml of *triethylamine* and pH adjusted to 4.0 with *orthophosphoric acid* in 1000 ml of *water*, 60 volumes of *acetonitrile*,
- flow rate: a gradient programme using the conditions given below,

Time (in min.)	Flow (ml)
0.0	0.7
7.0	0.7
8.0	2.0
35.0	2.0
36.0	0.7
40.0	0.7

- spectrophotometer set at 240 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the relative standard deviation of replicate injections for each peaks is not more than 2.0 per cent.

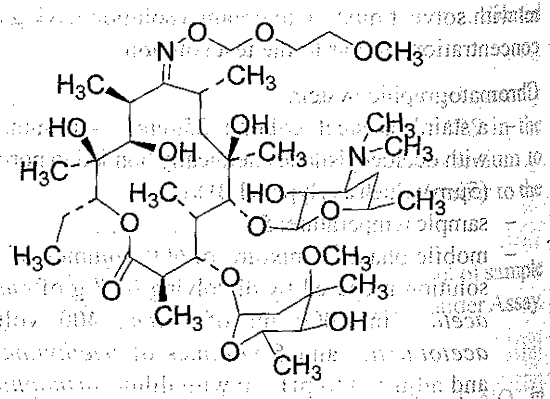
Inject reference solution (c) and the test solution.

Calculate the content of $C_{22}H_{28}FN_3O_6S$ and $C_{20}H_{21}ClO_4$ in the tablets.

Storage. Store protected from moisture at a temperature below 30°.

Labelling. The label states the strength in terms of the equivalent amount of rosuvastatin, and fenofibrate.

Roxithromycin



$C_{41}H_{76}N_2O_{15}$ Mol. Wt. 837.0

Roxithromycin is (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*S*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexo-

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pyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-10-[(*E*)-[(2-methoxyethoxy)methoxy]imino]-3,5,7,9,11; 13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl] oxy]oxacyclotetradecan-2-one; erythromycin 9-(*E*)-[*O*-[(2-methoxyethoxy)methyl]oxime].

Roxithromycin contains not less than 96.0 per cent and not more than 102.0 per cent of $C_{41}H_{76}N_2O_{15}$, calculated on the anhydrous basis.

Category. Antibacterial.

Description. A white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *roxithromycin IPRS* or with the reference spectrum of roxithromycin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Appearance of solution. A 1.0 per cent w/v solution in *methanol* is clear (2.4.1) and colourless (2.4.1).

Specific optical rotation (2.4.22). −96.0° to −93.0°, determined in a 1.0 per cent w/v solution in *acetone*.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Mix 30 volumes of *acetonitrile* and 70 volumes of a 4.8 per cent w/v solution of *ammonium dihydrogen phosphate* and adjust the pH to 5.3 with *dilute sodium hydroxide solution*.

Test solution. Dissolve 50.0 mg of the substance under examination in 25.0 ml of the solvent mixture.

Reference solution (a). A 0.2 per cent w/v solution of *roxithromycin IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Reference solution (c). Dissolve 2 mg of *erythromycin 9-(E)-[O-[[(2-methoxyethoxy)methoxy]methyl]oxime] (impurity I)* in 10 ml of reference solution (a). Further dilute 1.0 ml of the solution to 10.0 ml with reference solution (a).

Reference solution (d). Dilute 1.0 ml of *toluene* to 100.0 ml with *acetonitrile*. Dilute 0.2 ml of the solution to 200.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with spherical end-capped octadecylsilane bonded to porous silica (5 µm), with a 10 nm pore size and a carbon loading of about 19 per cent,
- column temperature: 15°,
- mobile phase: A. a mixture of 26 volumes of *acetonitrile* and 74 volumes of a 5.97 per cent w/v solution of *ammonium dihydrogen phosphate*, adjusted to pH 4.3 with *dilute sodium hydroxide solution*,
  B. a mixture of 30 volumes of *water* and 70 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
50	100	0
80	90	10
100	100	0

The relative retention time between roxithromycin and erythromycin 9-(E)-[O-[[(2-methoxyethoxy) methoxy]methyl] oxime] (roxithromycin impurity I) is about 1.15.

Inject reference solution (c). The test is not valid unless the peak-to-valley ratio H_p / H_v is not less than 2.0, where H_p = height above the baseline of the peak due to impurity I and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to roxithromycin.

Inject reference solution (b), (d) and the test solution. In the chromatogram obtained with the test solution the area of the impurity I peak obtained immediately after the peak obtained with roxithromycin is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). The area of any other individual impurity peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). The sum of the areas of all the peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent). Ignore any peak with an area 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent). Ignore any peak due to toluene identified using reference solution (d).

Heavy metals (2.3.13). Dissolve 2.0 g in a mixture of 15 volumes of *water* and 85 volumes of *acetone* and dilute to 20 ml with the same solvent mixture. This solution complies with the limit test for heavy metals, Method A (10 ppm), prepring the lead standard solution in the mixture of 15 volumes of *water* and 85 volumes of *acetone*.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

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Water (2.3.43). Not more than 3.0 per cent, determined on 0.2 g.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Mix 30 volumes of *acetonitrile* and 70 volumes of a 4.8 per cent w/v solution of *ammonium dihydrogen phosphate* and adjust the pH to 5.3 with *dilute sodium hydroxide solution.*

Test solution. Dissolve 50.0 mg of the substance under examination in 25.0 ml of the solvent mixture.

Reference solution (a). A 0.2 per cent w/v solution of *roxithromycin IPRS* in the solvent mixture.

Reference solution (b). Dissolve 2 mg of *erythromycin 9-(E)-[O-[[(2-methoxyethoxy)methoxy]methyl]oxime] (roxithromycin impurity I)* in 10.0 ml of reference solution (a). Further dilute 1.0 ml of the solution to 10.0 ml with reference solution (a).

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with spherical end-capped octadecylsilane bonded to porous silica (5 µm), with a 10 nm pore size and a carbon loading of about 19 per cent,
- column temperature 15°,
- mobile phase: mix 307 volumes of *acetonitrile* and 693 volumes of a 4.91 per cent w/v solution of *ammonium dihydrogen phosphate* adjusted to pH 5.3 with *dilute sodium hydroxide solution*,
- flow rate:1.5 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the peak-to-valley ratio H_p / H_v is not less than 1.5.

Inject reference solution (a) and the test solution

Calculate the content of $C_{14}H_{26}N_2O_{15}$.

Storage. Store protected from light and moisture.

Roxithromycin Tablets

Roxithromycin Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of roxithromycin, $C_{41}H_{76}N_2O_{15}$.

Usual strengths. 150 mg; 300 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *phosphate buffer pH 6.0*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. The filtrate obtained as given above.

Reference solution. A 0.0166 per cent w/v solution of *roxithromycin IPRS* in the dissolution medium.

Chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{41}H_{76}N_2O_{15}$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{41}H_{76}N_2O_{15}$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Mix 30 volumes of *acetonitrile* and 70 volumes of a 4.8 per cent w/v solution of *ammonium dihydrogen phosphate* and adjust the pH to 5.3 with *dilute sodium hydroxide solution.*

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.2 g of Roxithromycin, add 80 ml of the solvent mixture and mix. Dilute to 100.0 ml with the solvent mixture and filter.

Reference solution (a). A 0.2 per cent w/v solution of *roxithromycin IPRS* in the solvent mixture.

Reference solution (b). Dissolve 2 mg of *erythromycin 9-(E)-[O-[[(2-methoxyethoxy)methoxy]methyl]oxime] (roxithromycin impurity I)* in 10 ml of reference solution (a). Further dilute 1.0 ml of the solution to 10.0 ml with reference solution (a).

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with spherical end capped octadecylsilane bonded to porous silica (5 µm), with a 10 nm pore size and a carbon loading of about 19 per cent,
- column temperature: 15°,
- mobile phase: mix 307 volumes of *acetonitrile* and 693 volumes of a 4.91 per cent w/v solution of *ammonium dihydrogen phosphate* adjusted to pH 5.3 with *dilute sodium hydroxide solution*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

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Inject reference solution (b). The relative retention time between roxithromycin and erythromycin-(E)-[O-[[(2-methoxyethoxy)methoxy]methyl]oxime] (impurity I) is about 1.15. The test is not valid unless the peak-to-valley ratio H_p / H_v is not less than 1.5, where H_p = height above the baseline of the peak due to impurity I and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to roxithromycin.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

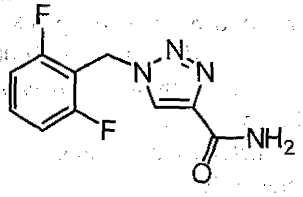
Inject reference solution (a) and the test solution.

Calculate the content of $C_{41}H_{76}N_2O_{15}$ in the tablets.

Storage. Store protected from light and moisture.

Labelling. If the tablets are dispersible, the label states that the tablets should be dispersed in water immediately before use.

Rufinamide



$C_{10}H_8F_2N_4O$ Mol Wt. 238.2

Rufinamide is 1-(2,6-Difluorobenzyl)-1*H*-[1,2,3]triazole-4-carboxamide.

Rufinamide contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{10}H_8F_2N_4O$, calculated on dried basis.

Category. Anticonvulsant.

Description. A white or off white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rufinamide IPRS* or with the reference spectrum of rufinamide.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 30 volumes of *water* and 70 volumes of *methanol.*

Test solution. Dissolve 25 mg of the substance under examination in the solvent mixture and dilute to 50.0ml with solvent mixture.

Reference solution. A 0.00025 per cent w/v solution of *rufinamide IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: A. a mixture of 90 volumes of buffer solution prepared to dissolve 6.9 g of *sodium dihydrogen phosphate monohydrate* in 1000 ml of *water* adjusted to pH 3.5 with ortho*phosphoric acid* and 10 volumes of *methanol,*

  B. a mixture of 10 volumes of buffer solution and 90 volumes of *methanol,*
- a gradient programme using the condition given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	05
10	90	10
20	90	10
35	65	35
45	55	45
60	55	45
65	95	05
75	95	05

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of areas of all the peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Assay. Determine by liquid chromatography (2.4.14).

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Solvent mixture. 30 volumes of buffer solution and 70 volumes of *acetonitrile.*

Test solution. Dissolve 25 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.0025 per cent w/v solution of *rufinamide IPRS* in the solvent mixture.

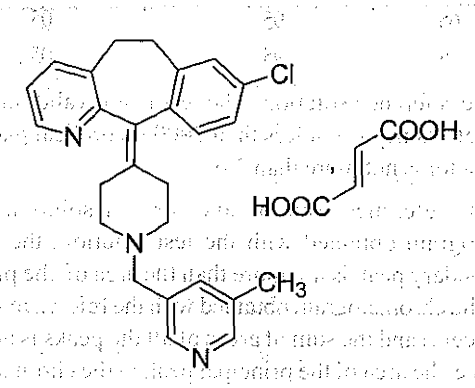
Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 75 volumes of buffer solution prepared to dissolving 3.48 g of *dipotassium hydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.5 with *orthophosphoric acid* , 10 volumes of *acetonitrile* and 15 volumes of *methanol*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_8F_2N_4O$.

Rupatadine Fumarate



$C_{30}H_{30}ClN_3O_4$ Mol. Wt. 532.0

Rupatadine Fumarate is 8-Chloro-6,11-dihydro-11-[1-[(5-methyl-3-pyridinyl)methyl]-4-piperidinylidene]-5*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridine fumarate.

Rupatadine Fumarate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{30}H_{30}ClN_3O_4$, calculated on the dried basis.

Category. Antiallergic.

Description. An off-white to pale yellow, powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *rupatadine fumarate IPRS* or with the reference spectrum of rupatadine fumarate.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 30 volumes of *acetonitrile*, 30 volumes of *methanol* and 40 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 2.8 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than the area of principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore the peak due to fumaric acid.

Fumaric acid. 20.0 to 25.0 per cent.

Weigh 0.3 g and dissolve in 60 ml of equal volumes of *methanol* and *water*. Titrate with *0.1 M sodium hydroxide* and determine the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.005803 of *fumaric acid.*

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

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Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.005 per cent w/v solution of *rupatadine fumarate IPRS* in the mobile phase.

Use chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{30}H_{30}ClN_3O_4$.

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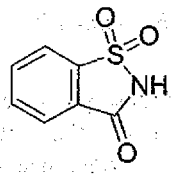
Saccharin



$C_7H_5NO_3S$ Mol. Wt. 183.2

Saccharin is 1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide.

Saccharin contains not less than 98.0 per cent and not more than 101.0 per cent of $C_7H_5NO_3S$, calculated on the dried basis.

Category. Pharmaceutical aid (sweetening agent).

Description. White crystals or a white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *saccharin IPRS* or with the reference spectrum of saccharin.

B. Mix 20 mg with 20 mg of *resorcinol*, add 0.5 ml of *sulphuric acid* and heat over a small flame until a dark green colour is produced; allow to cool and add 10 ml of *water* and an excess of *2 M sodium hydroxide*; a fluorescent green liquid is produced.

C. Dissolve 0.1 g in 5 ml of a 10 per cent w/v solution of *sodium hydroxide*, evaporate to dryness and gently fuse the residue over a small flame until ammonia is no longer evolved. Allow to cool, dissolve in 20 ml of *water*, make the solution just acidic to litmus paper, filter and add 0.05 ml of *ferric chloride solution*; a violet colour is produced.

D. A saturated solution is acidic to *litmus paper*.

Tests

Appearance of solution. Dissolve 5.0 g in 20 ml of a 20 per cent w/v solution of *sodium acetate* and dilute to 25 ml with the same solution; the solution is clear (2.4.1), and colourless (2.4.1).

Related substances. Determine by gas chromatography (2.4.13).

Internal standard solution. Dissolve 25 mg of *caffeine* in *dichloromethane* and dilute to 100 ml with the same solvent.

Test solution. Suspend 10.0 g of the substance under examination in 20 ml of *water* and dissolve using about 5 ml of *strong sodium hydroxide solution*. If necessary, adjust the pH of the solution to 7.8 with *1 M sodium hydroxide* or *1 M hydrochloric acid* and dilute to 50 ml with *water*. Shake the solution with 4 quantities, each of 50 ml, of *dichloromethane*. Combine the lower layers, dry over *anhydrous sodium sulphate* and filter. Wash the filter and the sodium sulphate with 10 ml of *dichloromethane*. Combine the solution and the washings and evaporate almost to dryness in a water-bath at a temperature not exceeding 40°. With a small quantity of *dichloromethane* transfer quantitatively the residue into a suitable 10-ml tube, evaporate to dryness in a current of *nitrogen* and dissolve the residue in 1.0 ml of the internal standard solution.

Blank solution. Evaporate 200 ml of *dichloromethane* to dryness in a water-bath at a temperature not exceeding 40°. Dissolve the residue in 1 ml of *dichloromethane*.

Reference solution. Dissolve 20 mg of *o-toluenesulphonamide* and 20 mg of *p-toluene sulphonamide* in *dichloromethane* and dilute to 100 ml with the same solvent. Dilute 5 ml of the solution to 50 ml with *dichloromethane*. Evaporate 5 ml of the final solution to dryness in a current of *nitrogen*. Dissolve the residue in 1 ml of the internal standard solution.

Chromatographic system
- a fused silica column 10 m × 0.53 mm packed with polymethylphenylsiloxane (film thickness 2 µm),
- temperature:
  column: 180°,
  inlet port and detector: 250°,
- flame ionization detector,
- flow rate: 10 ml per minute, using nitrogen as the carrier gas,
- split ratio: 1:2.

Inject 1 µl of each solution. The order of elution is *o*-toluenesulphonamide, *p*-toluenesulphonamide, caffeine.

The test is not valid unless the resolution between the peaks due to *o*-toluenesulphonamide and *p*-toluenesulphonamide in the chromatogram obtained with the reference solution is not less than 1.5 and the chromatogram obtained with the blank solution does not show any peak with the same retention times as the internal standard, *o*-toluenesulphonamide and *p*-toluenesulphonamide.

In the chromatogram obtained with the test solution the ratio of the area of the peak due to *o*-toluenesulphonamide to that of the internal standard is not more than the corresponding ratio in the chromatogram obtained with the reference solution (10 ppm).

In the chromatogram obtained with the test solution the ratio of the area of the peak due to *p*-toluenesulphonamide to that of the internal standard is not more than the corresponding ratio in the chromatogram obtained with the reference solution (10 ppm).

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Arsenic (2.3.10). Mix 5.0 g with 3.0 g of *anhydrous sodium carbonate*, add 10 ml of *bromine solution* and mix thoroughly. Evaporate to dryness on a water-bath, gently ignite and add to the cooled residue a mixture of 16 ml of *brominated hydrochloric acid AsT* and 5 ml of *bromine solution*. Add 40 ml of *water* and boil gently, adding sufficient bromine solution from time to time to maintain a slight excess. Filter and remove the excess of bromine with a sufficient quantity of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Readily carbonisable substances. Dissolve 0.2 g in 5 ml of *sulphuric acid (96 per cent w/w)* and maintain at about 50° for 10 minutes. The solution is not more intensely coloured than reference solution BYS6 (2.4.1).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

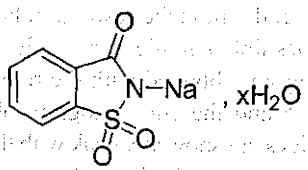
Assay. Weigh 0.5 g, dissolve in 40 ml of *ethanol (95 per cent)*, add about 40 ml of *water*. Titrate with *0.1 M sodium hydroxide* using *phenolphthalein solution* as indicator.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01832 g of $C_7H_5NO_3S$.

Storage. Store protected from moisture.

Saccharin Sodium

Soluble Saccharin



$C_7H_4NNaO_3S,xH_2O$ Mol. Wt. 205.2

Saccharin Sodium is the sodium salt of 1,2-benzisothiazol-3(2*H*)-3-one 1,1-dioxide.

Saccharin Sodium contains not less than 99.0 per cent and not more than 101.0 per cent of $C_7H_4NNaO_3S$, calculated on the anhydrous basis.

It may be anhydrous or contain a variable quantity of *water*.

Category. Pharmaceutical aid (sweetening agent).

Description. A white, crystalline powder or colourless crystals; efflorescent in dry air.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests B, C and D may be omitted if tests A and E are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *saccharin sodium IPRS* or with the reference spectrum of saccharin sodium.

B. Dissolve 0.1 g in 5 ml of a 10 per cent w/v solution of *sodium hydroxide*, evaporate to dryness and gently fuse the residue over a small flame until ammonia is no longer evolved. Allow to cool, dissolve in 20 ml of *water*, make the solution just acidic to *litmus paper*, filter and add 0.05 ml of *ferric chloride solution*; a violet colour is produced.

C. Mix 20 mg with 20 mg of *resorcinol*, add 0.5 ml of *sulphuric acid* and heat over a small flame until a dark green colour is produced; allow to cool and add 10 ml of *water* and an excess of *2 M sodium hydroxide*; a fluorescent green liquid is produced.

D. To 5 ml of a 10 per cent w/v solution add 3 ml of *2 M hydrochloric acid*; a white precipitate is produced. The precipitate, after washing with *water* and drying at 105° melts at 226° to 230° (2.4.21).

E. 0.5 ml of a 10 per cent w/v solution gives reaction (B) of sodium salts (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. Dissolve 5.0 g in sufficient *carbon dioxide-free water* to produce 50 ml (solution A). To 10 ml of solution A add 0.1 ml of *phenolphthalein solution*. The solution is neither pink nor red. Add 0.05 ml of *0.1 M sodium hydroxide*. The solution becomes pink or red.

Related substances. Determine by gas chromatography (2.4.13).

Internal standard solution. Dissolve 25 mg of *caffeine IPRS* in *dichloromethane* and dilute to 100 ml with the same solvent.

Test solution. Suspend 10.0 g of the substance under examination in 50 ml of *water*. If necessary, adjust the pH of the solution to 7.8 with *1 M sodium hydroxide* or *1 M hydrochloric acid* and dilute to 50 ml with *water*. Shake the solution with 4 quantities, each of 50 ml, of *dichloromethane*. Combine the lower layers, dry over *anhydrous sodium sulphate* and filter. Wash the filter and the sodium sulphate with 10 ml of *dichloromethane*. Combine the solution and the washings and evaporate almost to dryness in a water-bath at a temperature not exceeding 40°. With a small quantity of

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dichloromethane transfer quantitatively the residue into a suitable 10-ml tube, evaporate to dryness in a current of *nitrogen* and dissolve the residue in 1.0 ml of the internal standard solution.

Blank solution. Evaporate 200 ml of *dichloromethane* to dryness in a water-bath at a temperature not exceeding 40°. Dissolve the residue in 1 ml of *dichloromethane*.

Reference solution. Dissolve 20 mg of *o-toluenesulphonamide* and 20 mg of *p-toluene sulphonamide* in *dichloromethane* and dilute to 100 ml with the same solvent. Dilute 5 ml of the solution to 50 ml with *dichloromethane*. Evaporate 5 ml of the final solution to dryness in a current of *nitrogen*. Dissolve the residue in 1 ml of the internal standard solution.

Chromatographic system
- a fused silica column 10 m x 0.53 mm packed with polymethylphenylsiloxane (film thickness 2 µm),
- temperature:
  column:180°,
  inlet port and detector: 250°,
  flame ionization detector,
- flow rate: 10 ml per minute, using nitrogen as the carrier gas,
- split ratio: 1:2.

Inject 1 µl of each solution. The order of elution is *o*-toluenesulphonamide, *p*-toluenesulphonamide, caffeine.

The test is not valid unless the resolution between the peaks due to *o*-toluenesulphonamide and *p*-toluenesulphonamide in the chromatogram obtained with the reference solution is not less than 1.5 and the chromatogram obtained with the blank solution does not show any peak with the same retention times as the internal standard, *o*-toluenesulphonamide and *p*-toluenesulphonamide.

In the chromatogram obtained with the test solution the ratio of the area of the peak due to *o*-toluenesulphonamide to that of the internal standard is not more than the corresponding ratio in the chromatogram obtained with the reference solution (10 ppm).

In the chromatogram obtained with the test solution the ratio of the area of the peak due to *p*-toluenesulphonamide to that of the internal standard is not more than the corresponding ratio in the chromatogram obtained with the reference solution (10 ppm).

Arsenic (2.3.10). Mix 5.0 g with 3 g of *anhydrous sodium carbonate*, add 10 ml of *bromine solution* and mix thoroughly. Evaporate to dryness on a water-bath, gently ignite and add to the cooled residue a mixture of 16 ml of *brominated hydrochloric acid AsT* and 5 ml of *bromine solution*. Add 40 ml of water and boil gently, adding sufficient *bromine solution* from time to time to maintain a slight excess. Filter and remove the excess of bromine with a sufficient quantity of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Readily carbonisable substances. Dissolve 0.2 g in 5 ml of *sulphuric acid* and maintain at about 50° for 10 minutes. The solution is not more intensely coloured than reference solution BS6 (2.4.1).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

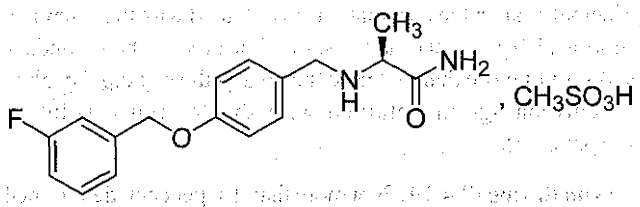
Water (2.3.43). Not more than 15.0 per cent, determined on 0.2 g.

Assay. Weigh 0.15 g, dissolve in 50 ml of *anhydrous glacial acetic acid*, with slight heating if necessary. Titrate with *0.1M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02052 g of $C_7H_4NNaO_3S$.

Storage. Store protected from moisture.

Safinamide Methane Sulphonate



$C_{17}H_{19}FN_2O_2CH_4SO_3$ Mol Wt. 398.5

Safinamide Methane Sulphonate is (*S*)-2-(4-(3-Fluorobenzyloxy)benzylamino)propanamide methane sulphonate.

Safinamide Methane Sulphonate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{17}H_{19}FN_2O_2CH_4SO_3$, calculated on dried basis.

Category. Antiparkinsonian.

Description. A white to off-white powder.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

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Test solution. Dissolve 50 mg of the substance under examination in mobile phase and diluted to 50.0 ml with mobile phase.

Reference solution. A 0.01 per cent w/v solution of *safinamide methane sulphonate IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: a mixture of 45 volumes of buffer solution prepared to dissolve 6.8 g of *potassium dihydrogen orthophosphate* and 2.22 g of *sodium octanesulfonate* in 1000 ml of *water*, 10 volumes of *acetonitrile* and 45 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with test solution the area of any secondary peak is not more than 0.1 time the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent) and the sum of the area all the secondary peaks is not more than 0.2 time the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent).

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in mobile phase and dilute to 50.0 ml with mobile phase.

Reference solution. A 0.1 per cent w/v solution of *safinamide methane sulphonate IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: a mixture of 45 volumes of buffer solution prepared to dissolve 6.8 g of *potassium dihydrogen orthophosphate* and 2.22 g of *sodium octanesulfonate* in 1000 ml of *water*, 10 volumes of *acetonitrile* and 45 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

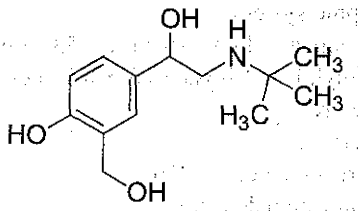
Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and tailing factor is not more than 2.0. and relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{19}FN_2O_2CH_4SO_3$.

Salbutamol

Albuterol



$C_{13}H_{21}NO_3$ Mol. Wt: 239.3

Salbutamol is (*RS*)-1-(4-hydroxy-3-hydroxymethylphenyl)-2-(*tert*-butylamino)ethanol.

Salbutamol contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{13}H_{21}NO_3$, calculated on the dried basis.

Category. Beta-adrenoceptor agonist.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *salbutamol IPRS* or with the reference spectrum of salbutamol.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.008 per cent w/v solution in *0.1 M hydrochloric acid* shows an absorption maximum only at about 276 nm; absorbance at about 276 nm, about 0.53 to 0.60.

C. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to salbutamol that in the chromatogram obtained with the reference solution.

D. Dissolve 10 mg in 50 ml of a 2 per cent w/v solution of *borax*, add 1 ml of a 3 per cent w/v solution of *4-aminophenazone*, 10 ml of a 2 per cent w/v solution of *potassium ferricyanide* and 10 ml of *chloroform*, shake and allow to separate; an orange-red colour is produced in the chloroform layer.

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Tests

Appearance of solution. A 2.0 per cent w/v solution in *methanol* is clear (2.4.1), and not more intensely coloured than reference solution BYS5 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in 50.0 ml of the mobile phase.

Reference solution. A solution containing 0.03 per cent w/v each of *salbutamol IPRS* and *(1RS)-2-[(1,1-dimethylethyl) amino]-1-(4- hydroxyphenyl)ethanol IPRS (salbutamol impurity A IPRS)* in the mobile phase. Dilute 2.0 ml of the solution to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 3.9 mm, packed with endcapped octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 22 volumes of *acetonitrile* and 78 volumes of a solution containing 0.29 per cent w/v of *sodium heptanesulphonate* and 0.25 per cent w/v of *potassium dihydrogen phosphate,* adjusted to pH 3.7 with *orthophosphoric acid,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

The relative retention time with reference to salbutamol for salbutamol impurity A is about 1.3.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to salbutamol and salbutamol impurity A is not less than 3.0.

Inject the reference solution and the test solution. Run the chromatogram 25 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.3 per cent) and the sum of areas of all the secondary peaks is not more than 3.3 times the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore any peak with an area less than 0.17 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Boron. To 50 mg add 5 ml of a solution containing 1.3 per cent w/v of *anhydrous sodium carbonate* and 1.7 per cent w/v of *potassium carbonate,* evaporate to dryness on a water-bath and dry at 120°. Ignite the residue rapidly until the organic matter has been destroyed, allow to cool, add 0.5 ml of *water* and 3.0 ml of a freshly prepared 0.125 per cent w/v solution of *curcumin* in *glacial acetic acid.* Evaporate to dryness and allow to cool. Add 3 ml of a mixture prepared by adding 5 ml of *sulphuric acid,* slowly and with stirring, to 5 ml of *glacial acetic acid.* Mix and allow to stand for 30 minutes. Add sufficient *ethanol (95 per cent)* to produce 100.0 ml, filter and measure the absorbance of the filtrate at 555 nm (2.4.7). Prepare a reference solution in the following manner. Dissolve 0.572 g of *boric acid* in 1000.0 ml of *water.* Dilute 1.0 ml to 100.0 ml with water. To 2.5 ml of the solution add 5 ml of a solution containing 1.3 per cent w/v of *anhydrous sodium carbonate* and 1.7 per cent w/v of *potassium carbonate* and treat this mixture in the same manner as described above beginning at the words "Evaporate to dryness...". The absorbance of the solution prepared from the substance under examination is not more than that of the reference solution (50 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh 0.2 g, dissolve in 30 ml of *anhydrous glacial acetic acid.* Titrate with *0.1 M perchloric acid,* determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02393 g of $C_{13}H_{21}NO_3$.

Storage. Store protected from light.

Salbutamol Inhalation

Salbutamol Inhalation Aerosol; Albuterol Inhalation Aerosol

Salbutamol Inhalation is a suspension of microfine Salbutamol or Salbutamol Sulphate in a suitable liquid in a suitable pressurised container. It may contain suitable pharmaceutical aids such as surfactants, stabilising agents etc.

Salbutamol Inhalation delivers not less than 80.0 per cent and not more than 120.0 per cent of the stated amount of salbutamol, $C_{13}H_{21}NO_3$, per inhalation, by actuation of the valve.

Usual strength. 100 µg in each metered-dose.

Identification

A. Discharge the container a sufficient number of times into a mortar to obtain about 2 mg of salbutamol, grind the residue thoroughly with 0.1 g of potassium bromide, add a further 0.2 g of potassium bromide and mix thoroughly.

On the resultant dispersion determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *salbutamol IPRS* or *salbutamol sulphate IPRS* or with the reference spectrum of salbutamol or salbutamol sulphate.

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B. In the test for Related substances, the principal spot in the chromatogram obtained with reference solution (a) corresponds to that in the chromatogram obtained with reference solution (b).

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 50 volumes of *ethyl acetate*, 30 volumes of *2-propanol*, 16 volumes of *water* and 4 volumes of *strong ammonia solution*.

Test solution. Discharge the inhaler a sufficient number of times into a small, dry beaker to obtain 10 mg of Salbutamol and dissolve the residue in 0.5 ml of *methanol*.

Reference solution (a). Dilute 1.0 volume of test solution (a) to 200 volumes with *methanol*.

Reference solution (b). A 0.010 per cent w/v solution of *salbutamol IPRS* in *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air until the odour of the solvent is no longer detectable, place it for a few minutes in an atmosphere saturated with *diethylamine* and spray with *diazotised sulphanilic acid solution*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a). Ignore any spot with an R_f value higher than 0.85.

Other tests. Comply with the tests stated under Inhalation Preparations (Pressurised metered-dose Preparations).

Follow the procedure described under Assay wherever the amount of active substance is to be determined in any test.

Assay. Carry out the test for Content of active ingredient delivered per actuation stated under Inhalation Preparations (Pressurised metered-dose Preparations).

Use 30 ml of *ethanol* for preparations containing salbutamol and 30 ml of a mixture of equal volumes of *ethanol* and *water* for preparations containing salbutamol sulphate and dilute with *ethanol* to produce a solution containing 10 µg of salbutamol per ml. To 20 ml of the solution in a separating funnel add 180 ml of *water* and in the following order, 4 ml of a 5 per cent w/v solution of *sodium bicarbonate*, 4 ml of *N,N-dimethyl-4-phenylenediamine sulphate solution* and 4 ml of a freshly prepared 8 per cent w/v solution of *potassium ferricyanide*. Mix, allow to stand for 15 minutes in subdued light and extract with two quantities, each of 10 ml, of *chloroform*. Filter the extracts through a plug of *cotton wool*, dilute to 25 ml with *chloroform* and measure the absorbance of the resulting solution at 605 nm (2.4.7). Calculate the content of $C_{13}H_{21}NO_3$ in the solution from the absorbance obtained by repeating the operation using a suitable quantity of a 0.001 per cent w/v solution of *salbutamol IPRS* in *ethanol*.

Calculate the amount of $C_{13}H_{21}NO_3$ delivered per actuation of the valve.

Determine the content of active ingredient a second and third time by repeating the procedure on the middle ten and on the last ten successive combined actuations of the valve. For each of the three determinations the average content of $C_{13}H_{21}NO_3$ delivered per actuation of the valve meets the requirements.

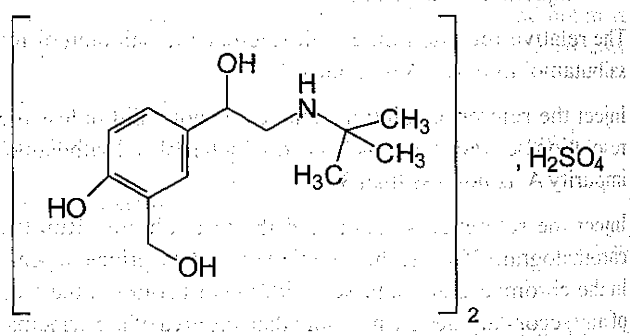
Storage. Store protected from light and moisture at a temperature not exceeding 30°.

Labelling. The label states whether the preparation contains Salbutamol or Salbutamol Sulphate.

When the active ingredient is Salbutamol Sulphate, the quantity is stated in terms of the equivalent amount of salbutamol.

Salbutamol Sulphate

Albuterol Sulphate



$(C_{13}H_{21}NO_3)_2,H_2SO_4$ Mol. Wt. 576.7

Salbutamol Sulphate is (*RS*)-1-(4-hydroxy-3-hydroxymethylphenyl)-2-(*tert*-butylamino)ethanol sulphate.

Salbutamol Sulphate contains not less than 98.0 per cent and not more than 101.0 per cent of $(C_{13}H_{21}NO_3)_2,H_2SO_4$, calculated on the dried basis.

Category. Beta-adrenoceptor agonist.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests B and C may be omitted if tests A, D and E are carried out.

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A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *salbutamol sulphate IPRS* or with the reference spectrum of salbutamol sulphate.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.008 per cent w/v solution in *0.1 M hydrochloric acid* shows an absorption maximum at about 276 nm is 0.44 to 0.51.

C. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to salbutamol that in the chromatogram obtained with the reference solution.

D. Dissolve 10 mg in 50 ml of a 2 per cent w/v solution of *borax*, add 1 ml of a 3 per cent w/v solution of *4-aminophenazone*, 10 ml of a 2 per cent w/v solution of *potassium ferricyanide* and 10 ml of *chloroform*, shake and allow to separate; an orange-red colour is produced in the chloroform layer.

E. It gives reaction (A) of sulphates (2.3.1).

Tests

Appearance of solution. A 1.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution BYS6 (2.4.1).

Acidity or alkalinity. To 10 ml of a 1.0 per cent w/v solution in *carbon dioxide-free water* add 0.15 ml of *methyl red solution* and 0.2 ml of *0.01 M sodium hydroxide*. The solution is yellow and not more than 0.4 ml of *0.01 M hydrochloric acid* is required to change the colour of the solution to red.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in 50.0 ml of the mobile phase.

Reference solution. A solution containing 0.03 per cent w/v each of *salbutamol IPRS* and *(1RS)-2-[(1,1-dimethylethyl) amino]-1-(4- hydroxyphenyl)ethanol IPRS (salbutamol impurity A IPRS)* in the mobile phase. Dilute 2.0 ml of the solution to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 3.9 mm, packed with endcapped octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 22 volumes of *acetonitrile* and 78 volumes of a solution containing 0.29 per cent w/v of *sodium heptanesulphonate* and 0.25 per cent w/v of *potassium dihydrogen phosphate*, adjusted to pH 3.7 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

The relative retention time with reference to salbutamol for salbutamol impurity A is about 1.3, for 5-[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-hydroxyethyl]-2-hydroxybenzaldehyde (salbutamol impurity D) is about 2.7 and for (1*RS*)-2-[(1,1-dimethylethyl)amino]-1-[4-(benzyloxy)-3- (hydroxymethyl) phenyl]ethanol (salbutamol impurity I) is about 23.2. The relative response factors to that of salbutamol impurity D is 2.63 and salbutamol impurity I is 1.47.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to salbutamol and salbutamol impurity A is not less than 3.0.

Inject the reference solution and the test solution. Run the chromatogram 25 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.3 per cent) and the sum of areas of all the secondary peaks is not more than 3.3 times the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore any peak with an area less than 0.17 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Boron. To 50 mg add 5 ml of a solution containing 1.3 per cent w/v of *anhydrous sodium carbonate* and 1.7 per cent w/v of *potassium carbonate*, evaporate to dryness on a water-bath and dry at 120°. Ignite the residue rapidly until the organic matter has been destroyed, allow to cool, add 0.5 ml of *water* and 3.0 ml of a freshly prepared 0.125 per cent w/v solution of *curcumin* in *glacial acetic acid*. Evaporate to dryness and allow to cool. Add 3 ml of a mixture prepared by adding 5 ml of *sulphuric acid*, slowly and with stirring, to 5 ml of *glacial acetic acid*. Mix and allow to stand for 30 minutes. Add sufficient *ethanol (95 per cent)* to produce 100.0 ml, filter and measure the absorbance of the filtrate at 555 nm (2.4.7). Prepare a reference solution in the following manner. Dissolve 0.572 g of *boric acid* in 1000.0 ml of *water*. Dilute 1.0 ml to 100.0 ml with *water*. To 2.5 ml of the solution add 5 ml of a solution containing 1.3 per cent w/v of *anhydrous sodium carbonate* and 1.7 per cent w/v of *potassium carbonate* and treat this mixture in the same manner as described above beginning at the words " Evaporate to dryness.....". The absorbance of the solution prepared from the substance under examination is not more than that of the reference solution (50 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh 0.4 g, dissolve in 5 ml of *anhydrous formic acid*, add 35 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

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1 ml of *0.1 M perchloric acid* is equivalent to 0.05767 g of $(C_{13}H_{21}NO_3)_2, H_2SO_4$.

Storage. Store protected from light.

Salbutamol Injection

Albuterol Sulphate Injection; Salbutamol Sulphate Injection

Salbutamol Injection is a sterile solution of Salbutamol Sulphate in Water for Injections containing suitable stabilising agents.

Salbutamol Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of salbutamol, $C_{13}H_{21}NO_3$.

Usual strengths. The equivalent of 250 µg of salbutamol per ml; the equivalent of 500 µg of salbutamol per ml; the equivalent of 5 mg of salbutamol in 5 ml (for intravenous infusion). (1 mg of Salbutamol Sulphate is approximately equivalent to 830 µg of salbutamol).

Identification

A. Dilute a volume with sufficient *0.1 M hydrochloric acid* to produce a solution containing 0.008 per cent w/v of salbutamol. When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows an absorption maximum only at about 276 nm.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*

Mobile phase. A mixture of 50 volumes of *ethyl acetate*, 30 volumes of *2-propanol*, 16 volumes of *water* and 4 volumes of *strong ammonia solution*.

Test solution. Evaporate a suitable volume of the injection to dryness using a rotary evaporator, wash the residue with four quantities, each of 5 ml, of *ethanol*, filter, evaporate the filtrate to dryness and dissolve the residue in sufficient *water* to produce a solution containing the equivalent of 0.1 per cent w/v of salbutamol.

Reference solution. A 0.12 per cent w/v solution of *salbutamol sulphate IPRS* in *water*.

Apply to the plate 2 µl of each solution. After development, dry the plate in air until the odour of solvent is no longer detectable, place for a few minutes in an atmosphere saturated with *diethylamine* and spray with *diazotised nitroaniline solution*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

C. Dilute a volume containing 0.5 mg of salbutamol to 50 ml with water, add 1 ml of *dilute ammonia solution*, 1 ml of a 3 per cent w/v solution of *4-aminophenazone*, 10 ml of a 2 per cent w/v solution of *potassium ferricyanide* and 10 ml of *chloroform*. Shake and allow to separate; an orange-red colour is produced in the chloroform layer.

D. A volume containing 1 mg of salbutamol gives the reactions of sulphates (2.3.1).

Tests

pH (2.4.24). 3.4 to 5.0.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of injection containing about 5 mg of Salbutamol in 100.0 ml of the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.0004 per cent w/v of *(1RS)-2-[(1,1-dimethylethyl)amino]-1-(4- hydroxyphenyl)ethanol IPRS (salbutamol impurity A IPRS)* and 0.0005 per cent w/v of *salbutamol sulphate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 3.9 mm, packed with endcapped octylsilane bonded to porous silica (5 µm) (Such as Waters Symmetry C8),
- mobile phase: a mixture of 22 volumes of *acetonitrile* and 78 volumes of a solution containing 0.29 per cent w/v of *sodium heptanesulphonate* and 0.25 per cent w/v of *potassium dihydrogen phosphate*, adjusted to pH 3.7 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 3.0.

Inject reference solution (a) and the test solution. Run the chromatogram 25 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

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Assay. Dilute a volume, measured, containing about 0.15 mg of salbutamol with sufficient *water* to produce 80 ml, add 4 ml of a 5 per cent w/v solution of *sodium bicarbonate*, 4 ml of *N,N-dimethyl-4-phenylenediamine sulphate solution* and 4 ml of a freshly prepared 8 per cent w/v solution of *potassium ferricyanide*. Mix, allow to stand for 15 minutes, protected from light. Extract with two quantities, each of 10 ml, of *chloroform*. Filter the extracts through a plug of cotton wool and dilute to 25.0 ml with *chloroform*. Measure the absorbance of the resulting solution at 605 nm (2.4.7).

Calculate the content of $C_{13}H_{21}NO_3$ from the absorbance obtained by repeating the operation using 10.0 ml of a 0.0018 per cent w/v solution of *salbutamol sulphate*.

Storage. Store protected from light, in single dose containers in which the air has been displaced by nitrogen or other suitable inert gas.

Labelling. The label states the strength in terms of the equivalent amount of salbutamol in a suitable dose-volume.

Salbutamol Syrup

Albuterol Sulphate Syrup; Salbutamol Sulphate Syrup

Salbutamol Syrup contains Salbutamol Sulphate equivalent to not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of salbutamol, $C_{13}H_{21}NO_3$.

Usual strength. The equivalent of 2 mg of salbutamol in 5 ml. (1 mg of Salbutamol Sulphate is approximately equivalent to 830 µg of salbutamol).

Identification

A. To 5 ml add 50 ml of a 2 per cent w/v solution of *borax*, 1 ml of a 3 per cent w/v solution of *4-aminophenazone*, 10 ml of a 2 per cent w/v solution of *potassium ferricyanide* and 10 ml of *chloroform*. Shake and allow to separate; an orange-red colour is produced in the chloroform layer.

B. To 5 ml add sufficient *1 M sodium hydroxide* to make the solution alkaline, add 1 ml of *alkaline borate buffer pH 9.2* and 1 ml of a 0.04 per cent w/v solution of *2,6-dichloroquinone chlorimide* in *ethanol (95 per cent)*; a blue colour develops.

Tests

pH (2.4.24): 3.4 to 4.5.

2-*tert*-butylamino-1-(4-hydroxy-3-methylphenyl)ethanol. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of the oral solution containing 5 mg of salbutamol in the mobile phase and dilute to 50 ml with the mobile phase, filter.

Reference solution. A 0.00005 per cent w/v solution of *2-tert-butylamino-1-(4-hydroxy-3-methylphenyl)ethanol sulphate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octylsilane bonded to porous silica (5 µm) (Such as Hypersil BDS C8),
- mobile phase: A. 1.5 volumes of *propan-2-ol* and 98.5 volumes of *0.1 M ammonium acetate*, adjusted to pH 4.5 with *glacial acetic acid*,
  B. *propan-2-ol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	100	0
20	86	14
30	86	14
35	100	0
40	100	0

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to 2-*tert*-butylamino-1-(4-hydroxy-3-methylphenyl)ethanol is not more than the area of the peak in the chromatogram obtained with the reference solution (0.5 per cent).

Other tests. Comply with the tests stated under Oral Liquids.

Assay. To a measured volume containing 4 mg of salbutamol add 25 ml of *0.05 M sulphuric acid* and extract with two quantities, each of 50 ml, of *ether*. Collect the aqueous layers into a 250-ml volumetric flask and combine the ether extracts. Wash the combined ether extracts with 50 ml of *water* and add the aqueous layer to the solution in the 250 ml volumetric flask. Discard the ether extracts and dilute the aqueous solution with sufficient *water* to produce 250.0 ml. To 10.0 ml of the solution add sufficient *water* to produce 80 ml and add 4 ml of a 5 per cent w/v solution of *sodium bicarbonate*, 4 ml of *N,N-dimethyl-4-phenylenediamine sulphate solution* and 4 ml of a freshly prepared 8 per cent w/v solution of *potassium ferricyanide*. Mix, allow to stand for 15 minutes, protected from light. Extract with two quantities, each of 10 ml, of *chloroform*. Filter the extracts through a plug of cotton wool and dilute to 25.0 ml with *chloroform*. Measure the absorbance of the resulting solution at 605 nm (2.4.7).

Calculate the content of $C_{13}H_{21}NO_3$ from the absorbance obtained by repeating the operation using 10.0 ml of a 0.0018 per cent w/v solution of *salbutamol sulphate*.

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Storage. Store protected from light.

Labelling. The label states the strength in terms of the equivalent amount of salbutamol in a suitable dose-volume.

Salbutamol Tablets

Albuterol Sulphate Tablets; Salbutamol Sulphate Tablets

Salbutamol Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of salbutamol, $C_{13}H_{21}NO_3$.

Usual strengths. The equivalent of 2 mg; 4 mg of salbutamol. (1 mg of Salbutamol Sulphate is approximately equivalent to 830 µg of salbutamol).

Identification

A. Carry out the method described under Related substances applying separately to the plate 2 µl of each of the following solutions. For the test solution shake a quantity of the powdered tablets containing 10 mg of salbutamol with 10 ml of *methanol (80 per cent)* and filter. The reference solution contains 0.12 per cent w/v of *salbutamol sulphate IPRS*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. Shake a quantity of the powdered tablets containing 8 mg of salbutamol with 50 ml of a 2 per cent w/v solution of *borax*, add 1 ml of a 3 per cent w/v solution of *4-aminophenazone*, 10 ml of a 2 per cent w/v solution of *potassium ferricyanide* and 10 ml of *chloroform*. Shake and allow to separate; an orange-red colour is produced in the chloroform layer.

C. Shake a quantity of the powdered tablets containing 4 mg of salbutamol with 10 ml of *water* and filter; the filtrate gives the reactions of sulphates (2.3.1).

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 50 volumes of *ethyl acetate*, 30 volumes of *2-propanol*, 16 volumes of *water* and 4 volumes of *strong ammonia solution*.

Test solution. Shake a quantity of the powdered tablets containing 10 mg of salbutamol with 1 ml of *water* for 15 minutes, centrifuge and use the supernatant liquid.

Reference solution. A 0.006 per cent w/v solution of *salbutamol sulphate IPRS* in *water*.

Apply to the plate 20 µl of each solution. After development, dry the plate in air until the odour of the solvent is no longer detectable, place it for a few minutes in an atmosphere saturated with *diethylamine* and spray with *diazotised sulphanilic acid solution*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution. Ignore any pink spot near the line of application.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14).

Test solution. Add 50 ml of *water* to one tablet, shake for 1 hour, add sufficient *water* to produce 100.0 ml, mix and centrifuge. Dilute further with *water*, if necessary, to produce a solution containing 0.002 per cent w/v of salbutamol.

Reference solution (a). A 0.0024 per cent w/v of *salbutamol sulphate IPRS* in *water*.

Reference solution (b). A 0.048 per cent w/v of *2-tert-butylamino-1-(4-hydroxy-3-methylphenyl) ethanol sulphate IPRS* and 0.048 per cent w/v of *salbutamol sulphate IPRS* in *methanol (10 per cent)*.

Chromatographic system

- a stainless steel column 20 cm x 5 mm, packed with spherical particles of silica, 5 µm in diameter, the surface of which has been modified with chemically-bonded nitrile groups (Such as Spherisorb CN),
- mobile phase: a mixture of 65 volumes of *water*, 30 volumes of *0.05 M ammonium acetate* and 5 volumes of *2-propanol*, adjusted to pH 4.5 with *glacial acetic acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 276 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless resolution between two principal peaks in the chromatogram obtained with reference solution (b) is at least 1.5.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{13}H_{21}NO_3$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 5 mg of salbutamol, transfer to a 250-ml volumetric flask, add 60 ml of *water* and shake for 60 minutes, dilute to volume with *water*. Mix and centrifuge 10 ml of the solution and use the supernatant liquid.

Reference solution (a). A 0.0024 per cent w/v of *salbutamol sulphate IPRS* in *water*.

Reference solution (b). A solution containing 0.0024 per cent w/v of *2-tert-butylamino-1-(4-hydroxy-3-methylphenyl)*

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ethanol sulphate IPRS and 0.0024 per cent w/v of *salbutamol sulphate IPRS in methanol (10 per cent)*.

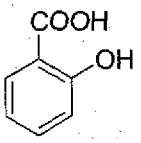
Use chromatographic procedure described under Uniformity of content. The test is not valid unless the resolution between the two principal peaks in the chromatogram obtained with reference solution (b) is not less than 1.5.

Calculate the content of $C_{13}H_{21}NO_3$ in the tablets.

Storage. Store protected from light.

Labelling. The label states the strength in terms of the equivalent amount of salbutamol.

Salicylic Acid



$C_7H_6O_3$ Mol. Wt. 138.1

Salicylic Acid is 2-hydroxybenzoic acid.

Salicylic Acid contains not less than 99.0 per cent and not more than 100.5 per cent of $C_7H_6O_3$, calculated on the dried basis.

Category. Keratolytic.

Description. White or colourless, acicular crystals or a white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Test B may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *salicylic acid IPRS* or with the reference spectrum of salicylic acid.

B. Dissolve about 30 mg in 5 ml of *0.05 M sodium hydroxide*, neutralise if necessary and dilute to 20 ml with *water*. 1 ml of the solution gives reaction (A) of salicylates (2.3.1).

C. Melting range. 158° to 161° (2.4.21).

Tests

Appearance of solution. Dissolve 1.0 g in 10 ml of *ethanol (95 per cent)*. The resulting solution is clear, and colourless (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.5 g of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). A 0.01 per cent w/v solution of *salicylic acid impurity C* in the mobile phase.

Reference solution (b). A 0.025 per cent w/v solution of *salicylic acid impurity B IPRS* in the mobile phase.

Reference solution (c). A 0.05 per cent w/v solution of *salicylic acid impurity A* in the mobile phase.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (e). Dilute a mixture of 1.0 ml of each of reference solution (a), (b) and (c) to 10.0 ml with the mobile phase.

Reference solution (f). Dilute a mixture of 0.1 ml of each of reference solution (a), (b) and (c) to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 1 volume of *glacial acetic acid*, 40 volumes of *methanol* and 60 volumes of *water*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 10 µl.

Name	Relative retention time
Salicylic acid impurity A[1]	0.7
Salicylic acid impurity B[2]	0.9
Salicylic acid impurity C[3]	1.0

[1]4-hydroxybenzoic acid,

[2]4-hydroxyisophthalic acid,

[3]Phenol.

Inject reference solutions (d) and (e). The test is not valid unless the 3rd peak in the chromatogram corresponds to the peak due to phenol in the chromatogram obtained with reference solution (d) and the resolution between the peaks due to impurities B and C is not less than 1.0 in the chromatogram obtained with reference solution (e).

Inject reference solution (f) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to salicylic acid impurity A is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.1 per cent). The area of any peak corresponding to salicylic acid impurity B is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.05 per cent). The area of any peak corresponding to salicylic acid

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impurity C is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.02 per cent), the area of any other secondary peak is not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (f) (0.05 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the peak due to impurity A in the chromatogram obtained with reference solution (f) (0.2 per cent). Ignore any peak with an area less than 0.01 times the area of the principal peak in the chromatogram obtained with reference solution (f).

Heavy metals (2.3.13). Dissolve 2.0 g in 15 ml of *ethanol (95 per cent)* and add 5 ml of *water*. 12 ml of the resulting solution complies with the limit test for heavy metals, Method D (20 ppm). Use *lead standard solution (100 ppm Pb)* diluted with a mixture of 3 volumes of *ethanol (95 per cent)* and 1 volume of *water* to contain 2 µg of Pb per ml to prepare the standard.

Iron (2.3.14). Boil 12.0 g with 14 ml of *dilute ammonia solution* and 35 ml of *water*. Cool and adjust the pH 5.0 to 6.0 by the dropwise addition of *dilute ammonia solution* or *dilute sulphuric acid* and dilute to 50 ml with *water*, if necessary. Any pink colour produced is not more intense than that obtained by boiling 2.0 g with 1 ml of *iron standard solution (20 ppm Fe)*, 2 ml of *dilute ammonia solution* and 45 ml of *water*, adjusting the pH 5.0 to 6.0 and diluting to 50 ml with *water* (2 ppm).

Chlorides (2.3.12). Dissolve 5.0 g in 50 ml of boiling *distilled water*, cool and filter (solution A). 20 ml of solution A complies with the limit test for chlorides (125 ppm).

Sulphates (2.3.17). 7.5 ml of solution A complies with the limit test for sulphates (200 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent, determined on 2.0 g.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying over *phosphorus pentoxide* in a desiccator.

Assay. Weigh 0.3 g, dissolve in 50 ml of *ethanol (95 per cent)*, add 20 ml of *water* and titrate with *0.1 M sodium hydroxide*, using *phenol red solution* as indicator, until a reddish violet colour is obtained.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01381 g of $C_7H_6O_3$.

Storage. Store protected from light.

Salicylic Acid Ointment

Salicylic Acid Ointment contains Salicylic Acid in a suitable water-emulsifying basis.

Salicylic Acid Ointment contains not less than 95.0 per cent and not more than 105.0 per cent w/w of the stated amount of salicylic acid, $C_7H_6O_3$.

Usual strengths. 2.0 per cent w/w; 6 per cent w/w; 12 per cent w/w.

Identification

Shake 1 g of ointment with 10 ml of *water*. Filter and add to the filtrate *ferric chloride solution*. An intense reddish violet colour is produced which persists on addition of *6 M acetic acid*. Further, addition of *2 M hydrochloric acid*, the colour disappears and a white, crystalline precipitate is produced.

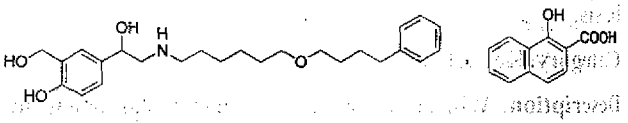
Tests

Other tests. Comply with the test stated under Ointments.

Assay. Weigh 10 g and dissolve in a mixture of 20 ml of *ethanol (95 per cent)* and 20 ml *ether* and titrate with *0.1 M sodium hydroxide* using *phenol red solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01381 g of $C_7H_6O_3$.

Salmeterol Xinafoate



$C_{25}H_{37}NO_4, C_{11}H_8O_3$ Mol. Wt. 603.7

Salmeterol Xinafoate is (*RS*)-4-hydroxy-α^1-[[[6-(4-phenylbutoxy)hexyl]amino]methyl]-1,3-benzenedimethanol 1-hydroxy-2-naphthoate.

Salmeterol Xinafoate contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{36}H_{45}NO_7$, calculated on the anhydrous basis.

Category. Bronchodilator.

Description. A white to off-white powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *salmeterol xinafoate IPRS* or with the reference spectrum of salmeterol xinafoate.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

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NOTE—Protect the solutions from light.

Solvent mixture. 50 volumes of *acetonitrile* and 50 volumes of *water*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 24 volumes of a 0.77 per cent w/v solution of *ammonium acetate* and 24 volumes of a 2.88 per cent w/v solution of *sodium dodecyl sulphate*, adjusted to pH 2.7 with *glacial acetic acid* and 52 volumes of *acetonitrile*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 2 ml per minute,
- spectrophotometer set at 278 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
16	100	0
36	30	70
45	30	70
50	100	0
60	100	0

Name	Relative retention time
Xinafoic Acid	0.2
Salmeterol impurity A[1]	0.3
Salmeterol impurity B[2]	0.5
Salmeterol impurity C[3]	0.7
Salmeterol impurity D[4]	0.8
Salmeterol impurity E[5]	0.9
Salmeterol (Retention time: about 13 minutes)	1.0
Salmeterol impurity F[6]	1.6
Salmeterol impurity G[7]	2.7

[1](1*RS*)-1-[4-hydroxy-3-(hydroxymethyl)phenyl]-2-[(4-phenylbutyl)amino] ethanol,

[2](1*RS*)-1-[4-hydroxy-3-(hydroxymethyl)phenyl]-2-[[6-(2-phenylethoxy)hexyl]amino]ethanol,

[3](1*RS*)-1-[4-hydroxy-3-(hydroxymethyl)phenyl]-2-[[6-(3-phenylpropoxy)hexyl] amino]ethanol,

[4]1-[4-[2-hydroxy-2-[4-hydroxy-3-(hydroxymethyl)phenyl]ethoxy]-3-(hydroxymethyl)phenyl]-2-[[6-(3-phenylbutoxy)hexyl]amino] ethanol,

[5]1-[4-hydroxy-3-(hydroxymethyl)phenyl]-2-[[6-(1-methyl-3-phenylpropoxy) hexyl]amino]ethanol,

[6](1*RS*)-1-(4-hydroxy-3-methylphenyl)-2-[[6-(4-phenylbutoxy)hexyl] amino]ethanol,

[7]1-[4-hydroxy-3-[[[2-hydroxy-2-[4-hydroxy-3-(hydroxymethyl) phenyl]ethyl][6-(4-phenylbutoxy)hexyl]amino]methyl]phenyl]-2-[[6-(4-phenylbutoxy)hexyl]amino]ethanol.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to salmeterol impurity D is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent). The area of any peak corresponding to salmeterol impurities A, F, G each of, is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 9 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.9 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent) and the peak due to xinafoic acid.

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.5 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 12.5 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution. A 0.025 per cent w/v solution of *salmeterol xinafoate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 24 volumes of a 0.77 per cent w/v solution of *ammonium acetate* with 24 volumes of a 2.89 per cent w/v solution of *sodium dodecyl sulphate*, adjusted to pH 2.7 with *glacial acetic acid* and 52 volumes of *acetonitrile*,

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- flow rate: 2 ml per minute,
- spectrophotometer set at 278 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{36}H_{45}NO_7$.

Storage. Store protected from light.

Salmeterol and Fluticasone Propionate Inhalation

Salmeterol and Fluticasone Propionate Inhalation is a suspension of microfine Salmeterol Xinafoate and Fluticasone Propionate in a suitable liquid filled in a suitable pressurised container. It may contain suitable pharmaceutical aids such as surfactants, stabilizing agents.

Salmeterol and Fluticasone Propionate Inhalation delivers not less than 80.0 per cent and not more than 120.0 per cent of the stated amounts of salmeterol, $C_{25}H_{37}NO_4$ and fluticasone propionate, $C_{25}H_{31}F_3O_5S$, per inhalation by actuation of the valve.

Usual strengths. Salmeterol, 25 µg and Fluticasone Propionate, 50 µg; Salmeterol, 25 µg and Fluticasone Propionate, 125 µg; Salmeterol, 25 µg and Fluticasone Propionate, 250 µg; Salmeterol, 50 µg and Fluticasone Propionate, 100 µg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with test solution correspond to the peaks in the chromatogram obtained with reference solution (c).

Tests

Other tests. Comply with the tests stated under Inhalation Preparations (Pressurised Metered-dose Preparations).

Follow the procedure described under Assay with suitable dilution of the reference solution wherever the amount of active substance is to be determined in any test.

Assay. Carry out the test for Content of active ingredient delivered per actuation stated under Inhalation Preparations (Pressurised Metered-dose Preparations).

Determine by liquid chromatography (2.4.14).

Test solution. Prepare using the mobile phase as described under the test for Content of active ingredient delivered per actuation stated under Inhalation Preparations (Pressurised Metered-dose Preparations).

Reference solution (a). A solution containing 0.5 mg of salmeterol per ml prepared by dissolving 15 mg of *salmeterol xinafoate IPRS* in 10 ml *acetonitrile* and adding sufficient of the mobile phase to produce 20 ml.

Reference solution (b). A solution containing 0.5 mg of fluticasone propionate per ml prepared by dissolving 10 mg of *fluticasone propionate IPRS* in 10 ml *acetonitrile* and adding sufficient of the mobile phase to produce 20 ml.

Reference solution (c). Dilute suitable volumes of reference solution (a) and reference solution (b) with the mobile phase to obtain a solution containing 5 µg Salmeterol and 50 µg Fluticasone propionate per ml or quantities as per the label claim.

Chromatographic system
- a stainless steel column 15 cm × 3.9 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 45 volumes of a buffer solution prepared by dissolving 1.15 g *ammonium dihydrogen orthophosphate* to 1000 ml of *water* and adjusted to pH 3.5 with *orthophosphoric acid*, 25 volumes of *acetonitrile* and 30 volumes of *methanol*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 100 µl or 200 µl.

Inject reference solution (c). The test is not valid unless the column efficiency for salmeterol and fluticasone propionate peak is not less than 1000 and 2500 theoretical plates respectively and the tailing factor is not more than 2.0 for each peak and the relative standard deviation for replicate injections for each component is not more than 2.0 per cent.

Inject reference solution (c) and the test solution.

Calculate the contents of $C_{25}H_{37}NO_4$ and $C_{25}H_{31}F_3O_5S$ in the solution and the contents of $C_{25}H_{37}NO_4$ and $C_{25}H_{31}F_3O_5S$ delivered per actuation of the valve.

Determine the contents of the active ingredients a second and third time by repeating the procedure on the middle ten and on the last ten successive combined actuations of the valve. For each of the three determinations the average contents of $C_{25}H_{37}NO_4$ and $C_{25}H_{31}F_3O_5S$ delivered per actuation of the valve meet the requirements.

Storage. Store protected from moisture at a temperature not exceeding 30°.

Labelling. The label states the amounts of active ingredients delivered per inhalation.

Salmeterol and Fluticasone Propionate Powder for Inhalation

Salmeterol and Fluticasone Propionate Powder for Inhalation consists of Fluticasone Propionate and Salmeterol Xinafoate in microfine powder either alone or admixed with Lactose in a pre-metered unit for use in a suitable powder inhaler.

Salmeterol and Fluticasone Propionate Powder for Inhalation contains not less than 90.0 per cent and not more than 125.0 per cent of the stated amounts of salmeterol $C_{25}H_{37}NO_4$ and fluticasone propionate, $C_{25}H_{31}F_3O_5S$ per unit dose.

Usual strengths. Salmeterol, 200 µg and Fluticasone Propionate, 125 µg; Salmeterol, 200 µg and Fluticasone Propionate, 250 µg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with reference solution (c).

Tests

Other tests. Comply with the tests stated under the Inhalation Preparations (Powders for Inhalation).

Follow the procedure described under Assay with suitable dilution of the reference solution wherever the amount of active substance is to be determined in any test.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve a quantity of the mixed contents of 20 capsules in sufficient of the mobile phase to get a solution containing 5 µg per ml of Salmeterol.

Reference solution (a). A solution containing 0.5 mg of salmeterol per ml prepared by dissolving 15 mg of *salmeterol xinafoate IPRS* in 10 ml *acetonitrile* and adding sufficient of the mobile phase to produce 20 ml.

Reference solution (b). A solution containing 0.5 mg of fluticasone propionate per ml prepared by dissolving 10 mg of *fluticasone propionate IPRS* in 10 ml *acetonitrile* and adding sufficient of the mobile phase to produce 20 ml.

Reference solution (c). Dilute suitable volumes of reference solution (a) and reference solution (b) with the mobile phase to obtain a solution containing 5 µg Salmeterol and 50 µg Fluticasone propionate per ml or quantities as per the label claim.

Chromatographic system
- a stainless steel column 15 cm x 3.9 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 45 volumes of a buffer solution prepared by dissolving 1.15 g *ammonium dihydrogen orthophosphate* to 1000 ml of *water* and adjusted to pH 3.5 with *orthophosphoric acid*, 25 volumes of *acetonitrile* and 30 volumes of *methanol*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 100 µl or 200 µl.

Inject reference solution(c). The test is not valid unless the column efficiency determined from the salmeterol and fluticasone propionate peak is not less than 1000 and 2500 theoretical plates respectively, the tailing factor for each of salmeterol and fluticasone propionate peaks is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

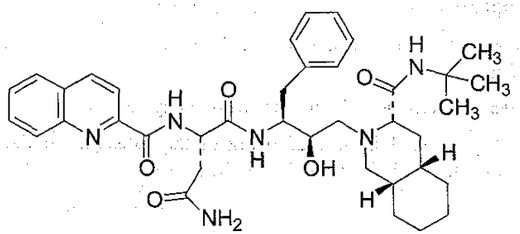
Inject reference solution (c) and the test solution.

Calculate the contents of $C_{25}H_{37}NO_4$ and $C_{25}H_{31}F_3O_5S$ per unit.

Storage. Store protected from moisture, at temperature not exceeding 30°.

Labelling. The label states the quantities of active ingredients per pre-metered unit.

Saquinavir



$C_{38}H_{50}N_6O_5$ Mol. Wt. 670.8

Saquinavir is (*S*)-*N*-[(α*S*)-α-{(1*R*)-2-[(3*S*,4a*S*,8a*S*)-3-(*tert*-butylcarbamoyl)octahydro-2(1*H*)-isoquinolyl]-1-hydroxyethyl}phenethyl]-2-quinaldamidosuccinamide.

Saquinavir contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{38}H_{50}N_6O_5$, calculated on the anhydrous basis.

Category. Antiretroviral.

Description. A white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6), Compare the spectrum with that obtained with *saquinavir IPRS* or with the reference spectrum of saquinavir.

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B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

C. When examined in the range 220 nm to 350 nm (2.4.7), a 0.002 per cent w/v solution in *methanol* shows absorption maxima at about 239 nm and 290 nm.

Tests

Specific optical rotation (2.4.22). −55.0° to −50.0°, determined in a 0.5 per cent w/v solution in *methanol*.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay using the test solution and reference solution (c).

Inject reference solution (c). Calculate the amount of related substance by area normalisation method. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent).

Heavy metals (2.3.13). 2.0 g complies with the limit tests for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 2.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25.0 mg of the substance under examination in 100.0 ml of the mobile phase.

Reference solution (a). A 0.025 per cent w/v solution of *saquinavir IPRS* in the mobile phase.

Reference solution (b). Dissolve suitable quantities of *saquinavir-related compound A IPRS* and *saquinavir IPRS* in the mobile phase to obtain a solution containing 2 µg per ml of saquinavir-related compound A and 0.25 mg per ml of saquinavir.

Reference solution (c). A 0.000025 per cent w/v solution of *saquinavir IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 20 volumes of *methanol*, 50 volumes of *acetonitrile* and 30 volumes of a buffer prepared by dissolving 4 g of *sodium dihydrogen phosphate* in 1000.0 ml of *water* to which 1 ml of *diethylamine* and 1 g of *sodium octane sulphonate* has been added,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

The relative retention times are about 0.89 for saquinavir-related compound A and about 1.0 for saquinavir.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to saquinavir-related compound A and saquinavir is not less than 1.5, the column efficiency determined from the saquinavir peak is not less than 500 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution. Run the chromatogram 3 times the retention time of the principal peak.

Calculate the content of $C_{38}H_{50}N_6O_5$.

Storage. Store protected from light.

Saquinavir Capsules

Saquinavir Capsules contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of saquinavir, $C_{38}H_{50}N_6O_5$.

Usual strength. 200 mg.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *saquinavir mesylate IPRS* or with the reference spectrum of saquinavir mesylate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of citrate buffer prepared by dissolving 5.82 g of *anhydrous dibasic sodium phosphate* and 16.7 g of *citric acid monohydrate* in *water* and dilute to 1000 ml with *water*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtered solution immediately, suitably diluted with the dissolution medium, if necessary, at the maximum at about 240 nm (2.4.7). Calculate the content of $C_{38}H_{50}N_6O_5$ in the medium from the absorbance obtained from a solution of known concentration of *saquinavir mesylate IPRS* in the same medium.

Q. Not less than 70 per cent of the stated amount of $C_{38}H_{50}N_6O_5$.

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Related substances. Determine by liquid chromatography (2.4.14) as described under Assay with the following modifications.

Inject the test solution. The area of any secondary peak is not more than 0.2 per cent and sum of areas of all the secondary peaks is not more than 1.0 per cent, calculated by area normalization.

Water (2.3.43). Not more than 3.0 per cent, determined on 1 g.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of mixed contents of Capsules containing 22 mg of Saquinavir in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution. A 0.025 per cent w/v solution of *saquinavir mesylate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 20°,
- mobile phase: a mixture of 140 volumes of 1.0 per cent v/v solution of *triethylamine* in *water*, adjusted to pH 2.5 with *orthophosphoric acid*, 50 volumes of *tetrahydrofuran* and 10 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

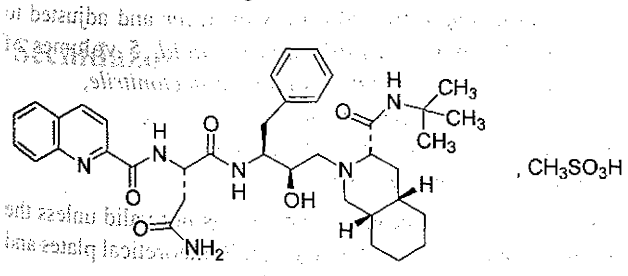
Inject the reference solution. The test is not valid unless the column efficiency is not less than 500 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{38}H_{50}N_6O_5$ in the capsules.

Storage. Store protected from moisture.

Saquinavir Mesylate



, CH_3SO_3H

$C_{38}H_{50}N_6O_5,CH_4O_3S$ Mol. Wt. 767.0

Saquinavir mesylate is (*S*)-*N*-[(a*S*)-a-{(1*R*)-2-[(3*S*,4a*S*,8a*S*)-3-(*tert*-butylcarbamoyl)octahydro-2(1*H*)-isoquinolyl]-1-hydroxyethyl}phenethyl]-2-quinaldamidosuccinamide methanesulphonate.

Saquinavir Mesylate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{38}H_{50}N_6O_5$, CH_4O_3S, calculated on the anhydrous basis.

Category. Antiretroviral.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6), Compare the spectrum with that obtained with *saquinavir mesylate IPRS* or with the reference spectrum of saquinavir mesylate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Specific optical rotation (2.4.22). –69.6° to –66.8°, determined in a 0.5 per cent w/v solution in *methanol* at 436 nm.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay using the test solution and reference solution (c).

Inject reference solution (c). Calculate the amount of related substances by area normalisation method. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Methanesulphonic acid. 11.9 to 13.1 per cent w/w, calculated on the anhydrous basis, determined by the following method. Weigh 0.1 g of the substance under examination, dissolve in 50 ml of *methanol*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentio-metrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.00961 g of CH_3SO_3H.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25.0 mg of the substance under examination in 100.0 ml of the mobile phase.

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Reference solution (a). A 0.025 per cent w/v solution of *saquinavir mesylate IPRS* in the mobile phase.

Reference solution (b). Dissolve suitable quantities of *saquinavir impurity A IPRS* and *saquinavir mesylate IPRS* in the mobile phase to obtain a solution containing 2 µg per ml of saquinavir impurity A and 0.25 mg per ml of saquinavir mesylate.

Reference solution (c). A 0.000025 per cent w/v solution of *saquinavir mesylate IPRS* in the mobile phase.

NOTE — Store the buffer solution protected from light. Make adjustments if necessary for system suitability.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 140 volumes of 1.0 per cent v/v solution of *triethylamine* in *water*, adjusted to pH 2.5 with *orthophosphoric acid*, 50 volumes of *tetrahydrofuran* and 10 volumes of *acetoritrile*.
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

The relative retention times are about 0.89 for saquinavir impurity A and about 1.0 for saquinavir mesylate.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to saquinavir impurity A and saquinavir mesylate is not less than 1.5, the column efficiency determined from the saquinavir mesylate peak is not less than 500 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution. Run the chromatograms 3 times the retention time of the principal peak.

Calculate the content of $C_{38}H_{50}N_6O_5$, CH_4O_3S.

Storage. Store protected from light.

Saquinavir Mesylate Tablets

Saquinavir Mesylate Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of saquinavir $C_{38}H_{50}N_6O_5$.

NOTE —Perform the tests and assay using low-actinic glassware.

Usual strength. 200 mg.

Identification

A. In the Assay, the principal spot in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. When examined in the range 200 nm to 400 nm (2.4.7), 1 ml of a 0.1 per cent w/v solution in *methanol* diluted to 100 ml with *citrate buffer pH 3.0* (see under Dissolution), shows absorption maxima at the same wavelengths as shown by the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *citrate buffer pH 3.0* prepared by dissolving 5.82 g of *anhydrous dibasic sodium phosphate* and 16.7 g of *citiric acid monohydrate* in 1000 ml of *water* and adjusted to pH 3.0 with *orthophosphoric acid*.

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter promptly. Use the filtrate, dilute if necessary, with the dissolution medium. Measure the absorbance (2.4.7) of the resulting solution at the maximum at about 240 nm. Calculate the content of $C_{38}H_{50}N_6O_5$ in the medium from the absorbance obtained from a solution of known concentration of *saquinavir mesylate IPRS*.

Q. Not less than 75 per cent of the stated amount of $C_{38}H_{50}N_6O_5$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets Disperse a quantity of the powder containing 100 mg of saquinavir in 100 ml of the mobile phase and filter.

Reference solution (a). A 0.1 per cent w/v solution of *saquinavir mesylate IPRS* equivalent to *saquinavir* in the mobile phase.

Reference solution (b). Dilute 1 ml of reference solution (a) to 100 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 14 volumes of *triethylamine phosphate solution* prepared by diluting 10 ml of *triethylamine* to 1000 ml with *water* and adjusted to pH 2.5 with *orthophosphoric acid*, 5 volumes of tetrahydrofuran and 1 volume of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 3000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram for 5 times the retention time (about 12 minutes) of the principal peak. In the chromatogram obtained with the

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test solution, the area of any secondary peak is not more than 0.2 times the area of the peak in the chromatogram obtained with the reference solution (b) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than the area of the peak in the chromatogram obtained with the reference solution (b) (1.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Water (2.3.43). Not more than 6.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 100 mg of saquinavir, dissolve in 100.0 ml of mobile phase and filter. Dilute 5.0 ml of the filtrate to 20.0 ml with the mobile phase.

Reference solution. A 0.1 per cent w/v solution of *saquinavir mesylate IPRS* equivalent to *saquinavir* in the mobile phase. Dilute 5.0 ml of the solution to 20.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 14 volumes of a solution prepared by diluting 10.0 ml of *triethylamine* to 1000 ml with *water*, adjusted to pH 2.5 with *orthophosphoric acid* and filtering, 5 volumes of *tetrahydrofuran* and 1 volume of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency in not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

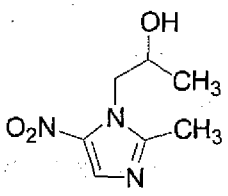
Inject the reference solution and the test solution.

Calculate the content of $C_{38}H_{50}N_6O_5$ in the tablets.

Storage. Store protected from moisture.

Labelling. The label states the strength in terms of the equivalent amount of saquinavir.

Secnidazole



$C_7H_{11}N_3O_3$ Mol. Wt. 185.2

Secnidazole is (*RS*)-1-(2-methyl-5-nitroimidazol-1-yl) propan-2-ol.

Secnidazole contains not less than 98.0 per cent and not more than 101.0 per cent of $C_7H_{11}N_3O_3$, calculated on the anhydrous basis.

Category. Antiamoebic.

Description. A white to yellowish white, crystalline powder.

Identification

A. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *0.1 M hydrochloric acid* shows an absorption maximum at about 277 nm, 0.325 to 0.355.

B. Heat about 10 mg in a water-bath with 10 mg of *zinc powder*, 1 ml of *water* and 0.25 ml of *2 M hydrochloric acid* for 5 minutes and cool. The solution gives the reaction of primary aromatic amines (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution in *1 M hydrochloric acid* is not more opalescent than opalescence standard OS2 (2.4.1) and not more intensely coloured than reference solution GYS4 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 100.0 ml of the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the same solvent.

Reference solution (b). A solution containing 0.005 per cent w/v each of *2-methyl-5-nitroimidazole IPRS* and *secnidazole IPRS* in the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the same solvent.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of *methanol* and 65 volumes of 0.14 per cent w/v solution of *potassium dihydrogen phosphate*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 318 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the resolution between 2-methyl-5-nitroimidazole and secnidazole is not less than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 2 times the area of the peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of areas of all the secondary peaks

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is not more than 5 times the area of the peak in the chromatogram obtained with the reference solution (a) (0.5 per cent). Disregard any peak which is 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.01 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method A (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). 4.0 to 6.0 per cent, determined on 0.4 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in 25.0 ml of the mobile phase. Dilute 5.0 ml of the solution to 100.0 ml with the same solvent.

Reference solution. A 0.005 per cent w/v solution of *secnidazole IPRS* in mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of *methanol* and 65 volumes of 0.14 per cent w/v solution of *potassium dihydrogen phosphate*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 318 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_7H_{11}N_3O_3$.

Storage. Store protected from light and moisture.

Secnidazole Tablets

Secnidazole Tablets contain not less than 95.0 per cent and not more than 110.0 per cent of the stated amount of secnidazole, $C_7H_{11}N_3O_3$.

Usual strengths. 1 g; 2 g.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1 M hydrochloric acid*.

Speed and time. 100 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter promptly through a membrane filter disc having an average pore diameter not greater than 1.0 µm, rejecting the first 1 ml of the filtrate. Dilute the filtrate, if necessary, with the same solvent. Measure the absorbance of the resulting solution at the maximum at about 277 nm (2.4.7). Calculate the content of $C_7H_{11}N_3O_3$ in the medium from the absorbance obtained from a solution of known concentration of *secnidazole IPRS* in the same medium.

Q. Not less than 80 per cent of the stated amount of $C_7H_{11}N_3O_3$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of powdered tablets containing 50 mg of Secnidazole, disperse in 100 ml of mobile phase and filter.

Reference solution (a). A 0.05 per cent w/v solution of *secnidazole IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with mobile phase.

Chromatographic system as described under Assay.

Inject reference solution (a). The test is not valid unless the tailing factor is not more than 2.0 and the column efficiency in not less than 2000 theoretical plates.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and the sum of areas of all the secondary peaks is not more than 2.0 times the area of the peak in the chromatogram obtained with the reference solution (b) (2.0 per cent).

Water (2.3.43). Not more than 6.5 per cent, determined on 0.5 g.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powdered tablet containing 50 mg of Secnidazole, disperse in 100.0 ml of mobile phase and filter. Dilute 5.0 ml of the filtrate to 50.0 ml with mobile phase.

Reference solution. A 0.005 per cent w/v solution of *secnidazole IPRS* in mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane chemically bonded to porous silica (5 µm),
- mobile phase: a mixture of 85 volumes of *0.01 M potassium dihydrogen orthophosphate* and 15 volumes of *acetonitrile*,

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– flow rate: 1 ml per minute,
– spectrophotometer set at 228 nm,
– injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation of replicate injections is not more than 2.0 per cent.

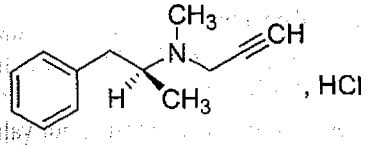
Inject the reference solution and the test solution.

Calculate the content of $C_7H_{11}N_3O_3$.

Storage. Store protected from light and moisture.

Labelling. The label states the strength of secnidazole.

Selegiline Hydrochloride



$C_{13}H_{17}N,HCl$ Mol. Wt. 223.7

Selegiline Hydrochloride is(*R*)-Methyl(α-methylphenethyl) prop-2-ynylamine hydrochloride.

Selegiline Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{13}H_{17}N,HCl$, calculated on the dried basis.

Category. Antiparkinsonian.

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *selegiline hydrochloride IPRS* or with the reference spectrum of selegiline hydrochloride.

B. It gives reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 3.5 to 4.5, determined in a 2.0 per cent w/v solution in *carbon dioxide-free water.*

Specific optical rotation (2.3.22). –12.0° to –10.0°, determined on a 10.0 per cent w/v solution in *carbon dioxide-free water.*

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). A solution containing 0.1 per cent w/v of *selegiline hydrochloride IPRS* and 0.02 per cent w/v of *butyl parahydroxybenzoate* in the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system
– a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
– mobile phase: a mixture of 500 volumes of *acetonitrile* and 500 volumes of butylammonium acetate buffer solution pH 6.5 prepared by dissolving 4 ml of *butylamine* in 900 ml of *water*, adjusted to pH 6.5 with *acetic acid* and dilute to 1000 ml with *water*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 215 nm,
– injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the resolution between the peaks corresponding to selegiline and butyl parahydroxybenzoate is not less than 3.0.

Inject reference solution (b) and the test solution. Run the chromatogram 1.7 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

***(S)*- Selegiline.** Determine by liquid chromatography (2.4.14).

Solvent mixture. 10 µl of *butylamine* and 1 ml of *2-propanol.*

Test solution. Dissolve 20 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 8 mg of *(RS)-selegiline hydrochloride IPRS* in the solvent mixture and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 0.5 ml of reference solution (a) to 20.0 ml with the mobile phase.

Chromatographic system
– a stainless steel column 25 cm x 4.6 mm, packed with silica gel OD for chiral separations (5 µm),
– mobile phase: a mixture of 0.2 volume of *2-propanol* and 99.8 volumes of *cyclohexane*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 220 nm,
– injection volume: 20 µl.

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The retention time of *(S)*-selegiline is about 10 minutes.

Inject reference solution (a). The test is not valid unless the resolution between the peaks corresponidng to *(S)*-selegiline and *(R)*-selegiline is not less than 1.5.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to *(S)*-selegiline is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying at 60° at a pressure not exceeding 0.5 kPa.

Assay. Dissolve 0.18 g in 50 ml of *acetic anhydride*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02237 g of $C_{13}H_{18}ClN$.

Storage. Store protected from light.

Selegiline Tablets

Selegiline Hydrochloride Tablets

Selegiline Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of selegiline hydrochloride, $C_{13}H_{18}ClN$.

Usual strengths. 5 mg; 10 mg.

Identification

A. Shake a quantity of the powdered tablets containing 0.1 g of Selegiline Hydrochloride with 2 ml of *water* and 1 ml of *1M sodium hydroxide*. Extract with 10 ml of *dichloromethane*, filter the extract through *anhydrous sodium sulphate* and evaporate the filtrate to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *selegiline hydrochloride IPRS*, treated in the same manner or with the reference spectrum of selegiline.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 500 ml of *water*,
Speed and time. 50 rpm and 20 minutes.

Withdraw a suitable volume of the medium and centrifuge at 3500 rpm for 10 minutes.

Determine by liquid chromatography (2.4.14).

Test solution. Use the clear supernatant, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *selegiline hydrochloride IPRS* in the dissolution medium to obtain a solution having the same concentration as that to the test solution.

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 80 volumes of *0.1 M monobasic ammonium phosphate*, adjusted to pH 3.1 with *orthophosphoric acid* and 20 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 15 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{13}H_{17}N,HCl$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{13}H_{17}N,HCl$.

(*S*)-Selegiline. Determine by liquid chromatography (2.4.14).

Solvent mixture. 1 ml of *propan-2-ol* and 10 µl of *butylamine*.

Test solution. Disperse a quantity of the powdered tablets containing 20 mg of Selegiline Hydrochloride with the solvent mixture and dilute to 10.0 ml with the mobile phase, shake thoroughly, filter.

Reference solution (a). Dissolve 8 mg of (RS)-*selegiline hydrochloride IPRS* in the solvent mixture and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 0.5 ml of reference solution (a) to 20.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with silica gel OD for chiral separation (5 µm) (Such as Chiralcel OD),
- mobile phase: a mixture of 0.2 volume of *propan-2-ol* and 99.8 volumes of *cyclohexane*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

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The retention time of (*S*)-selegiline is about 10 minutes.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks corresponding to (*S*)-selegiline and (*R*)-selegiline in the chromatogram obtained with reference solution (a) is not less than 1.5.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to (*S*)-selegiline is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 20 mg of Selegiline Hydrochloride with 10.0 ml of a mixture of equal volumes of *methanol* and *acetonitrile*, filter. Dilute 5.0 ml of the filtrate to 10.0 ml with *water*.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). A 0.001 per cent w/v solution of *methylamphetamine hydrochloride* in the mobile phase.

Reference solution (c). A solution containing 0.005 per cent w/v of *selegiline hydrochloride IPRS* and 0.001 per cent w/v of *nortriptyline hydrochloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: dilute a mixture of 250 ml of *methanol* and 250 ml of *acetonitrile* to 1000 ml with a solution prepared by dissolving 4 ml of *butylamine* in 900 ml of *water*, adjusted to pH 6.5 with *acetic acid* and diluted to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the resolution between the two principal peaks is not less than 3.0.

Inject reference solution (a), (b) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 2.5 times the area of the peak in the chromatogram obtained with reference solution (a) (0.5 per cent), the area of not more than two such peaks is more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of any such peaks is not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.2 per cent).

If, in the chromatogram obtained with the test solution, the area of any peak corresponding to methylamphetamine is more than 0.5 times the area of the peak in the chromatogram obtained with reference solution (b), the following limits apply. The area of any peak corresponding to methylamphetamine is not more than the area of the peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and the sum of areas of all peak is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of the areas of any such peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than 0.1 times that of the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14) as described under Assay using following test solution.

Test solution. Disperse 1 tablet in 50 ml of equal volumes of *methanol* and *acetonitrile* and dilute with water to obtain a solution containing 0.005 per cent w/v solution of Selegiline Hydrochloride.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 10 mg of Selegiline Hydrochloride with 100.0 ml of a mixture of equal volumes of *methanol* and *acetonitrile*, filter. Dilute 50.0 ml of the solution to 100.0 ml with *water*.

Reference solution (a). A 0.005 per cent w/v solution of *selegiline hydrochloride IPRS* in the mobile phase.

Reference solution (b). A solution containing 0.005 per cent w/v of *selegiline hydrochloride IPRS* and 0.001 per cent w/v of *nortriptyline hydrochloride IPRS* in the mobile phase.

Use chromatographic system as described under Related substances.

Inject reference solution (b). The assay is not valid unless the resolution between the two principal peaks is not less than 3.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{13}H_{18}ClN$ in the tablets.

Storage. Store protected from light and moisture.

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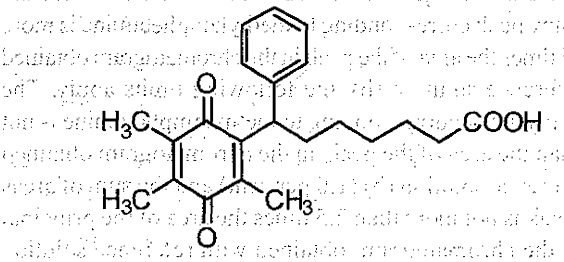
Seratrodast



$C_{22}H_{26}O_4$ Mol Wt. 354.4

Seratrodast is 7-Phenyl-7-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)heptanoic acid.

Seratrodast contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{22}H_{26}O_4$, calculated on the dried basis.

Description. A pale yellow to orange powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *seratrodast IPRS* or with the reference spectrum of seratrodast.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 20 volumes of *water* and 80 volumes of *acetonitrile*.

Test solution. Dissolve 25 mg of the substance under examination in the solvent mixture and dilute to 25.0 ml with solvent mixture.

Reference solution. A 0.0003 per cent w/v solution of *seratrodast IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a 0.1 per centv/v solution of *orthophosphoric acid* in *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	40	60
20	40	60
30	25	75
40	25	75
50	40	60
65	40	60

Inject reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.3 per cent) and the sum of areas of all the peaks is not more than 3.3 times the area of the principal peak in the chromatogram obtained with reference solution (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g by drying in an oven at 105°, for 3 hours.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 20 volumes of *water* and 80 volumes of *acetonitrile*.

Test solution. Dissolve 0.1g of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *seratrodast IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of 0.1 per cent v/v solution of *orthophosphoric acid* in *water* and 65 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215nm,
- injection volume: 20 µl.

Inject reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution and the test solution.

Calculate the content of $C_{22}H_{26}O_4$.

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Serratiopeptidase

Serratiopeptidase is a proteolytic enzyme for oral use which is produced by bacteria of genus Serratia.

Serratiopeptidase contains not less than 2000 units and not more than 2600 units of serratiopeptidase activity per mg.

Category. Proteolytic enzyme.

Description. A grayish white to pale brown powder.

Identification

Dissolve 0.4 g of serratiopeptidase in 100 ml of *acetic acid sodium acetate buffer solution pH 5.0*, transfer exactly 1 ml of the solution into 3 test tubes, refer them as A, B and C. Add 1 ml of *water* in tube A. In tube B and C add 1 ml of *0.04 M disodium edetate solution*. Mix gently and allow them to stand at 4±1°, for 1 hour. Add 2 ml of *water* in tube A and C. Add 2 ml of *0.04 M zinc chloride* solution in tube B. Mix gently and allow them to stand at 4±1°, for 1 hour. Pipette 1 ml of A and B and make up the volume to 200 ml with *borate buffer pH 9.0*. Pipette 1 ml of C and make up the volume to 50 ml with *borate buffer pH 9.0*. Proceed with these solutions as sample solutions as directed in the assay. The activities of solution A and B are almost same and the activity of C is not more than 5 per cent of that of activity of A.

Tests

Arsenic (2.3.10). Dissolve 2.0 g in 50 ml of *water*, and add 10 ml of *stannated hydrochloric acid*. The resulting solution complies with the limit test for arsenic (5 ppm).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated Ash (2.3.18). Not more than 7.0 per cent.

Loss on drying (2.4.19). Not more than 7.0 per cent, determined on 1 g by drying in an oven at 105° for 4 hours.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 10^3 CFU per g and total fungal count is not more than 10^2 CFU per g determined by plate count. 1 g is free from *Escherichia coli* and 10 g is free from *Salmonella* and *Shigella*.

Assay. Unit definition: one serratiopeptidase unit is defined as the amount of enzyme required to liberate 1 µg of free tyrosine per minute under the specified assay conditions.

sodium borate hydrochloric acid buffer pH 9.0. Dissolve 19 g *sodium borate* in 900 ml of *water*. Adjust the pH 9.0 with *1 M hydrochloric acid* and dilute with *water* to 1000 ml.

Substrate solution. Dissolve 1.2 g dried casein in 100 ml of *sodium borate hydrochloric acid buffer pH 9.0*. Keep it on boiling water-bath for 1-2 minutes to get clear solution. Cool and filter through cotton and dilute with *sodium borate hydrochloric acid buffer pH 9.0* to 200 ml.

Protein precipitating solution. To 18 g of *trichloroacetic acid*, add 30 g of *sodium acetate* and 20 ml of *glacial acetic acid*, and dilute with *water* to 1000 ml.

Dilute folin's reagent. 1 ml of *folin's reagent*, add 2 ml of *water*.

Reference tyrosine solution. Dissolve 10 mg of *tyrosine* in 1 ml of *1 M hydrochloric acid* and dilute to 100 ml with *sodium borate hydrochloric acid buffer pH 9.0*.

Reference tyrosine curve. To 1, 2, 3, 4 and 5 ml of *reference tyrosine solution*, add 5, 4, 3, 2 and 1 ml of *sodium borate hydrochloric acid buffer pH 9.0* respectively. Add 5 ml of *protein precipitating reagent* in each tube. To 2 ml of these solutions, add 5 ml of 6.0 per cent w/v *sodium carbonate solution*. Add 1 ml of diluted *folin's reagent* mix and allow to stand at 37° for 30 minutes, measure the absorbance at 660 nm (2.4.7). Plot a graph of µmol of tyrosine per system against the absorbance.

Stock test solution. Weigh about 0.1 g of the substance under examination, dissolve in 100 ml of *sodium borate hydrochloric acid buffer pH 9.0* (solution A). Mix and keep it for 5 minutes. Take 1 ml of solution A and dilute to 200 ml with *sodium borate hydrochloric acid buffer pH 9.0* (solution B).

Test solution. Pipet 1.0 ml of *stock test solution*, allow to stand in a water-bath at 37° for 5 minutes. Add 5 ml of substrate solution shake immediately and allow to stand in water-bath at 37° for 20 minutes. Add 5 ml of *protein precipitating solution*. Mix and allow to stand in water-bath at 37° for 30 minutes and filter. Take 2 ml of the filtrate, add 5 ml of 6.0 per cent w/v *sodium carbonate solution*. Add 1 ml of *diluted folin's reagent* mix and allow to stand at 37° for 30 minutes. Measure the absorbance at 660 nm (2.4.7).

Blank solution. Pipet 1.0 ml of *stock test solution*, allow to stand in a water-bath at 37° for 5 minutes, add 5 ml of *protein precipitating solution*, shake immediately and allow to stand in water-bath at 37° for 20 minutes. Add 5 ml of substrate solution. Mix and allow to stand in water-bath at 37° for 30 minutes and filter. Take 2 ml of the filtrate, add 5 ml of 6.0 per cent w/v *sodium carbonate solution*. Add 1 ml of *diluted folin's reagent* mix and allow stand at 37° for 30 minutes. Measure the absorbance at 660 nm (2.4.7).

Calculate the serratiopeptidase IU/mg by using concentration of tyrosine from graph considering the reaction time and dilution.

Storage. Store protected from light and moisture, at temperature not exceeding 30°.

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Serratiopeptidase Tablets

Serratiopeptidase Tablets contain not less than 90.0 per cent of the stated amount of Serratiopeptidase.

Usual strengths. 5 mg; 10 mg.

Tests

Other tests. Comply with the tests stated under Tablets.

Assay. The tyrosine units are dissolved in *sodium carbonate solution*, which is alkaline in nature. The *Folin's ciocalteau reagent* helps in colour development where the tyrosine units bind to the copper molecule in the reagent and causes the reduction of phosphomolybdate which is present in the reagent. There is formation of tyrosine-copper molybdate coloured complex. The intensity of colour depends upon the tyrosine units present which is read at 660 nm.

Disodium tetraborate buffer. Dissolve 19.0 gm of *disodium tetraborate* in 900 ml of *water*, adjusted to pH 9.0 with *1 M hydrochloric acid* and dilute to 1000 ml with *water*.

Casein hammerstein substrate. Dissolve 1.2 gm of *Casein Hammerstein* to 100 ml of the sodium tetraborate buffer. Allow the casein to dissolve and form homogeneous solution. Boil the solution in boiling water-bath for 2 minutes. After removing from boiling water-bath, cool the casein substrate immediately in ice cold water. Filter the solution through cotton plug to get clear solution and dilute to 200 ml with *water*.

NOTE—Always add casein to the buffer while stirring. Do not add buffer to casein as this will cause lumps and not go into solution completely and giving incorrect values.

Trichloroacetic acid reagent. Dissolve 18 gm of *trichloroacetic Acid*, 30 gm of *anhydrous sodium acetate* and 20 ml of *glacial acetic acid* in 1000 ml of *water*.

NOTE—TCA should be in the crystal form and not in liquid form as this will effect the quantity weighed and thereby interfere with the complete precipitation of casein. Use Sodium acetate anhydrous because if, sodium acetate trihydrate should be used, the trihydrate forms gives lower results.

Diluted Folin's Ciocalteau Reagent. Dilute 1 ml of Folin's Ciocalteau reagent with 2 ml of *water* to get 3 ml of the reagent.

NOTE—Folin's Ciocalteau reagent should be of the protein estimation grade and not the indicator grade. Folin's reagent should be diluted to just before addition to the tubes since it is sensitive to light. Do not prepare this solution at the beginning of the assay.

Tyrosine reference solution. Dissolve 160 mg of *tyrosine* in 100 ml of *0.2 M hydrochloric acid.*

Dilute 1 ml of the solution to 100 ml with *0.2 M hydrochloric acid.* Use 2 ml of the solution for colour development.

Trisodium phosphate buffer pH 6.8. Weigh 19 g of *trisodium phosphate* and 6.4 ml of *hydrochloric acid* in 1000 ml of *water*, adjusted to pH 6.8.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 120 mg of Serratiopeptidase with 100 ml of *trisodium phosphate buffer pH 6.8.* Stir on magnetic stirrer for 1 hour. Further dilute 5.0 ml to 25 ml with *5.0 per cent ammonium sulphate solution,* stir for 5 minutes and filter. Dilute 2 ml of the filtrate to 100 ml with *sodium tetraborate buffer*. 1 ml of resulting solution is used for analysis. Set the water bath to 37°.

For each sample, keep four test tubes. Mark two test tubes as test and two as blank. Add 5 ml of Casein Substrate to the tubes marked as test and 5 ml. of TCA to the test tube marked as blank. Prewarm these tubes for five minutes along with the tubes containing the sample dilutions. Add 1 ml of enzyme solution to all these tubes and vertex the tubes for exactly 10 seconds. Keep the test tubes in the water-bath at 37° for 20 minutes for the reaction. After exactly 20 minutes, add 5 ml of TCA to tubes marked as test and 5 ml of Casein substrate to the tube marked as blank Vortex all the tubes exactly for 30 seconds. Keep the tubes in the water-bath at 37° for 30 minutes for precipitation, filter.

NOTE— For tablets, the settlement may take a longer time than 30 minutes. So filter the solution after the settlement is clearly seen (Vortex in between for better precipitation).

Mark tubes as test, blank, tyrosine and tyrosine blank.

Add the following respectively;

Test – 2 ml of the test filtrate.

Blank – 2 ml of blank filtrate.

Tyrosine – 2 ml of tyrosine standard solution.

Tyrosine blank – 0.2 M hydrochloric acid.

Add 5 ml of 6 per cent sodium carbonate solution to all tubes.

Add 1 ml of diluted Folin's reagent to all the tubes. Keep the tubes at 37° for 30 minutes for colour development. Measure the absorbance at about 660 nm (2.4.7).

$$\text{Serratiopeptidase Units/Tablet} = \frac{A_1 - A_2}{A_3 - A_4} \times 176 \times \frac{T}{0.160} \times \frac{1}{20} \times \frac{100}{\text{Spl. wt. (g)}} \times \frac{25}{5} \times \frac{100}{2} \times \text{Avg. wt. in g}$$

$$\text{Percentage of L.A.} = \frac{\text{Serratiopeptidase Units/Tablet}}{\text{L.A.}} \times 100$$

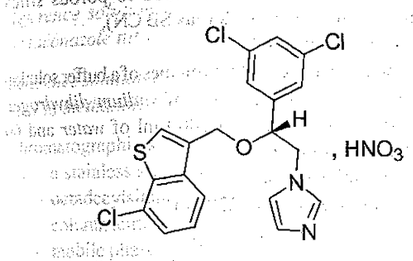
where, 176 = Conversion Co-efficient of Tyrosine to Serratiopeptidase,

T = Actual weight of Tyrosine taken (g),
0.160 = Theoretical weight of Tyrosine to be taken
20 = Reaction time (minutes),
A_1 = Absorbance of test solution,
A_2 = Absorbance of blank solution,
A_3 = Absorbance of tyrosine reference solution,
A_4 = Absorbance of 0.2M hydrochloric acid,
L.A. = Labelled amount/tablet.

Storage. Store protected from moisture, at a temperature not exceeding 25°.

Labelling. The label states the strength in terms of the equivalent to Enzyme activity units of Serratiopeptidase.

Sertaconazole Nitrate



$C_{20}H_{16}Cl_3N_3O_4S$ Mol. Wt. 500.8

Sertaconazole Nitrate is (*RS*)-1-[2-[(7-Chloro-1-benzothiophen-3-yl)methoxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole nitrate.

Sertaconazole Nitrate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{20}H_{16}Cl_3N_3O_4S$ calculated on the anhydrous basis.

Category. Antifungal.

Description. A white or almost white powder.

Identification

Test A and C may be omitted if tests B, D and E are carried out. Tests B, D and E may be omitted if test A and C are carried out.

A. Melting point (2.4.21). 156° to 161°.

B. When examined in the range 240 nm to 320 nm (2.4.7), a 0.01 per cent w/v solution in *methanol* gives absorption maxima at 260 nm, 293 nm and 302 nm and the absorbance ratio (A_{302}/A_{293}) is 1.16 to 1.28.

C. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sertaconazole nitrate IPRS* or with the reference spectrum of sertaconazole nitrate.

D. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 1 volume of *concentrated ammonia,* 40 volumes of *toluene* and 60 volumes of *dioxane.*

Solvent mixture. 10 volumes of *concentrated ammonia* and 90 volumes of *methanol.*

Test solution. Dissolve 40 mg of the substance under examination in the solvent mixture and dilute to 10 ml with the solvent mixture.

Reference solution (a). A 0.4 per cent w/v solution of *sertaconazole nitrate IPRS* in the solvent mixture.

Reference solution (b). A 0.4 per cent w/v solution of *miconazole nitrate IPRS* in reference solution (a).

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 15 cm. After development, dry the plate in air for 15 minutes and expose to iodine vapour for 30 minutes. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The spot in the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

E. It gives the reactions of nitrates (2.3.1).

Tests

Appearance of solution. A 1.0 per cent w/v solution in *ethanol (95 per cent)* is clear (2.4.1), and not more intensely coloured than reference solution YS5 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 10 mg of the substance under examination in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.0005 per cent w/v each of *sertaconazole nitrate IPRS* and *miconazole nitrate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with nitrile silica (10 µm),
- mobile phase: a mixture of 37 volumes of *acetonitrile* and 63 volumes a buffer solution prepared by dissolving 1.5 g of *sodium dihydrogen phosphate* in 1000 ml of *water*.

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- flow rate: 1.6 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

The retention time of peaks due to nitrate ion is about 1 minute, miconazole about 17 minutes and sertaconazole about 19 minutes.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to miconazole and sertaconazole is not less than 2.0.

Inject reference solution (a) and the test solution. Run the chromatogram 1.3 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding sertaconazole impurity A, B and C are not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent), the relative retention time of impurity C, impurity B and impurity A are about 0.12, 0.18 and 0.3 respectively, the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak which is 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent) and due to nitrate ion.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 0.5 g.

Assay. Dissolve 0.4 g in 50 ml mixture of equal volumes of *anhydrous acetic acid* and *methyl ethyl ketone*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.05008 g of $C_{20}H_{16}Cl_3N_3O_4S$.

Storage. Store protected from light.

Sertaconazole Nitrate Cream

Sertaconazole Nitrate Cream contains Sertaconazole Nitrate in a suitable cream base.

Sertaconazole Nitrate Cream contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of sertaconazole nitrate, $C_{20}H_{15}Cl_3N_2OS$ in a suitable cream base.

Usual strength. 2 per cent w/w.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 3.0 to 4.5

Other tests. Comply with the tests stated under Creams.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the cream containing about 20 mg of Sertaconazole Nitrate in 70 ml of the *methanol* and sonicate for 15 minutes with intermittent shaking. Cool to room temperature and dilute to 100.0 ml with *methanol* and filter. Further dilute 5.0 ml of the solution to 25.0 ml with *methanol*.

Reference solution. A 0.004 per cent w/v solution of *sertaconazole nitrate IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with nitrile groups chemically-bonded to porous silica particles (5 µm), (Such as Zorbax SB CN),
- column temperature: 40°,
- mobile phase: a mixture of 40 volumes of a buffer solution prepared by dissolving 1.5 g of *sodium dihydrogen phosphate monohydrate* in 1000 ml of *water* and 60 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{15}Cl_3N_2O_5$ in the cream.

Storage. Store at a temperature not exceeding 25°. Do not freeze.

Sertaconazole Nitrate and Beclomethasone Dipropionate Cream

Sertaconazole Nitrate and Beclomethasone Dipropionate Cream in a suitable bases.

Sertaconazole Nitrate and Beclomethasone Dipropionate Cream contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of Sertaconazole Nitrate, $C_{20}H_{15}Cl_3N_2OS$ and Beclomethasone Dipropionate, $C_{28}H_{37}ClO_7$.

Usual strength. 2.0 per cent w/w. Sertaconazole Nitrate and 0.025 per cent w/w Beclomethasone Dipropionate.

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Identification

In the Assay, the retention time of the principal peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24).3.0 to 4.5.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the cream containing about 50 mg of Sertaconazole Nitrate in 30.0 ml of *methanol*, vortex to disperse the cream completely, ultrasound for 15 minutes with intermittent swirling. Cool to room temperature and dilute to 50.0 ml with *methanol* and filter.

Reference solution (a). A 0.0005 per cent w/v solution of *sertaconazole nitrate IPRS* in *methanol*.

Reference solution (b). A 0.0125 per cent w/v solution of *nitric acid* in *methanol*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. dissolve 1.5 g of *sodium dihydrogen phosphate monohydrate* in 1000 ml of *water* and filter, B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.6 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile Phase B (per cent v/v)
0	60	40
10	60	40
20	40	60
35	40	60
42	10	90
55	10	90
60	60	40
65	60	40

The relative retention time of Beclomethasone Dipropionate is about 0.9 with respect to Sertaconazole.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injection is not more than 5.0 per cent.

Inject reference solution (a) and (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent) Ignore the peak corresponding to the peak in the chromatogram obtained with reference solution (b) and any peak with an area less than 0. 1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Other tests. Comply with the tests stated under Creams

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the cream containing about 50.0 mg of sertaconazole nitrate and 0.625 mg of beclomethasone dipropionate in 30 ml of the *methanol*, vortex to disperse the cream completely, ultrasound for 15 minutes with intermittent shaking. Cool to room temperature and dilute to 50.0 ml with *methanol* and filter. (use it for the analysis of beclomethasone dipropionate). Futher dilute 5.0 ml to 50.0 ml with *methanol*, (sertaconazole).

Reference solution. A solution containing 0.01 per cent w/v of *sertaconazole nitrate IPRS* and 0.00125 per cent w/v of *beclomethasone dipropionate IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 40 volumes of a buffer solution prepared by dissolving 1.5 g of *sodium dihydrogen phosphate dihydrate* in 1000 ml of *water*, and 60 volumes of *acetonitrile*,
- flow rate: 1.6 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency in not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{15}Cl_3N_2OS$ and $C_{28}H_{37}ClO_7$ in the cream.

Storage. Store below 25°. Do not refrigerate.

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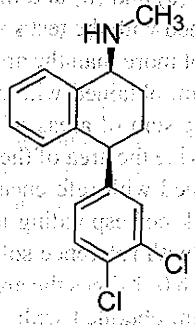
Sertraline Hydrochloride



$C_{17}H_{17}Cl_2N,HCl$ Mol. Wt. 342.7

Sertraline Hydrochloride is (1S,4S)-4-(3,4-dichlorophenyl)-*N*-methyl-1,2,3,4-tetrahydronaphthalen-1-amine hydrochloride.

Sertraline Hydrochloride contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{17}H_{17}Cl_2N,HCl$, calculated on the anhydrous basis.

Category. Antidepressant.

Description. A white or almost white, crystalline powder. It shows polymorphism (2.5.11).

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sertraline hydrochloride IPRS* or with the reference spectrum of sertraline hydrochloride.

B. Dissolve 10 mg in 5 ml of *ethanol* and add 5 ml of *water*. The solution gives reaction (A) of chlorides (2.3.1).

Tests

Specific optical rotation (2.4.22). +38.8° to +43.0°, determined in a 1.0 per cent w/v solution in a mixture of 1 volume of 10.3 per cent w/v solution of *hydrochloric acid* and 19 volumes of *methanol*.

Enantiomeric purity. Determine by liquid chromatography (2.4.14).

Solvent mixture. 1 volume of *diethylamine*, 40 volumes of *hexane* and 60 volumes of *2-propanol*.

Test solution. Dissolve 60 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). Dissolve the contents of a vial of *sertraline for system suitability IPRS* (containing sertraline impurity G) in 1.0 ml of the solvent mixture.

Reference solution (b). Dilute 0.5 ml of the test solution to 100.0 ml with the solvent mixture.

Chromatographic system.

- a stainless steel column 25 cm x 4.6 mm, packed with silica gel AD for chiral separation (5 µm),
- mobile phase: a mixture of 30 volumes of *hexane* and 70 volumes of a mixture of 1 volume of *diethylamine*, 25 volumes of *2-propanol* and 975 volumes of *hexane*,
- flow rate: 0.4 ml per minute.
- spectrophotometer set at 275 nm,
- injection volume: 20 µl.

The elution order of the peaks is sertraline and then sertraline impurity G ((1R,4R)-4-(3,4-dichlorophenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine).

Inject reference solution (a) and (b). The test is not valid unless the signal-to-noise ratio for the peak corresponding to sertraline is not less than 10 in the chromatogram obtained with reference solution (b) and the resolution between the peaks corresponding to sertraline impurity G and sertraline is not less than 1.5 in the chromtaogram obtained with reference solution (a).

Inject reference solution (b) and the test solution. Run the chromatogram for about 30 minutes. In the chromatogram obtained with the test solution, the area of the peak corresponding to sertraline impurity G is not more than 3 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent).

Impurity E. Determine by liquid chromatography (2.4.14).

Solvent mixture. 50 volumes of mobile phase A and 50 volumes of mobile phase B.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). A 0.0002 per cent w/v solution of *sertraline impurity E (mandelic acid)* in the solvent mixture.

Reference solution (b). A solution containing 0.0004 per cent w/v of *benzoic acid* and 0.0008 per cent w/v of *mandelic acid* (sertraline impurity E) in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (3 µm),
- mobile phase: A. dissolve 1.0 g of *sodium lauryl sulphate* in 800 ml of *water* and add 200 ml of *acetonitrile*, add 1.0 ml of *orthophosphoric acid*,

  B. dissolve 1.0 g of *sodium lauryl sulphate* in 100 ml of *water* and add 900 ml of *acetonitrile*, add 1.0 ml of *orthophosphoric acid*,

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– a gradient programme using the conditions given below,
– flow rate: 1 ml per minute,
– spectrophotometer set at 220 nm,
– injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	60	40
8	60	40
9	10	90
16	10	90
24	10	90
27	60	40
35	60	40

The relative retention time with reference to sertraline (retention time: about 18 minutes) for sertraline impurity E is about 0.2 and for benzoic acid is about 0.3.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to sertraline impurity E and benzoic acid is not less than 5.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of the peak corresponding to sertraline impurity E is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent).

Related substances. Determine by gas chromatography (2.4.13).

Test solution. Introduce 0.25 g of the substance under examination into a 15 ml stoppered centrifuge tube, add 2.0 ml of *methanol* and 0.2 ml of a 25 per cent w/v solution of *potassium carbonate* and mix in a vortex mixer for 30 seconds. Add 8.0 ml of *dichloromethane*, stopper the tube and mix in a vortex mixer for 60 seconds. Add 1 g of *anhydrous sodium sulphate*, mix well and then centrifuge for about 5 minutes.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with *dichloromethane*. Dilute 1.0 ml of the solution to 20.0 ml with *dichloromethane*.

Chromatographic system
– a fused silica column 30 m x 0.53 mm, coated with polymethylphenylsiloxane (film thickness 1.0 µm),
– temperature:

column	time (in min)	temperature (°)
	0-1	200
	1-31	200 → 260
	31-39	260

– inlet port: 250° and detector at 280°,
– flame ionization detector,
– flow rate: 9 ml per minute, using nitrogen as the carrier gas,
– split ratio: 1:10

Name	Relative retention time
Sertraline impurity B[1]	0.5
Sertraline impurity C[2]	0.7
Sertraline impurity D[3]	0.7
Sertraline (Retention time: about 24 minutes)	1.0
Sertraline impurity A[4]	1.05
Sertraline impurity F[5]	1.1

[1](1RS,4RS)-N-methyl-4-phenyl-1,2,3,4-tetrahydronaphthalen-1-amine,

[2](1RS,4RS)-4-(4-chlorophenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine,

[3](1RS,4RS)-4-(3-chlorophenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine,

[4](1RS,4SR)-4-(3,4-dichlorophenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine,

[5](4R)-4-(3,4-dichlorophenyl)-3,4-dihydronaphthalen-1(2H)-one.

Inject 1 µl of the reference solution and the test solution. In the chromatogram obtained with the test solution, the sum of areas of any peaks corresponding to sertraline impurities C and D is not more than 0.8 per cent. The area of the peak corresponding to sertraline impurities A, B and F is not more than 0.2 per cent. The area of any other secondary peak is not more than 0.1 per cent and the sum of areas of all the secondary peaks is not more than 1.5 per cent. Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Heavy metals (2.3.13). A 5.0 per cent w/v solution of the substance under examination in *ethanol (95 per cent)*. 12 ml of the solution complies with the limit test for heavy metals, Method D (20 ppm) using 10 ml of *lead standard solution (1 ppm Pb)*.

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). Not more than 0.5 per cent, determined on 2.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 55 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution. A 0.0055 per cent w/v solution of *sertraline hydrochloride IPRS* in the mobile phase.

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Chromatographic system
- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (4 µm),
- mobile phase: a mixture of 15 volumes of *methanol*, 40 volumes of buffer solution prepared by dissolving 28.6 ml of *glacial acetic acid*, 34.8 ml of *triethylamine*, and dilute to 100 ml with *water*. Dilute 10 ml of the solution to 1000 ml with *water* and 45 volumes of *acetonitrile*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{17}Cl_2N$,HCl.

Storage. Store protected from light.

Sertraline Tablets

Sertraline Hydrochloride Tablets

Sertraline Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sertraline, $C_{17}H_{17}Cl_2N$.

Usual strength. 25 mg; 50 mg; 100 mg.

Identification

Shake a quantity of the powdered tablets containing 0.25 g of Sertraline Hydrochloride with 10 ml of *ethanol* for 10 minutes, filter and evaporate the filtrate to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sertraline hydrochloride IPRS* or with the reference spectrum of sertraline hydrochloride.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *sodium acetate buffer solution pH 4.5*,
Speed and time. 75 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate as given above.

Reference solution. A solution of *sertraline hydrochloride IPRS* of the concentration similar to the test solution in the dissolution medium.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (4 µm),
- mobile phase: a mixture of 15 volumes of *methanol*, 40 volumes of a solution containing 0.286 per cent v/v *glacial acetic acid* and 0.348 per cent v/v *triethylamine* and 45 volumes of *acetonitrile*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 20 µl.

Calculate the content of $C_{17}H_{17}Cl_2N$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{17}H_{17}Cl_2N$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 30 volumes of *acetonitrile* and 70 volumes of *water*.

Test solution. Disperse a quantity of whole tablets containing 500 mg of Sertraline Hydrochloride with 150 ml of the solvent mixture with the aid of ultrasound and dilute to 200 ml with the solvent mixture and filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 500.0 ml with the solvent mixture.

Reference solution (b). A 0.001 per cent w/v solution of (R)-*mandelic acid (sertraline impurity E)* in the solvent mixture.

Reference solution (c). A 0.25 per cent w/v solution of *sertraline hydrochloride impurity IPRS* in solvent mixture.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 2.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a 0.272 per cent w/v solution of *potassium dihydrogen orthophosphate*, adjusted to pH 3.0 with *orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute.
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
30	70	30
40	60	40
41	70	30
51	70	30

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Inject reference solution (c). For peak identification of sertraline hydrochloride impurity.

Inject reference solution (a), (b), (d) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to sertraline impurity C (1RS,4RS)-4-(4-chlorophenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine) is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent). The area of any peak corresponding to (*R*)-mandelic acid is not more than the area of any corresponding peak in the chromatogram obtained with reference solution (b) (0.4 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of areas of all the secondary peaks other than sertraline impurity C is not more than 7.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent). Ignore any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

Enatiomeric purity. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of whole tablets containing 400 mg of Sertraline Hydrochloride with 140 ml of a mixture of 1 volume of *acetonitrile* and 2 volumes of *water* with the aid of ultrasound and dilute to 200.0 ml with a mixture of 1 volume of *acetonitrile* and 2 volumes of *water*, filter.

Reference solution (a). A 0.003 per cent w/v solution of *sertraline hydrochloride cis-isomer IPRS* in *methanol (10.0 per cent).*

Reference solution (b). A solution containing 0.0002 per cent w/v each of *sertraline hydrochloride IPRS* and *sertraline hydrochloride cis-isomer ((1R,4R)-4-(3,4-dichloro-phenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine) IPRS* in *methanol (10.0 per cent).*

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with silica gel OD for chiral sepearation (Such as ChromTech Chiral-AGP),
- mobile phase: a mixture of 10 volumes of *propan-2-ol* and 90 volumes of a 0.23 per cent w/v solution of *ammonium dihydrogen orthophosphate*,
- flow rate: 0.7 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 40 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 2.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to sertraline cis-isomer is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.2 g of Sertraline Hydrochloride with 150 ml of the mobile phase with the aid of ultrasound for 45 minutes, cool and dilute to 200 ml with *water*, filter. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

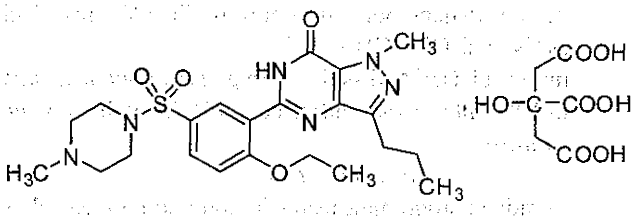
Reference solution. A 0.005 per cent w/v solution of *sertraline hydrochloride IPRS* in the mobile phase.

Use chromatographic system as described under Dissolution.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{17}Cl_2N$ in the tablets.

Sildenafil Citrate



$C_{28}H_{38}N_6O_{11}S$ Mol. Wt. 666.7

Sildenafil Citrate is 5-[2-ethoxy-5-(4-methylpiperazinylsulphonyl)phenyl]-1-methyl-3-*n*-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one citrate.

Sildenafil Citrate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{28}H_{38}N_6O_{11}S$, calculated on the anhydrous basis.

Category. Used in erectile dysfunction.

Description. A white to off white powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum that obtained with *sildenafil citrate IPRS* or with the reference spectrum of sildenafil citrate.

Tests

Citric acid. 28.0 per cent to 34.0 per cent.

Weigh 0.2 g of the substance under examination and transfer into a 250-ml conical flask, add 50 ml of *methanol* and sonicate

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to dissolve, then add 50 ml of *water*. Titrate with *0.1 M sodium hydroxide solution* using *phenolphthalein* indicator until the colour changes from colourless to pink.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.006403 g of $C_6H_8O_7$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Use freshly prepared solution.

Solvent mixture. 30 volumes of *water* and 70 volumes of *acetonitrile*.

Test solution. Dissolve 50 mg of the substance under examination in 100.0 ml of the solvent mixture.

Reference solution (a). A 0.05 per cent w/v solution of *sildenafil citrate IPRS* in the solvent mixture.

Reference solution (b). Dilute 10.0 ml of reference solution (a) to 100.0 ml with the solvent mixture. Further dilute 3.0 ml of the solution to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS C18),
- mobile phase: A, dissolve 3.85 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 7.5 with *ammonia solution*,

  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cen v/v)	Mobile phase B (per cent v/v)
0	65	35
15	65	35
30	20	80
40	20	80
45	65	35
50	65	35

The relative retention time with reference to sildenafil for citric acid is about 0.11, for N-oxide is about 0.29 and for chlorosulphonyl is about 1.6.

Inject reference solution (b). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with test solution the area of the peak due to N-oxide impurity is not more than 1.67 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of peak due to chlorosulphonyl is not more than 0.67 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent) and the sum of areas of all the secondary peaks is not more than 3.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore any peak corresponding to citric acid.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 2.5 per cent, determined on 0.2 g.

Assay. Determine by liquid chromatography (2.4.14).

NOTE—Use freshly prepared solution.

Solvent mixture. 30 volumes of *water* and 70 volumes of *acetonitrile*.

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.1 per cent w/v solution of *sildenafil citrate IPRS* in the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS C18),
- mobile phase: a mixture of 50 volumes of a buffer solution prepared by dissolving 3.85 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 7.5 with *ammonia solution* and 50 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the theoretical plates is not less than 2000, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{28}H_{38}N_6O_{11}S$.

Storage. Store protected from light and moisture.

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Sildenafil Tablets

Sildenafil Citrate Tablets

Sildenafil Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of sildenafil, $C_{22}H_{30}N_6O_4S$.

Usual strengths. 25 mg; 50 mg; 100 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.01 M Hydrochloric acid*,
Speed and time. 100 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtered solution, suitably diluted with the medium if necessary, at the maximum at about 294 nm (2.4.7). Calculate the content of $C_{22}H_{30}N_6O_4S$ in the medium from the absorbance obtained from a solution of known concentration of *sildenafil citrate IPRS* in the same medium.

Q. Not less than 70 per cent of the stated amount of $C_{22}H_{30}N_6O_4S$.

Related substances. Determine by liquid chromatography (2.4.14)

Solvent mixture. 50 volumes of mobile phase A and 50 volumes of *methanol*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 50 mg of sildenafil with 35 ml of the solvent mixture, sonicate for 20 minutes and dilute to 50 ml with the solvent mixture, filter.

Reference solution (a). A 0.028 per cent w/v solution of *sildenafil citrate IPRS* in the solvent mixture.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane chemically bonded to porous silica (5 µm) (Such as Zorbax Eclipse XDB C18),
- column temperature: 40°,
- mobile phase: A. a solution containing 3.85 g of *ammonium acetate* and 1.0 ml of *triethylamine* in 1000 of *water*, adjusted to pH 5.5 with *acetic acid*,
  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 294 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile Phase A (per cent /v/v)	Mobile Phase B (per cent /v/v)
0	47	53
20	47	53
35	10	90
45	10	90
50	47	53
60	47	53

Inject reference solution (b). The test is not valid unless the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent), the area of peak corresponding to N-oxide impurity at relative retention time 0.54 is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and the sum of areas of all the secondary peaks is not more than 2.0 times the area of the peak in the chromatogram obtained with reference solution (b) (2.0 per cent). Ignore the peak due to citric acid at retention time about 2.4 minutes.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 50 volumes of mobile phase and 50 volumes of *methanol*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 50 mg of sildenafil with 35 ml of the solvent mixture, sonicate for 20 minutes and dilute to 50.0 ml with the solvent mixture, filter.

Reference solution. A 0.14 per cent w/v solution of *sildenafil citrate IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with silica (5 µm) (Such as Zorbax Eclipse XDB C18),
- mobile phase: a mixture of 30 volumes of solution containing 3.85 g of *ammonium acetate* and 1.0 ml of *triethylamine* in 1000 ml of *water*, adjusted to pH 5.5 with *acetic acid* and 70 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 294 nm,
- injection volume: 20 µl.

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Inject the reference solution. The test is not valid unless the theoretical plates is not less than 2000, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution the test solution.

Calculate the content of $C_{22}H_{30}N_6O_4S$ in the tablet.

Storage. Store protected from moisture.

Labelling. The label states the strength in terms of the equivalent amount of sildenafil.

Colloidal Silicon Dioxide

Colloidal Anhydrous Silica

SiO_2 Mol. Wt. 60.1

Colloidal Silicon Dioxide is a submicroscopic fumed silica prepared by the vapour-phase hydrolysis of a silicon compound.

Colloidal Silicon Dioxide contains not less than 99.0 per cent and not more than 100.5 per cent of SiO_2, calculated on the ignite basis.

Category. Pharmaceutical aid (tablet excipient).

Description. A light, fine, white, amorphous powder. It has a particle size of about 15 nm.

Identification

About 20 mg gives the reaction of silicates (2.3.1).

Tests

pH (2.4.24). 3.5 to 5.5, determined in a suspension of 1.0 g in 30 ml of *carbon dioxide-free water*.

Arsenic (2.3.10). To 2.5 g contained in a round-bottomed flask add 50 ml of *3 M hydrochloric acid* and heat under a reflux condenser for 30 minutes. Cool, filter with the aid of suction and transfer the filtrate to a 100-ml volumetric flask. Wash the filter with several portions of hot water and add the washings to the volumetric flask. Cool, dilute to volume with *water* and mix. To 50.0 ml of the solution add 3 ml of *hydrochloric acid*; the resulting solution complies with the limit test for arsenic (8 ppm).

Heavy metals (2.3.13). Suspend 2.5 g in sufficient *water* to produce a semi-fluid slurry and dry at 140°. When the dried substance is white, break up the mass using a glass rod, add 25 ml of *1 M hydrochloric acid*, boil gently for 5 minutes, stirring frequently with the glass rod, centrifuge for 20 minutes and filter the supernatant liquid through a membrane filter. To the residue in the centrifuge tube add 3 ml of *2 M hydrochloric acid* and 9 ml of *water*, boil, centrifuge for 20 minutes and filter the supernatant liquid through the same membrane filter. Wash the residue with small quantities of *water*, combine the filtrates and washings and dilute to 50.0 ml with *water*. To 20.0 ml of the solution add 50 mg of *L-ascorbic acid* and 1 ml of *strong ammonia solution*, neutralise with *2 M ammonia* and dilute to 25 ml with *water*. 12 ml of the solution complies with the limit test for heavy metals, Method D (25 ppm). Use *lead standard solution (1 ppm Pb)* to prepare the standard.

Chlorides (2.3.12). To 1.0 g add a mixture of 20 ml of *2 M nitric acid* and 30 ml of *water*, heat on a water-bath for 15 minutes, shaking frequently, dilute to 50 ml with *water* if necessary, filter and cool. The filtrate complies with the limit test for chlorides (250 ppm).

Loss on ignition (2.4.20). Not more than 5.0 per cent, determined on 0.2 g by igniting at 900° in a platinum crucible for 2 hours.

Assay. To the residue obtained in the test for Loss on ignition add 0.2 ml of *sulphuric acid* and sufficient *ethanol (95 per cent)* to moisten the residue completely, add 6 ml of *hydrofluoric acid* and evaporate to dryness on a hot plate at 95° to 105°, avoiding loss from sputtering. Wash the sides of the dish with 6 ml of *hydrofluoric acid*, evaporate to dryness in a well-ventilated hood, ignite at 1000°, allow to cool in a desiccator and weigh. The difference between the weight of the final residue and that of the residue obtained in the test for Loss on ignition represents the amount of SiO_2 in the amount of the substance taken for the test for Loss on ignition.

Storage. Store protected from light.

Silver Nitrate

$AgNO_3$ Mol. Wt. 169.9

Silver Nitrate contains not less than 99.0 per cent and not more than 100.5 per cent of $AgNO_3$.

Category. Local anti-infective.

Description. Colourless crystals or a white, crystalline powder.

Identification

A. It gives the reactions of silver salts (2.3.1).

B. 10 mg gives reaction (A) of nitrates (2.3.1).

Tests

Appearance of solution. A 4.0 per cent w/v solution (solution A) is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. To 2 ml of solution A add 0.1 ml of *bromocresol green solution*; the solution is blue. To 2 ml of the solution add 0.1 ml of *phenol red solution*; the solution is yellow.

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Aluminium, bismuth, copper and lead. Dissolve 1.0 g in a mixture of 4 ml of *strong ammonia solution* and 6 ml of *water*; the resulting solution is clear (2.4.1), and colourless (2.4.1).

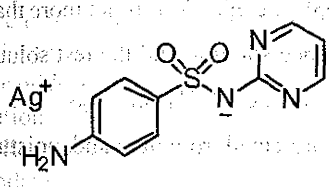
Foreign salts. Not more than 0.3 per cent, determined by the following method. To 30 ml of solution A add 7.5 ml of *2 M hydrochloric acid*, shake vigorously, heat for 5 minutes on a water-bath, filter and evaporate 20 ml of the filtrate to dryness on a water-bath. Dry the residue at 105° and weigh.

Assay. Weigh 0.3 g, dissolve in 50 ml of *water*, add 2 ml of *2 M nitric acid* and 2 ml of *ferric ammonium sulphate solution* and titrate with *0.1 M ammonium thiocyanate* until a reddish yellow colour is produced.

1 ml of *0.1 M ammonium thiocyanate* is equivalent to 0.01699 g of $AgNO_3$.

Storage. Store protected from light and moisture, in non-metallic containers.

Silver Sulphadiazine



$C_{10}H_9AgN_4O_2S$ Mol. Wt. 357.1

Silver Sulphadiazine is benzenesulphonamide, 4-amino-*N*-2-pyrimidinyl, monosilver(1+) salt.

Silver Sulphadiazine contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{10}H_9AgN_4O_2S$, calculated onthe dried basis.

Category. Antiinfective.

Description. A white to creamy-white crystalline powder. Stable in air, but turns yellow on exposure to light.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *silver sulphadiazene IPRS* or with the reference spectrum of silver sulphadiazene.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

C. Dissolve 1 g in 15 ml of *ammonium hydroxide* and 15 ml of *water*, dilute to 50 ml with *water* the solution complies with the tests for silver (2.3.1).

Tests

Nitrate. Not more than 0.1 per cent.

Test solution. Transfer 2.0 g of the substance under examination to a beaker, add 30.0 ml of *water* stir for 20 minutes, and filter through a suitable, nitrate-free filter.

Reference solution. A 0.033 per cent w/v solution of *potassium nitrate* in *water*.

Pipet 3.0 ml of the test solution and of deionized *water* to provide the blank into separate test tubes. Pipet 1.0 ml of the reference solution and 2.0 ml of *water* into a third test tube. Cool the three test tubes in an ice-bath. Slowly add 7.0 ml of cold chromotropic acid solution prepared by dissolving 50 mg of *chromotropic acid* in 100 ml of cold *sulphuric acid*, to each test tube, while swirling, and allow the test tubes to remain in the ice-bath for 3 minutes after the addition of the chromotropic acid solution. Remove the test tubes from the ice-bath and allow to stand for 30 minutes. Determine the absorbances of the reference solution and the test solution at the maximum at about 408 nm (2.4.7) against the blank solution. Calculate the nitrate content.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 5 volumes of *acetonitrile*, 6 volumes of *ammonium hydroxide* and 90 volumes of *water*.

Test solution. Dissolve 100 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution (a). A 0.0001 per cent w/v solution of *silver sulphadiazine IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.1 per cent w/v of *silver sulphadiazine IPRS* and 0.0001 per cent w/v, each of, *sulphanilic acid IPRS, sulphanilamide IPRS* and *sulphaguanidine* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as YMC-Pack Pro C18),
- mobile phase: A. a buffer solution prepared by dissolving 0.77 g of *ammonium acetate* in 900 ml of *water*, adjusted to pH 5.2 with *glacial acetic acid* and dilute to 1000 ml with *water*,

  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 275 nm,
- injection volume: 10 µl.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	98	2
4	98	2
16	40	60
20	40	60
22	20	80
32	20	80
34	98	2
40	98	2

Name	Relative retention time	Correction factor
Sulphanilic acid	0.27	3.7
Sulphaguanidine[1,2]	0.65	1.18
Sulphanilamide[2]	0.71	—
2-Aminopyrimidine[3]	0.82	2.08
Sulphadiazine	1.0	—
Acetylsulphadiazine[4]	1.14	—

[1]4-Amino-*N*-carbamimidoylbenzenesulphonamide,

[2]These impurities are controlled using the limit for any other secondary peak,

[3]Pyrimidin-2-amine,

[4]*N*-{4-[*N*-(Pyrimidin-2-yl) sulphamoyl]phenyl}acetamide.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to sulphaguanidine and sulphanilamide is not less than 2.0 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to sulphanilic acid and 2-aminopyrimidine, each of, is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent), the area of any peak corresponding to acetylsuphadiazine is not more than twice the area of principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of areas of all peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore the peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Silver content. 29.3 per cent to 30.5 per cent.

Transfer 500 mg to a beaker, add 150 ml of *water* and 50 ml of *nitric acid* and stir for 15 minutes. Titrate with *0.1M ammonium thiocyanate*, determining the end-point potentiometrically (2.4.25) using a silver-based indicator electrode and a double-junction reference electrode.

1 ml of *0.1 M ammonium thiocyanate* is equivalent to 0.01079 g of silver.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 1 hour.

Assay. Determine by liquid chromatography (2.4.14), using the chromatographic system and solvent mixture as described under Related substances.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution. A 0.005 per cent w/v solution of *silver sulphadiazine IPRS* in the solvent mixture.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_9AgN_4O_2S$.

Storage. Store protected from light and moisture.

Silver Sulphadiazine Cream

Silver Sulphadiazine Cream contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of $C_{10}H_9AgN_4O_2S$.

Usual strength. 1 per cent w/w.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *chloroform*, 40 volumes of *methanol* and 10 volumes of *ammonium hydroxide*.

Test solution. Transfer a quantity of cream containing 50 mg of Silver Sulphadiazine to a 50-ml conical flask, add 4 ml of *ammonium hydroxide* and mix until a smooth paste has been obtained. Add 16 ml of *methanol*, shake for 5 minutes, and filter.

Reference solution. Dissolve 35 mg of *silver sulphadiazine IPRS* in 4 ml of *ammonium hydroxide* and add 16 ml of *methanol*.

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Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 10 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.0 to 7.0, determined in the supernatant obtained from 5 per cent w/v of cream in *water*.

Microbial contamination (2.2.9). 1.0 g is free from *E. coli*, *Staphylococcus species*, *Pseudomonas aeruginosa* and 10 g is free from *Salmonella species* and *Shigella*.

Other tests. Comply with the tests stated under Creams.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 100 ml of *ammonium hydroxide* in *water* and dilute to 1000 ml with *water*.

Test solution. Transfer a portion of cream containing 10 mg of Silver Sulphadiazine to a 50-ml round-bottom centrifuge tube. Add about 30 ml of *methanol*, tightly seal the tube with a cap containing an inert line and mix using a vortex mixer for about 15 seconds. Centrifuge for 15 minutes. Aspirate, and discard the methanol *(Note — Care should be taken to avoid aspirating any of the residue)*. Transfer 5.0 ml of the internal standard solution into the tube and add 20 ml of *ethanol (95 per cent)*. Replace the cap and mix. Heat in a water-bath at 60° with periodic mixing for 15 minutes to melt and disperse the cream. While the mixture is still hot, transfer 10.0 ml of the solvent mixture to the tube, mix and cool to room temperature. Transfer 2.0 ml of the solution to another tube, add 40 ml of mobile phase, mix and filter.

Internal standard solution. A 0.2 per cent w/v solution of sulphamerazine in the solvent mixture.

Reference solution (a). A 0.1 per cent w/v solution of *silver sulphadiazine IPRS* in the solvent mixture.

Reference solution (b). Pipet 10.0 ml of reference solution (a) into a 50-ml test tube. Add 20 ml of *ethanol (95 per cent)* and 5.0 ml of the internal standard solution. Pipet 2.0 ml of the solution into a 50-ml test tube, add 40 ml of mobile phase and mix.

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 900 volumes of *water*, 99 volumes of *acetonitrile* and 1 volume of *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

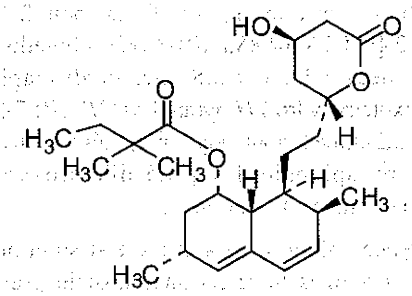
Inject reference solution (b). The test is not valid unless the resolution between the peaks due to silver sulphadiazine and sulphamerazine is not less than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (b) and the test solution.

Calculate the content of silver sulphadiazine in the cream.

Storage. Store protected from light and moisture.

Simvastatin



$C_{25}H_{38}O_5$ Mol.Wt. 418.6

Simvastatin is (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronapthalen-1-yl 2,2-dimethylbutyrate.

Simvastatin contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{25}H_{38}O_5$, calculated on the dried basis. A suitable antioxidant may be added.

Category. Antihyperlipidaemic.

Description. A white or almost white, crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *simvastatin IPRS* or with the reference spectrum of simvastatin.

Tests

Appearance of solution. A 1.0 per cent w/v solution in *methanol* is clear (2.4.1) and not more intensely coloured than reference solution BYS6 (2.4.1).

Specific optical rotation (2.4.22). +285° to +300°, determined in a 0.5 per cent w/v solution in *acetonitrile*.

Related substances. Determine by liquid chromatography (2.4.14), as described under the Assay.

The relative retention time with reference to simvastatin for (3*R*,5*R*)-7-[(1*S*,2*S*,6*R*,8*S*,8a*R*)-8-[(2,2-dimethylbutanoyl)oxy]-2,6-dimethyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]-3,5-

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dihydroxyheptanoic acid (hydroxy acid) (simvastatin impurity A) is about 0.45, for lovastatin (simvastatin impurity E) and epilovastatin (simvastatin impurity F) is about 0.6, for (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbut-3-enoate (simvastatin impurity G) is about 0.8, for (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-(acetyloxy)-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate (acetate ester) (simvastatin impurity B) is about 2.38, for (1*S*,3*R*,7*S*,8*S*,8a*R*)-3,7-dimethyl-8-[2-[(2*R*)-6-oxo-3,6-dihydro-2*H*-pyran-2-yl]ethyl]-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate (anhydro-simvastatin) (simvastatin impurity C) is about 2.42 and for (2*R*,4*R*)-2-[2-[(1*S*,2*S*,6*R*,8*S*,8a*R*)-8-[(2,2-dimethylbutanoyl) oxy]-2,6-dimethyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]ethyl]-6-oxotetrahydro-2*H*-pyran-4-yl (3*R*, 5*R*)-7-[(1*S*,2*S*,6*R*, 8*S*,8a*R*)-8-[(2,2-dimethylbutanoyl) oxy]-2,6-dimethyl-1,2,6,7,8, 8a-hexahydronaphthalen1-yl]-3,5-dihydroxyheptanoate (simvastatin impurity D) is about 3.8.

Inject reference solution (b) and the test solution. Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of the secondary peaks corresponding to lovastatin and epilovastatin is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), the area of any secondary peak other than lovastatin and epilovastatin is not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent) and the sum of the areas of all the secondary peaks other than lovastatin and epilovastatin is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13).). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 60° for 3 hours under high vacuum.

Assay. Determine by liquid chromatography (2.4.14).

NOTE—Prepare the solutions immediately before use.

Solvent mixture. 40 volumes of a 0.14 per cent w/v solution of *potassium dihydrogen phosphate*, adjusted to pH 4.0 with *orthophosphoric acid* and 60 volumes of *acetonitrile*.

Test solution. Dissolve 75 mg of the substance under examination in 50.0 ml of the solvent mixture.

Reference solution (a). Dissolve 1 mg each of *simvastatin IPRS* and *lovastatin IPRS* in 50.0 ml of the solvent mixture.

Reference solution (b). Dilute 0.5 ml of the test solution to 100.0 ml with the solvent mixture.

Reference solution (c). Dissolve 75 mg of *simvastatin IPRS* in 50.0 ml of the solvent mixture.

Chromatographic system

- a stainless steel column 3.3 cm x 4.6 mm, packed with end-capped octadecylsilane bonded to porous silica (3 µm),
- mobile phase: A. a mixture 50 volumes of *acetonitrile* and 50 volumes of a 0.1 per cent v/v solution of *orthophosphoric acid*,

  B. 0.1 per cent v/v solution of *orthophosphoric acid* in *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 3.0 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 5 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
4.5	100	0
4.6	95	5
8.0	25	75
11.5	25	75
11.6	100	0
13	100	0

Inject reference solution (a). The test is not valid unless resolution between the peaks due to lovastatin and epilovastatin and simvastatin is not less than 4.0.

Inject reference solution (c) and the test solution.

Calculate the content of simvastatin.

Storage. Store protected from light and moisture, under nitrogen.

Simvastatin Tablets

Simvastatin Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of simvastatin, $C_{25}H_{38}O_5$.

Usual strengths. 10 mg; 20 mg; 40 mg.

Identification

Shake a quantity of the powdered tablets containing about 50 mg of Simvastatin with 20 ml of *dichloromethane*, filter and

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evaporate the filtrate to dryness using a rotary evaporator and a water-bath at 40°. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *simvastatin IPRS* or with the reference spectrum of simvastatin.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.01 M sodium dihydrogen orthophosphate* containing 0.5 per cent w/v of *sodium dodecyl sulphate*, adjusted to pH 7.0 with *1 M sodium hydroxide*,

Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary in the dissolution medium.

Reference solution. Dissolve a quantity of *simvastatin IPRS* in *methanol* and dilute with the dissolution medium to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Use the chromatographic system as described under Assay.

Q. Not less than 70 per cent of the stated amount of $C_{25}H_{38}O_5$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Prepare the solutions immediately before use.

Buffer solution. A 0.51 per cent w/v solution of *sodium dihydrogen orthophosphate*, adjusted to pH 4.5 with 25 per cent v/v solution of *orthophosphoric acid*.

Solution A. A mixture of 1 volume of 0.3 per cent v/v solution of *glacial acetic acid*, adjusted to pH 4.0 with *1 M sodium hydroxide* and 4 volumes of *acetonitrile*.

Test solution. Shake a quantity of the powdered tablets containing about 25 mg of Simvastatin with 20 ml of solution A for 15 minutes, dilute to 100 ml and filter.

Reference solution (a). Dilute 1 ml of the test solution to 500 ml with solution A.

Reference solution (b). A solution of 0.002 per cent w/v each of *simvastatin IPRS* and *lovastatin IPRS* in solution A.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (3 µm) (Such as Hypersil ODS),
- mobile phase: A. a mixture of 40 volumes of *acetonitrile* and 60 volumes of buffer solution,

  B. a mixture of 20 volumes of buffer solution and 80 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
5	85	15
30	0	100
45	0	100
46	85	15
50	85	15

The relative retention time with reference to simvastatin for simvastatin hydroxy acid (simvastatin impurity A) is about 0.52, for lovastatin (simvastatin impurity E) is about 0.71, for epilovastatin (simvastatin impurity F) is about 0.74, for anhydrosimvastatin (simvastatin impurity C) is about 1.74 and for simvastatin dimer (simvastatin impurity D) is about 2.42.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to simvastatin and lovastatin is not less than 3.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any peaks corresponding to lovastatin, epilovastatin and anhydrosimvastatin is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent), the area of any peak corresponding to simvastatin impurity A is not more than 7.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent), the area of any other secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent) and the sum of the areas of all the secondary peaks other than any peaks corresponding to lovastatin, epilovastatin, anhydrosimvastatin and simvastatin impurity A, is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than 0.25 times of the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solution A. Add 3 ml of *glacial acetic acid* to 900 ml of *water*, adjusted to pH 4.0 with *1 M sodium hydroxide* and add sufficient *water* to produce 1000 ml. Mix 1 volume of this solution with 4 volumes of *acetonitrile*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 100 mg of

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Simvastatin, disperse in 100 ml of solution A and filter. Dilute 5.0 ml isf the solution to 50.0 ml with solution A.

Reference solution. A 0.01 per cent w/v solution of *simvastatin IPRS* in solution A.

Chromatographic system

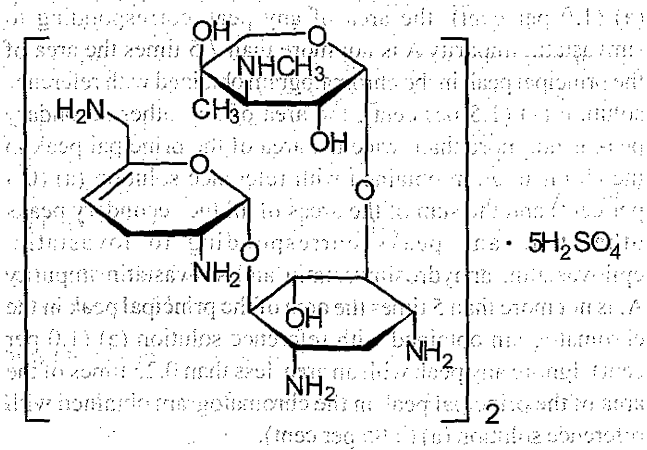
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- column temperature: 45°,
- mobile phase: a mixture of 7 volumes of a buffer solution prepared by dissolving 5.1 g of *sodium dihydrogen orthophosphate* in 900 ml of *water*, adjusted to pH 4.5 with *orthophosphoric acid* or *1 M sodium hydroxide* and add sufficient *water* to produce 1000 ml and 13 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor of the principal peak is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of $C_{25}H_{38}O_5$ in the tablets.

Sisomicin Sulphate



$(C_{19}H_{37}N_5O_7)_2,5H_2SO_4$ Mol. Wt. 1385.5

Sisomicin Sulphate is 4-*O*-[(2*R*,3*R*)-*cis*-3-Amino-6-aminomethyl-3,4-dihydro-2*H*-pyran-2-yl]-2-deoxy-6-*O*-(3-deoxy-4-*C*-methyl-3-methylamino-β-*L*-arabinopyranosyl) streptamine sulphate.

Sisomicin Sulphate has a potency not less than 580 µg of sisomicin per mg, calculated on the dried basis.

Category. Antibacterial.

Description. A off-white powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 60 volumes of *methanol*, 30 volumes of *ammonium hydroxide* and 25 volumes of *chloroform*.

Test solution. A 1.0 per cent w/v solution of the substances under examination.

Reference solution (a). A 1.0 per cent w/v solution of *sisomicin IPRS*.

Reference solution (b). A mixture of equal volumes of the test solution and reference solution (a).

Apply to the plate 5 µl of each solution. After development, allow the plate to dry in air, heat it at 110° for 15 minutes and immediately spray it with a 1.0 per cent solution of *ninhydrin* in *butanol* containing 1.0 per cent of *pyridine*. The principal brown spot in the chromatogram obtained with the test solution corresponds to those in the chromatograms obtained with reference solution (a) and (b).

B. It gives reaction (A) of sulphates (2.3.1).

Tests

pH (2.4.24). 3.5 to 5.5 determined in a 4.0 per cent w/v solution.

Specific optical rotation (2.4.22). +100° to +110°, determined in a 1.0 per cent w/v solution in *water*.

Loss on drying (2.4.19). Not more than 15.0 per cent, determined on 0.1 g by drying in an oven at 110° at a pressure of 5 mm of mercury for 3 hours.

Residue on ignition. Not more than 1.0 per cent, the charred residue being moistened with 2 ml of *nitric acid* and 5 drops of *sulphuric acid*.

Assay. Determine by the microbiological assay of antibiotics, Method A (2.2.10), and express the result in µg of sisomicin per mg.

Storage. Store protected from moisture.

Sisomicin Sulphate Injection

Sisomicin Sulphate Injection is a sterile solution of Sisomicin Sulphate in Water for Injection. It may contain one or more suitable buffers, chelating agents and preservatives.

Sisomicin injection contains not less than 90.0 per cent and not more than 120.0 per cent of stated amount of sisomicin, $C_{19}H_{37}N_5O_7$.

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Usual strengths. 10 mg per ml; 50 mg per ml.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 60 volumes of *methanol*, 30 volumes of *ammonium hydroxide* and 25 volumes of *chloroform*.

Test solution. Dilute a suitable volume of injection (if required) to contain 1.0 per cent w/v solution of sisomicin sulphate.

Reference solution (a). A 1.0 per cent w/v solution of *sisomicin IPRS*.

Reference solution (b). A mixture of equal volumes of the test solution and reference solution (a).

Apply to the plate 5 µl of each solution. After development, allow the plate to dry in air, heat it at 110° for 15 minutes and immediately spray it with a 1.0 per cent solution of *ninhydrin* in *butanol* containing 1.0 per cent of *pyridine*. The principal brown spot in the chromatogram obtained with the test solution corresponds to those in the chromatograms obtained with reference solution (a) and (b).

Tests

pH (2.4.24). 2.5 to 5.5.

Bacterial endotoxins (2.2.3). Not more than 0.50 Endotoxin Unit per mg of sisomicin.

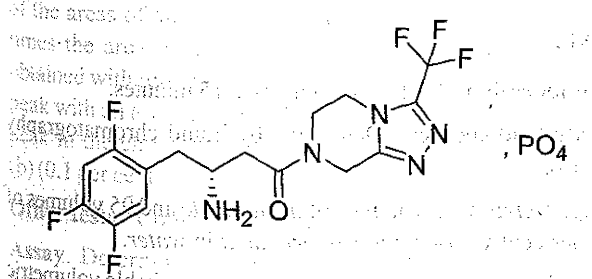
Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine the microbiological assay of antibiotics, Method A (2.2.10) and express the result in mg of sisomicin, $C_{19}H_{37}N_5O_7$ per ml.

Storage. Store in single dose or in multiple dose container. Preferable of type 1 glass.

Sitagliptin Phosphate

Sitagliptin Phosphate Monohydrate



$C_{16}H_{18}F_6N_5O_5P, H_2O$ Mol. Wt 523.3

Sitagliptin Phosphateis is (3R)-3-amino-1-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8H)-yl]-4-(2,4,5-trifluorophenyl)butan-1-one phosphate monohydrate.

Sitagliptin Phosphate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{16}H_{18}F_6N_5O_5P$, calculated on the anhydrous basis.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sitagliptin phosphate IPRS* or with the reference spectrum of sitagliptin phosphate.

B. In the Enantiomeric purity test, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with reference solution (b).

C. It gives reaction (A) of phosphates (2.3.1).

Tests

Enantiomeric purity. Determine by liquid chromatography (2.4.14).

Solvent mixture. 10 volumes of *water* and 90 volumes of *methanol*.

Test solution. Dissolve 80 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution (b). A 0.8 per cent w/v solution of *sitagliptin containing impurity A {(3S)-3-amino-1-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-4-(2,4,5-trifluorophenyl)butan-1-one(S-enantiomer)} IPRS* in the solvent mixture.

Chromatographic system
- a column 25 cm x 4.6 mm packed with amylose derivative of silica gel for chiral separation (5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 1 volume of *water*, 1 volume of *diethylamine*, 400 volumes of *heptane*, and 600 volumes of *ethanol*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 268 nm,
- injection volume: 10 µl.

The relative retention time with reference to sitagliptin for sitagliptin impurity A is about 0.9.

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Inject reference solution (b). The test is not valid unless the resolution between the peaks due to sitagliptin and sitagliptin impurity A is not less than 1.5.

Inject reference solution (a) and the test solution. Run the chromatogram 1.6 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to sitagliptin impurity A is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 5 volumes of *acetonitrile* and 95 volumes of a 0.1 per cent v/v solution of *orthophosphoric acid*.

Test solution. Dissolve 25 mg of the substance under examination in the solvent mixture and dilute to 250.0 ml with the solvent mixture.

Reference solution (a). A 0.01 per cent w/v solution of *sitagliptin phosphate IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Reference solution (c). In order to prepare sitagliptin fumarate adduct in-situ, place 10 mg of *sitagliptin phosphate IPRS* and 1 mg of *sodium stearyl fumarate* in a vial, add 1 ml of *water* and tightly seal the vial. Heat at 80° for 48 hours. Transfer the contents of the vial into a 100-ml volumetric flask using a small amount of the solvent mixture. Rinse the vial three times with the solvent mixture. Transfer the rinsates into the flask and dilute to 100.0 ml with the solvent mixture and centrifuge. Use the supernatant liquid.

Chromatographic system

- a column 15 cm x 4.6 mm packed with endcapped cyanopropylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 15 volumes of *acetonitrile* and 85 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in about 900 ml of *water*, adjusted to pH 2.0 with *orthophosphoric acid* and dilute to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

The relative retention time with reference to the sitagliptin peak for the fumarate adduct is about 1.2.

Inject reference solution (c). The test is not valid unless the resolution between sitagliptin and the fumarate adduct is not less than 1.0.

Inject reference solution (a), (b) and the test solution. Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.2 per cent, determined in platinum crucible.

Water (2.3.43). 3.3 per cent to 3.7 per cent, determined on 0.3 g.

Assay. Determine by liquid chromatography (2.4.14) as described under test for Related substances.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 1.5 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{16}H_{18}F_6N_5O_5P$.

Storage. Store at a temperature not exceeding 30°.

Sitagliptin Tablets

Sitagliptin Phosphate Tablets

Sitagliptin Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sitagliptin, $C_{16}H_{15}F_6N_5O$.

Usual strengths. 25 mg; 50 mg; 100 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Disintegration (2.5.1). Not more than 15 minutes.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 5 volumes of *acetonitrile* and 95 volumes of 0.1 per cent v/v *orthophosphoric acid* in *water*.

Test solution. Transfer 10 intact tablets to a suitable volumetric flask and dilute to volume with the solvent mixture to obtain a

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concentration of 0.1 per cent w/v of sitagliptin. Stir vigorously for 1 hour. Transfer 8.0 ml of the solution to a 100-ml volumetric flask and dilute with the solvent mixture to volume and mix. Centrifuge a portion of the solution until clear and use the supernatant.

Reference solution (a). A 0.01 per cent w/v solution of *sitagliptin phosphate IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Reference solution (c). In order to prepare sitagliptin fumarate adduct in-situ, place 10 mg of *sitagliptin phosphate IPRS* and 1 mg of *sodium stearyl fumarate* in a vial, add 1 ml of *water* and tightly seal the vial. Heat at 80° for 48 hours. Transfer the contents of the vial into a 100-ml volumetric flask using a small amount of the solvent mixture. Rinse the vial three times with the solvent mixture. Transfer the rinsates into the flask and dilute to 100.0 ml with the solvent mixture and centrifuge. Use the supernatant liquid.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with cyanopropylsilane bonded to porous silica (5 µm) (Such as Supelco Discovery Cyano),
- mobile phase: a mixture of 15 volumes of *acetonitrile* and 85 volumes of buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in about 900 ml of *water*, adjusted to pH 2.0 with *orthophosphoric acid* and dilute to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

The relative retention time with reference to the sitagliptin peak for the fumarate adduct is about 1.2.

Inject reference solution (c). The test is not valid unless the resolution between sitagliptin and the fumarate adduct is not less than 1.0.

Inject reference solution (b) and the test solution. Run the chromatogram about 6 times the retention time of the principal peak. The area of any secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14.) as described under test for Related substances with the following modifications.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{16}H_{15}F_6N_5O$ in the tablets.

Storage. Store protected from moisture.

Labelling. The label states the quantity of active ingredients in terms of the equivalent amount of sitagliptin.

Sodium Acetate

$CH_3COONa,3H_2O$

$C_2H_3NaO_2, 3H_2O$ Mol. Wt. 136.1

Sodium Acetate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_2H_3NaO_2$, calculated on the dried basis.

Category. Pharmaceutical aid (for peritoneal dialysis fluids).

Description. Colourless crystals or a white, crystalline powder.

Identification

Dissolve 10.0 g in sufficient *carbon dioxide-free water* to produce 100.0 ml (solution A). 1 ml of solution A gives reaction (B) of acetates and reaction (A) of sodium salts (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

pH (2.4.24). 7.5 to 9.0, determined in a 5.0 per cent w/v solution.

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water* and add 15 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Calcium and magnesium. Not more than 50 ppm, calculated as Ca, determined by the following method. Mix 200 ml of *water* with 10 ml of *ammonia buffer pH 10.0*, 0.1 g of *mordant black 11 mixture* and 2 ml of *0.05 M zinc chloride*. Add dropwise *0.02 M disodium edetate* until the colour changes from violet to blue. To this solution add 10 g of the substance under examination, shake to dissolve and titrate with *0.02 M disodium edetate* until the blue colour is restored. Not more than 0.65 ml of *0.02 M disodium edetate* is required.

Heavy metals (2.3.13). 12 ml of solution A complies with the limit test for heavy metals, Method D (10 ppm), using 10 ml of *lead standard solution (1 ppm Pb)*.

Iron (2.3.14). 20 ml of solution A complies with the limit test for iron (20 ppm).

Chlorides (2.3.12). 10 ml of solution A complies with the limit test for chlorides (250 ppm).

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Sulphates (2.3.17). 7.5 ml of solution A complies with the limit test for sulphates (200 ppm).

Reducing substances. Dissolve 1.0 g in 100 ml of boiling *water*, add 5 ml of *1 M sulphuric acid* and 0.5 ml of *0.002 M potassium permanganate*, mix and boil gently for 5 minutes; the pink colour is not completely discharged.

Loss on drying (2.4.19). 39.0 to 40.5 per cent, determined on 0.2 g by drying in an oven at 130°. Place the substance under examination in the oven while the oven is still cold.

Assay. Dissolve 0.25 g in 50 ml of *anhydrous glacial acetic acid*, add 5 ml of *acetic anhydride*, mix and allow to stand for 30 minutes. Titrate with *0.1 M perchloric acid*, using 0.3 ml of *1-naphtholbenzein solution* as indicator, until a green colour is produced. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.00820 g of $C_2H_3NaO_2$.

Sodium Acetate intended for use in the preparation of dialysis solutions complies with the following additional requirement.

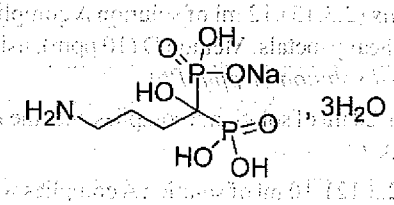
Aluminium. Dissolve 20 g in 100 ml of *water* and adjust to pH 6.0 by the addition of about 10 ml of *1 M hydrochloric acid*. Extract with successive quantities of 20, 20 and 10 ml of a 0.5 per cent w/v solution of *8-hydroxyquinoline* in *chloroform* and dilute the combined extracts to 50 ml with *chloroform*. Use as the standard solution a mixture of 2.0 ml of *aluminium standard solution (2 ppm Al)*, 10 ml of *acetate buffer pH 6.0* and 98 ml of *water* treated in the same manner and as the blank solution a mixture of 10 ml of *acetate buffer pH 6.0* and 100 ml of *water* treated in the same manner. Measure the fluorescence (2.4.5) of the test solution and the standard solution, using an excitation wavelength of about 392 nm and emission wavelength of about 518 nm, and setting the instrument to zero with the blank solution in each case. The fluorescence of the test solution is not more than that of the standard solution (0.2 ppm).

Storage. Store protected from light.

Labelling. The label states whether or not the material is intended for use in the manufacture of dialysis solutions.

Sodium Alendronate Trihydrate

Sodium Alendronate



$C_4H_{12}NNaO_7P_2, 3H_2O$ Mol. Wt. 325.1

Sodium Alendronate Trihydrate is monosodium trihydrogen (4-amino-1-hydroxybutylidene)bisphosphonate trihydrate.

Sodium Alendronate Trihydrate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_4H_{12}NNaO_7P_2$, calculated on the dried basis.

Category. Bisphosphonate; treatment of osteoporosis.

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sodium alendronate IPRS* or with the reference spectrum of sodium alendronate.

B. It gives reaction (a) of sodium salt (2.3.1).

Tests

Appearance of solution. A 1.0 per cent w/v solution is clear and not more intensely coloured than reference solution BYS7 (2.4.1).

pH (2.4.24). 4.0 to 5.0 determined in a 1.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Solution A. Dissolve 29.4 g of *sodium citrate* in *water* and dilute to 1000 ml with the same solvent.

Solution B. Dissolve 19.1 g of *disodium tetraborate* in *water* and dilute to 1000 ml with the same solvent.

Solution C. Dissolve 0.200 g of *(9-fluorenyl) methyl chloroformate* in *acetonitrile* and dilute to 50.0 ml with the same solvent.

NOTE — Prepare solutions immediately before use.

Test solution. Dissolve 30 mg of the substance under examination in 50 ml of solution A. Transfer 5.0 ml of the solution to a 50 ml polypropylene screw cap centrifuge tube containing 5.0 ml of solution B. Add 5.0 ml of *acetonitrile* and 5.0 ml of solution C. Shake and allow to stand at room temperature for 30 minutes. Add 20 ml of *methylene chloride* and shake vigorously for one minute. Centrifuge for 5-10 minutes and use the clear upper layer.

Reference solution (a). Dissolve 15 mg of *4-aminobutanoic acid* (impurity A) in 100.0 ml of solution A. Dilute 10.0 ml of the solution to 50.0 ml with solution A. Transfer 5.0 ml of the solution to a 50 ml polypropylene screw cap centrifuge tube containing 5.0 ml of solution B. Add 5.0 ml of *acetonitrile* and 5.0 ml of solution C. Shake and allow to stand at room temperature for 30 minutes. Add 20 ml of *methylene chloride* and shake vigorously for one minute. Centrifuge for 5-10 minutes and use the clear upper layer.

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Reference solution (b). Dissolve 3 mg of *sodium alendronate IPRS* (containing impurity D) and dilute to 5.0 ml with solution A. Transfer this solution to a 50 ml polypropylene screw cap centrifuge tube containing 5.0 ml of solution B. Add 5.0 ml of *acetonitrile* and 5.0 ml of solution C. Shake and allow to stand at room temperature for 30 minutes. Add 20 ml of *methylene chloride* and shake vigorously for one minute. Centrifuge for 5 to 10 minutes and use the clear upper layer.

Blank solution. Transfer 5 ml of solution A to a 50 ml polypropylene screw cap centrifuge tube containing 5.0 ml of solution B. Add 5.0 ml of *acetonitrile* and 5.0 ml of solution C. Shake and allow to stand at room temperature for 30 minutes. Add 20 ml of *methylene chloride* and shake vigorously for a minute. Centrifuge for 5 to 10 minutes and use the clear upper layer.

Chromatographic system

- a stainless steel column 25 cm x 4.1 mm, packed with styrene-divinylbenzene copolymer (10 µm)
- column temperature: 45°,
- mobile phase: A. 15 volumes of *acetonitrile* and 85 volumes of a buffer solution prepared by dissolving 2.84 g of *anhydrous disodium hydrogen phosphate* and 5.88 g of *sodium citrate* in 2000 ml *water*, adjusted to pH 8.0 with *orthophosphoric acid* or *sodium hydroxide*,
  B. 70 volumes of *acetonitrile* and 30 volumes of a buffer solution prepared by dissolving 2.84 g of *anhydrous disodium hydrogen phosphate* and 5.88 g of *sodium citrate* in 2000 ml *water*, adjusted to pH 8.0 with *orthophosphoric acid* or *sodium hydroxide*,
- a gradient programme using the conditions given below,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 266 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
3	100	0
18	50	50
28	0	100
35	100	0

The retention time of alendronate is about 7 minutes.

The relative retention time with reference to alendronate for impurity D and impurity A is about 1.4 and 1.9 respectively.

Inject reference solution (a) and reference solution (b) to identify the peak due to impurity A and impurity D.

Inject the reference solution (b). The test is not valid unless the resolution between the peak due to alendronate and impurity D is not less than 5.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to impurity A multiply by correction factor 0.4 is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent), the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent), calculated by area normalization.

Impurities B and C. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50.0 mg of the substance under examination in *water* and dilute to 25.0 ml with *water*.

Reference solution (a). Dissolve 50.0 mg of *sodium alendronate trihydrate IPRS* in *water* and dilute to 25.0 ml with the *water*.

Reference solution (b). Dissolve 3.0 g of *phosphoric acid* in *water* and dilute to 100.0 ml with the *water*. Dilute 1.0 ml of the solution to 100.0 ml with *water*.

Reference solution (c). Dissolve 2.5 g of *phosphorous acid* in *water* and dilute to 100.0 ml with the *water*. Dilute 1.0 ml of the solution to 100.0 ml with *water*.

Reference solution (d). Mix 2.0 ml of reference solution (b) and 2.0 ml of reference solution (c) and dilute to 50.0 ml with *water*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with anion-exchange resin containing quaternary ammonium groups [$CH_2N^+(CH_3)_3$] attached to a lattice consisting of methacrylate (7 µm),
- column temperature: 35°,
- mobile phase: a solution of 0.2 ml of *anhydrous formic acid* in 1000 ml of *water*, adjusted the pH to 3.5 with *2 M sodium hydroxide*,
- flow rate: 1.2 ml per minute,
- refractometer,
- injection volume: 100 µl.

The retention time of alendronate is about 16 minutes.

The relative retention time with reference to alendronate for impurity B and impurity C is about 1.3 and 1.6 respectively.

Inject reference solution (b), (c). Run the chromatogram twice the retention time of the principal peak. Identify the peak due to impurity B and impurity C.

Inject reference solution (d) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to impurity B is not more than 0.83 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent) and impurity C is not

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more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Between 16.1 per cent to 17.1 per cent, determined on 1.0 g by drying in an oven at 140° to 145°.

Assay. Determine by liquid chromatography (2.4.14), as described under Impurities B and C with the following modifications.

Inject the reference solution (a) and the test solution.

Calculate the content of $C_4H_{12}NNaO_7P_2$.

Sodium Alendronate Tablets

Alendronic Acid Tablets; Alendronate Sodium Tablets

Sodium Alendronate Tablets contains Sodium Alendronate Trihydrate.

Sodium Alendronate Tablets contain sodium alendronate equivalent to not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of alendronic acid , $C_4H_{13}NO_7P_2$.

Usual strength. 10 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with *water*, to produce a solution containing 0.00084 per cent w/v of alendronic acid.

Reference solution. A 0.0011 per cent w/v solution of *sodium alendronate IPRS* in *water*.

Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with anion exchange resin (7 µm) (Such as Allsep Anion),
- column temperature: 35°,
- mobile phase: a 0.02 per cent v/v solution of *formic acid*, pH adjusted to 3.5 with *2 M sodium hydroxide*,
- flow rate: 1.2 ml per minute,
- refractive index detector, maintained at 40°,
- injection volume: 100 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_4H_{13}NO_7P_2$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_4H_{13}NO_7P_2$.

Phosphate and phosphite. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets containing 0.1 g of alendronic acid with 20 ml of *water* and mix with the aid of ultrasound for 30 minutes. Add sufficient *water* to produce 25.0 ml and filter.

Reference solution. A solution containing 0.0024 per cent w/v of *orthophosphoric acid* and 0.002 per cent w/v of *phosphorous acid* in *water*.

Use the chromatographic system as described under Dissolution.

Run the chromatogram twice the retention time of the principal peak.

Inject the reference solution. The relative retention time with reference to alendronic acid (retention time, about 17 minutes) are: phosphate, about 1.3; phosphite, about 1.6.

Inject the reference solution. The test is not valid unless the signal to noise ratios of the peaks due to phosphate and phosphite are not less than 10.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of each peak due to phosphate and phosphite is not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.5 per cent).

4-Aminobutanoic acid. Determine by liquid chromatography (2.4.14).

Buffer solution. A solution containing 0.294 per cent w/v of *sodium citrate* and 0.142 per cent w/v of *anhydrous disodium hydrogen orthophosphate*, adjusted to pH 8.0 using *orthophosphoric acid* and filter.

Test solution. Shake a quantity of the powdered tablets containing 23 mg of alendronic acid with a 2.94 per cent w/v solution of *sodium citrate* and dilute to 50.0 ml with the same solvent, mix and filter; discard the first 5 ml of filtrate. To 5 ml of the filtrate in a screw cap centrifuge tube, add 5 ml of a 1.91 per cent w/v solution of *sodium borate*, 10 ml of a 0.2 per cent w/v solution of (9-fluorenyl)methyl chloroformate in *acetonitrile*, shake for 1 minute and allow to stand at room

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temperature for 30 minutes, add 20 ml of *dichloromethane* and shake vigorously for 1 minute; centrifuge and use the aqueous layer.

Reference solution (a). Dilute 5.0 ml solution containing 0.0003 per cent w/v of *4-aminobutanoic acid* in a 2.94 per cent w/v solution of *sodium citrate* in a screw cap centrifuge tube add 5 ml of a 1.91 per cent w/v solution of *sodium borate*, 10 ml of a 0.2 per cent w/v solution of (9-fluorenyl)methyl chloroformate in *acetonitrile*, shake for 45 seconds and allow to stand at room temperature for 30 minutes, add 20 ml of *dichloromethane* and shake vigorously for 1 minute, centrifuge and use the aqueous layer.

Reference solution (b). Dilute 5.0 ml solution containing 0.06 per cent w/v of *sodium alendronate IPRS* and 0.01 per cent w/v of *4-aminobutanoic acid* in a 2.94 per cent w/v solution of *sodium citrate* in a screw cap centrifuge tube add 5 ml of a 1.91 per cent w/v solution of *sodium borate*, 10 ml of a 0.2 per cent w/v solution of (9-fluorenyl)methyl chloroformate in *acetonitrile*, shake for 45 seconds and allow to stand at room temperature for 30 minutes, add 20 ml of *dichloromethane* and shake vigorously for 1 minute; centrifuge and use the aqueous layer.

Chromatographic system

- a stainless steel column 25 cm × 4.1 mm, packed with styrene-divinylbenzene co-polymer (10 µm) (Such as Hamilton PRP-1),
- column temperature: 45°,
- mobile phase: A. a mixture of 15 volumes of *acetonitrile* and 85 volumes of buffer solution;
  B. a mixture of 70 volumes of *acetonitrile* and 30 volumes of buffer solution,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 266 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
15	100	0
25	50	50
27	0	100
32	100	0
40	100	0

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to sodium alendronate and aminobutanoic acid, is not less than 10.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of peak due to 4-aminobutanoic acid is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent).

Uniformity of content. Complies with tests stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay with the following modification.

Test solution. Disperse 1 tablet in 10 ml of *water*, with the aid of ultrasound for 20 minutes and dilute to 25.0 ml with *water* and filter.

Calculate the content of $C_4H_{13}NO_7P_2$ in the tablet.

Other tests. Comply with the tests stated under Tablet.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of alendronic acid with 20 ml of *water* for 30 minutes with the aid of ultrasound and occasional shaking and agitate until cool, add sufficient *water* to produce 25.0 ml, mix and filter

Reference solution. A 0.25 per cent w/v solution of *sodium alendronate IPRS* in *water*.

Use chromatographic system as described under Dissolution.

Inject the reference solution and the test solution.

Calculate the content of $C_4H_{13}NO_7P_2$ in the tablets.

1 mg of $C_4H_{12}NNaO_7P_2,3H_2O$ is equivalent to 0.7662 mg of alendronic acid, $C_4H_{13}NO_7P_2$.

Labelling. The label states the strength in terms of the equivalent amount of alendronic acid.

Sodium Alginate

Sodium Polymannuronate

Sodium Alginate consists mainly of the sodium salt of alginic acid which is a mixture of polyuronic acids $[(C_6H_8O_6)_n]$ composed of residues of D-mannuronic acid and L-guluronic acids and is obtained mainly from algae belonging to the order Phaeophyceae.

Category. Pharmaceutical aid (viscosity-increasing agent).

Description. A cream-coloured to pale yellowish brown powder.

Identification

A. Dissolve 0.2 g with shaking in 20 ml of *water* and to 5 ml of the resulting solution add 1 ml of *calcium chloride solution*; a voluminous gelatinous precipitate is produced.

B. To 10 ml of the solution obtained in test A add 1 ml of *1 M sulphuric acid*; a gelatinous mass is produced.

C. To 5 mg add 5 ml of *water*, 1 ml of a freshly prepared 1 per cent w/v solution of *naphthalene-1,3-diol* in *ethanol (95 per*

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cent) and 5 ml of *hydrochloric acid*. Boil for 3 minutes, cool, add 5 ml of *water* and shake with 15 ml of *di-isopropyl ether*. The upper layer exhibits a deeper bluish red colour than the upper layer obtained by repeating the procedure without the substance under examination.

D. The residue obtained in the test for Sulphated ash gives reaction (A) of sodium salts (2.3.1).

Tests

Heavy metals (2.3.13). 0.5 g complies with the limit test for heavy metals, Method B (40 ppm), using *nitric acid* instead of *sulphuric acid* for wetting the sample.

Chloride. Not more than 1.0 per cent, determined by the following method. Shake 2.5 g with 50 ml of *2 M nitric acid* for 1 hour, dilute to 100 ml with *2 M nitric acid* and filter. To 50 ml of the filtrate add 10.0 ml of *0.1 M silver nitrate* and 5 ml of *toluene*. Titrate with *0.1 M ammonium thiocyanate* using 2 ml of *ferric ammonium sulphate solution* as indicator and shaking vigorously towards the end-point. Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of Cl.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 10^3 CFU per g. 1 g is free from *Escherichia coli* and 10 g is free from *Salmonella* and *Shigella*.

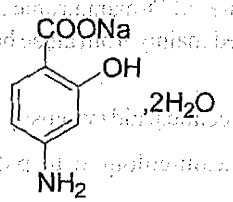
Sulphated ash (2.3.18). 30.0 to 36.0 per cent, determined on 0.1 g and calculated on the dried basis.

Loss on drying (2.4.19). Not more than 15.0 per cent, determined on 0.2 g by drying in an oven at 105° for 4 hours.

Storage. Store protected from light.

Sodium Aminosalicylate

Sodium PAS



$C_7H_6NNaO_3,2H_2O$. Mol. Wt. 211.2

Sodium Aminosalicylate is sodium 4-amino-2-hydroxybenzoate dihydrate.

Sodium Aminosalicylate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_7H_6NNaO_3$, calculated on the anhydrous basis.

Category. Antitubercular.

Description. A white to cream coloured crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sodium aminosalicylate IPRS* or with the reference spectrum of sodium aminosalicylate.

B. A 5 per cent w/v solution complies with the tests for sodium salts (2.3.1).

Tests

pH (2.4.24). 6.5 to 8.5, determined in a 2.0 per cent w/v solution.

3-aminophenol. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50.0 mg of the substance under examination in 100 ml of the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *3-aminophenol IPRS* in 100.0 ml of the mobile phase (solution A). Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase. Dilute 5.0 ml of the resulting solution to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 25.0 mg of *sodium aminosalicylate IPRS* in 100.0 ml of the mobile phase. Dilute 5.0 ml of the solution and 5 ml of solution A to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous base deactivated silica (5 µm) (Such as Hypersil BDS),
- mobile phase: dissolve 6.0 g of *disodium hydrogen orthophosphate* and 6.6 g of *sodium dihydrogen orthophosphate dihydrate* in 1600 ml with *water*, add 19 ml of *tetra n-butyl ammonium hydroxide (20 per cent solution)* and make the volume to 1700 ml with *water* and add 300 ml of *methanol*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (a). The relative standard deviation for replicate injections is not more than 5.0 per cent

Inject reference solution (b). The test is not valid unless the resolution between *m*-aminophenol and sodium aminosalicylate is at least 5.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of the peak corresponding to 3-aminophenol is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.25 per cent).

Hydrogen Sulphide and Sulphur dioxide. Dissolve 0.5 g in 5 ml of *1 M sodium hydroxide*, add 6 ml of *3 M hydrochloric acid* and stir vigorously. No odour of hydrogen sulphide or sulphur dioxide is perceptible, and not more than a faint odour of Amyl Alcohol is perceptible. A piece of moistened *lead*

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acetate paper held over the mixture does not become discoloured.

Arsenic (2.3.10). Mix 5.0 g with 10 ml of *bromine solution* and evaporate to dryness on a water-bath, ignite gently, dissolve the cooled residue, ignoring any carbon, in 50 ml of *water* and 14 ml of *brominated hydrochloric acid AsT* and remove the excess bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). 0.66 g complies with the limit test for heavy metals, Method B (30 ppm).

Chlorides (2.3.12). Dissolve 1.0 g in 10 ml of *water*, add 3 ml of *acetic acid* and filter, dilute the filtrate to 50 ml with *water*. 5.0 ml of the solution complies with the limit test for chlorides (0.25 per cent).

Sulphates (2.3.17). 0.1 g complies with the limit test for sulphates (0.15 per cent).

Loss on drying (2.4.19). 16.0 to 17.5 per cent, determined on 0.5 g by drying in an oven at 105°.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50.0 mg of the substance under examination in 100.0 ml of the mobile phase. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *sodium aminosalicylate IPRS* in 100.0 ml of the mobile phase (solution A). Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 25.0 mg of *3-aminophenol IPRS* in 100.0 ml of the mobile phase. Dilute 5.0 ml of the solution and 5 ml of solution A to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 6.0 g of *disodium hydrogen orthophosphate* and 6.6 g of *sodium dihydrogen orthophosphate dihydrate* in 1600 ml with *water*, add 19 ml of *tetra n-butyl ammonium hydroxide (20 per cent solution)* and make the volume to 1700 ml with *water* and add 300 ml of *methanol*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the tailing factor is not more than 2.0 and the column efficiency in not less than 3000 theoretical plates. The relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (b). The test is not valid unless the resolution between 3-aminophenol and sodium aminosalicylate is not less than 5.0.

Inject the reference solution and the test solution.

Calculate the content of $C_7H_6NNaO_3$.

Storage. Store protected from light and moisture.

Sodium Aminosalicylate Tablets

Sodium PAS Tablets

Sodium aminosalicylate tablet contains not less than 95.0 per cent not more than 105.0 per cent of $C_7H_6NNaO_3,2H_2O$.

Usual strengths. 500 mg; 1 g.

Identification

Digest a quantity of powdered tablets containing about 3.0 g of sodium aminosalicylate, with 40 ml of *water*, and filter. Add to the filtrate 15 ml of *1M acetic acid*, and allow it to stand until precipitation has occurred. Collect the precipitate on a filter, wash well with *water* and dry at 105°C for 30 min. The residue complies with the following tests.

A. Place about 1 g of the residue in a small, round-bottom flask, and add 10 ml of *acetic anhydride*. Heat the flask on a steam bath for 30 minutes, and add 40 ml of *water*, mix, filter, cool, and allow to stand until diacetyl derivative crystallizes, wash well with *water*, and dry at 105° for 1 hour. The diacetyl derivative so obtained melts at 191° to 197°.

B. Shake 0.1 g of the residue with 10 ml of *water*, and filter. To 5 ml of the filtrate add 1 drop of *ferric chloride solution*. A violet colour is produced.

C. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml *water*,
Speed and time. 100 rpm for 45 minutes.

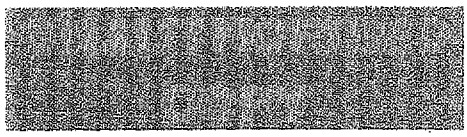
Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. The filtrate diluted with the mobile phase to produce a 0.055 per cent w/v solution.

Reference solution. A 0.055 per cent w/v solution of *sodium aminosalicylate IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),



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- mobile phase: dissolve 6.0 g of *disodium hydrogen orthophosphate* and 6.6 g of *sodium dihydrogen orthophosphate dihydrate* in 1600 ml with *water*, add 19 ml of *tetra n-butyl ammonium hydroxide (20 per cent aqueous solution)* and make the volume to 1700 ml with *water*, add 300 ml of *methanol*, mix well and degas,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent

Inject the reference solution and the test solution.

Calculate the content of $C_7H_6NNaO_3.2H_2O$ in the medium

Q. Not less than 75 per cent of the stated amount of $C_7H_6NNaO_3.2H_2O$.

3-aminophenol. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 50 mg of Sodium Aminosalicylate and dissolve in 100 ml of the mobile phase.

Reference solution (a). A 0.024 per cent w/v solution of *m-aminophenol IPRS* in the mobile phase. Dilute 5 ml to 100 ml with the mobile phase. Dilute 5 ml of the resulting solution to 50 ml with the mobile phase.

Reference solution (b). A 0.024 per cent w/v solution of *sodium aminosalicylate IPRS* in the mobile phase.

Reference solution (c). Mix 5 ml each of reference solution (a) and reference solution (b) and dilute to 100 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous base deactivated silica (5 µm) (Such as Hypersil BDS),
- mobile phase: dissolve 6.0 g of *disodium hydrogen orthophosphate* and 6.6 g of *sodium dihydrogen orthophosphate dihydrate* in 1600 ml with *water*, add 19 ml of *tetra n-butyl ammonium hydroxide (20 per cent aqueous solution)* and make the volume to 1700 ml with *water*, add 300 ml of *methanol*, mix well and degas,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject reference solution (c). The resolution between 3-aminophenol and sodium aminosalicylate is not less than 5.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of the peak corresponding to 3-aminophenol is not more than the area of the peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 0.5 g of sodium aminosalicylate, add sufficient mobile phase to produce 100.0 ml and filter. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.05 per cent w/v solution of *sodium aminosalicylate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 6.0 g of *disodium hydrogen orthophosphate* and 6.6 g of *sodium dihydrogen orthophosphate dihydrate* in 1600 ml with *water*, add 19 ml of *tetra n-butyl ammonium hydroxide (20 per cent aqueous solution)* and make the volume to 1700 ml with *water*, add 300 ml of *methanol*, mix well and degas,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

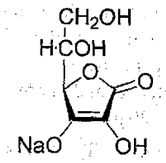
Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent

Inject the reference solution and the test solution.

Calculate the content of $C_7H_6NNaO_3.2H_2O$ in the tablets.

Storage. Store protected from moisture.

Sodium Ascorbate



$C_6H_7NaO_6$ Mol. Wt. 198.1

Sodium Ascorbate is L-ascorbic acid monosodium salt.

Sodium Ascorbate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_6H_7NaO_6$, calculated on the dried basis.

Category. Vitamin C.

Description. White or faintly yellow crystals or a crystalline powder. It darkens gradually on exposure to light.

Identification

Test A may be omitted if tests B, C and D are carried out. Test B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sodium ascorbate IPRS.*

B. To 4 ml of a 2 per cent w/v solution add 1 ml of *0.1 M hydrochloric acid*, add a few ml of *2,6-dichlorophenol-indophenol solution*; the solution is decolorised.

C. To 4 ml of a 2 per cent w/v solution add 1 ml of *0.1 M hydrochloric acid*, add 1 drop of a freshly prepared 5 per cent w/v solution of *sodium nitroprusside* and 2 ml of *dilute sodium hydroxide solution*. Add 0.6 ml of *hydrochloric acid* dropwise and stir; the yellow colour turns blue.

D. A 2 per cent w/v solution gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1) and not more intensely coloured than reference solution BYS7 (2.4.1).

pH (2.4.24). 7.0 to 8.0, determined in a 10.0 per cent w/v solution.

Specific optical rotation (2.4.22). + 103° to + 108°, determined in a 10.0 per cent w/v solution.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

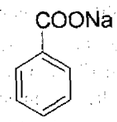
Loss on drying (2.4.19). Not more than 0.25 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.2 g in a mixture of 100 ml of *carbon dioxide-free water* and 25 ml of *1 M sulphuric acid.* Titrate immediately with *0.05 M iodine*, using 1 ml of *starch solution* as indicator, until a persistent violet-blue colour is obtained.

1 ml of *0.05 M iodine* is equivalent to 0.009905 g of $C_6H_7NaO_6$.

Storage. Store protected from light.

Sodium Benzoate



$C_7H_5NaO_2$ Mol. Wt. 144.1

Sodium Benzoate contains not less than 99.0 per cent and not more than 100.5 per cent of $C_7H_5NaO_2$, calculated on the dried basis.

Category. Pharmaceutical aid (preservative).

Description. A white, crystalline or granular powder or flakes; hygroscopic.

Identification

A. To a 10 per cent w/v solution add *ferric chloride test solution*; a buff coloured precipitate is formed. Add *dilute hydrochloric acid*; a white crystalline precipitate is produced.

B. Gives the reactions of sodium salts and reactions (B) and (C) of benzoates (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution YS6 (2.4.1).

Acidity or alkalinity. To 20 ml of a 5.0 per cent w/v solution in *carbon dioxide-free water* add 0.2 ml of *phenolphthalein solution*. Not more than 0.2 ml of *0.1 M hydrochloric acid* or 0.2 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution.

Arsenic (2.3.10). Mix 5.0 g with 10 ml of *bromine solution* and evaporate to dryness on a water-bath. Ignite gently, dissolve the cooled residue, ignoring any carbon, in 50 ml of *water* and 14 ml of *brominated hydrochloric acid AsT*, and remove the excess of *bromine* with 2 ml of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Chlorinated compounds. Dissolve 0.33 g in 5 ml of *0.5 M sodium carbonate*, evaporate to dryness and heat the residue until completely charred, keeping the temperature below 400°. Extract the residue with a mixture of 10 ml of *water* and 12 ml of *dilute nitric acid* and filter; the filtrate complies with the limit test for chlorides (2.3.12).

Loss on drying (2.4.19). Not more than 2.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh 0.25 g, dissolve in 20 ml of *anhydrous glacial acetic acid*, warming to 50° if necessary, cool. Titrate with *0.1 M perchloric acid*, using 0.05 ml of *1-naphtholbenzein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01441 g of $C_7H_5NaO_2$.

Storage. Store protected from light.

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Sodium Bicarbonate

Sodium Hydrogen Carbonate

$NaHCO_3$ Mol. Wt. 84.0

Sodium Bicarbonate contains not less than 99.0 per cent and not more than 101.0 per cent of $NaHCO_3$.

Category. Electrolyte replenisher; systemic alkaliser.

Description. A white, crystalline powder or small, opaque, monoclinic crystals. It gradually forms sodium carbonate on heating in the dry state or in solution.

Identification

A. To 5 ml of a 5.0 per cent w/v solution in *carbon dioxide-free water* (solution A) add 0.1 ml of *phenolphthalein solution*; a pale pink colour is produced. On heating, a gas is evolved and the solution becomes red.

B. It gives reaction (A) of bicarbonates (2.3.1).

C. Solution A gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water*, add 15 ml of *brominated hydrochloric acid AsT* and remove the excess of *bromine* with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Calcium. A 2.0 per cent w/v solution, when boiled for 5 minutes, is clear.

Heavy metals (2.3.13). Mix 4.0 g with 5 ml of *water* and 18 ml of *dilute hydrochloric acid*, heat to boiling and maintain that temperature for 1 minute. Add 0.05 ml of *phenolphthalein solution* and sufficient *5 M ammonia* dropwise to give the solution a faint pink colour, cool and dilute to 25 ml with *water*. The solution complies with the limit test for heavy metals, Method A (5 ppm).

Iron (2.3.14). Dissolve 2.0 g in 20 ml of *water* and 4 ml of *hydrochloric acid* and dilute to 40 ml with *water*; the resulting solution complies with the limit test for iron (20 ppm).

Carbonate. pH of solution A, when freshly prepared, not more than 8.6.

Chlorides (2.3.12). 1.25 g dissolved in 15 ml of *water* and 2 ml of *nitric acid* complies with the limit test for chlorides (200 ppm).

Sulphates (2.3.17). Suspend 1.0 g in 10 ml of *distilled water*, neutralise with *hydrochloric acid* and dilute to 15 ml with *distilled water*. The resulting solution complies with the limit test for sulphates (150 ppm).

Assay. Weigh 1.5 g, dissolve in 50 ml of *carbon dioxide-free water* and titrate with *1 M hydrochloric acid* using 0.2 ml of *methyl orange solution* as indicator.

1 ml of *1 M hydrochloric acid* is equivalent to 0.08401 g of $NaHCO_3$.

Storage. Store protected from moisture.

Sodium Bicarbonate Injection

Sodium Bicarbonate Intravenous Infusion

Sodium Bicarbonate Injection is a sterile solution of Sodium Bicarbonate in Water for Injections.

Sodium Bicarbonate Injection contains not less than 94.0 per cent and not more than 106.0 per cent of the stated amount of sodium bicarbonate, $NaHCO_3$.

Category. Electrolyte replenisher; systemic alkaliser.

Usual strengths. 1.4, 4.2, 5.0, 7.5 and 8.4 per cent w/v.

Description. A clear, colourless solution.

Identification

A. The residue on evaporation, when moistened with *hydrochloric acid* and introduced on a platinum wire into a flame, imparts a yellow colour to the flame.

B. It gives reaction (A) of sodium salts and the reactions of bicarbonates (2.3.1).

Tests

pH (2.4.24). 7.0 to 8.5.

Bacterial endotoxins (2.2.3). Not more than 1.7 Endotoxin Units per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Titrate a volume containing 1.5 g of Sodium Bicarbonate with *1 M hydrochloric acid* using *methyl orange solution* as indicator.

1 ml of *1 M hydrochloric acid* is equivalent to 0.08401 g of $NaHCO_3$.

Storage. Store in single dose containers.

Labelling. The label states (1) the strength as the percentage w/v of Sodium Bicarbonate; (2) the approximate concentrations, in millimoles per litre, of the sodium ions and the bicarbonate ions; (3) that an injection containing visible particles in the solution should not be used.

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Sodium Carbonate

Na_2CO_3 Mol. Wt. 106.0 (anhydrous)

Na_2CO_3,H_2O Mol. Wt. 124.0 (monohydrate)

Sodium Carbonate is anhydrous or contains one molecule of water of hydration.

Sodium Carbonate contains not less than 99.5 per cent and not more than 100.5 per cent of Na_2CO_3, calculated on the dried basis.

Category. Pharmaceutical aid (alkalising agent)

Description. *Anhydrous* — A white or almost white, slightly granular powder, hygroscopic.

Monohydrate — A white, crystalline powder or colourless crystals.

Identification

A. Dissolve 1 g in *water* and dilute to 10 ml with *water*; the solution is strongly alkaline.

B. The solution prepared for test A gives reaction (A) of carbonates and reactions of sodium salts (2.3.1).

Tests

Appearance of solution. Dissolve 2.0 g in 10 ml of *water*. The resulting solution is clear and not more intensely coloured than reference solution YS6 (2.4.1).

Alkali hydroxides and bicarbonates. Dissolve 0.4 g in 20 ml of *water*, add 20 ml of *barium chloride solution* and filter. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution*. The solution does not become red. Heat the remainder of the filtrate to boiling for 2 minutes. The solution remains clear.

Heavy metals (2.3.13). Dissolve 2.0 g in a mixture of 5 ml of *hydrochloric acid* and 25 ml of *water*. Heat the solution to boiling and cool. Add *dilute sodium hydroxide solution* until the solution is neutral. Dilute to 50 ml with *water* (solution A). 10 ml of the resulting solution complies with the limit test for heavy metals, Method A (50 ppm).

Iron (2.3.14). Dilute 20 ml of solution A to 40 ml with *water*. The solution complies with the limit test for iron (50 ppm).

Chlorides (2.3.12). Dissolve 2.0 g in *water*, add 20 ml of *dilute nitric acid* and dilute to 25 ml with *water*. The solution complies with the limit test for chlorides (125 ppm).

Sulphates (2.3.17). 15 ml of solution A complies with the limit test for sulphates (250 ppm).

Loss on drying (2.4.19). Not more than 1.0 per cent (for anhydrous form) or between 12.0 per cent and 15.0 per cent (for monohydrate form), determined on 2.0 g by drying in an oven at 300°.

Assay. Dissolve 1.0 g in 25 ml of *water* and titrate with *1 M hydrochloric acid* using *methyl orange solution* as indicator.

1 ml of *1 M hydrochloric acid* is equivalent to 0.05299 g of Na_2CO_3.

Storage. Store in tightly-closed, non-metallic containers.

Labelling. The label states whether the material is anhydrous or monohydrate.

Sodium Chloride

NaCl Mol. Wt. 58.4

Sodium Chloride contains not less than 99.0 per cent and not more than 100.5 per cent of NaCl, calculated on the dried basis.

Category. Pharmaceutical aid (tonicity agent); fluid and electrolyte replenisher.

Description. White or colourless crystals or a white crystalline powder.

Identification

A. It gives the reactions of chlorides (2.3.1).

B. A 20 per cent w/v solution in *carbon dioxide-free water* prepared from *distilled water* (solution A) gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. To 20 ml of solution A add 0.1 ml of *bromothymol blue solution*; not more than 0.5 ml of *0.01 M hydrochloric acid* or of *0.01 M sodium hydroxide* is required to change the colour of the solution.

Arsenic (2.3.10). Dissolve 10.0 g in 50 ml of *water* and 12 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (1 ppm).

Barium. Dissolve 2 g in 10 ml of *water*, and add 2 ml of *dilute sulphuric acid*; no turbidity is produced within 2 hours.

Bromide. To 0.5 ml of solution A add 4.0 ml of *water*, 2.0 ml of *phenol red reagent* and 1.0 ml of 0.01 per cent w/v solution of *chloramine T* and mix immediately. After exactly 2 minutes, add 0.15 ml of *0.1 M sodium thiosulphate*, mix and dilute to 10.0 ml with *water*. The absorbance of the solution measured at about 590 nm (2.4.7), using *water* as the blank, is not more than that of the standard solution prepared at the same time and in the same manner, starting with the addition of " 2.0 ml

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of *phenol red reagent*.....", using 5.0 ml of a 0.0003 per cent w/v solution of *potassium bromide* (100 ppm).

Calcium and magnesium. Not more than 50 ppm, calculated as Ca, determined by the following method. Dissolve 20.0 g in 200 ml of *water*, and add 0.1 ml of *hydrochloric acid*, 5 ml of *strong ammonia-ammonium chloride solution*, 5 drops of *eriochrome black T solution* and titrate with *0.005 M disodium edetate* to a blue end-point.

1 ml of *0.005 M disodium edetate* is equivalent to 0.0002004 g of Ca.

Ferrocyanide. Dissolve 2.0 g in 6 ml of *water* and add 0.5 ml of a mixture of 5 ml of a 1 per cent w/v solution of *ferric ammonium sulphate* in a 0.25 per cent w/v solution of *sulphuric acid*, and 95 ml of a 1 per cent w/v solution of *ferrous sulphate*; no blue colour is produced within 10 minutes.

Heavy metals (2.3.13). 4.0 g in 2 ml of *dilute acetic acid* and sufficient *water* to produce 25 ml. The solution complies with the limit test for heavy metals, Method A (5 ppm).

Iodide. Moisten 5 g by adding dropwise, a solution freshly prepared by mixing 25 ml of *iodide-free starch solution* 2 ml of *0.5 M sulphuric acid*, 0.15 ml of *sodium nitrite solution* and 25 ml of *water* and examine the mixture in daylight; the substance shows no blue colour after 5 minutes.

Iron (2.3.14). 2.0 g dissolved in 20 ml of *water* complies with the limit test for iron (20 ppm).

Sulphates (2.3.17). 2.5 ml of solution A diluted to 15 ml with *water* complies with the limit test for sulphates (300 ppm).

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 0.1 g in 50 ml of *water* in a glass-stoppered flask. Add 50.0 ml of *0.1 M silver nitrate*, 5 ml of *2 M nitric acid* and 2 ml of *dibutyl phthalate*, shake well and titrate with *0.1 M ammonium thiocyanate* using 2 ml of *ferric ammonium sulphate solution* as indicator, until the colour becomes reddish yellow.

1 ml of *0.1 M silver nitrate* is equivalent to 0.005844 g of NaCl.

Sodium Chloride intended for use in the manufacture of parenteral preparations or in the manufacture of dialysis solutions complies with the following additional requirement.

Potassium. Not more than 0.1 per cent, determined by flame photometry (2.4.4) or by method A for atomic absorption spectrophotometry (2.4.2), using a 1.0 per cent w/v solution and measuring at 767 nm. Use suitable dilutions in water of *potassium solution FP* or *potassium solution AAS* respectively, for the standard solution.

Sodium Chloride intended for use in the preparation of dialysis solutions complies with the following additional requirement.

Aluminium. Not more than 0.2 ppm, determined by the following method. Dissolve 20 g in 100 ml of *water* and add 10 ml of *acetate buffer pH 6.0*. Extract the resulting solution with successive quantities of 20, 20 and 10 ml of a 0.5 per cent w/v solution of *8-hydroxyquinoline* in *chloroform* and dilute the combined extracts to 50 ml with *chloroform*. Use as the blank solution a mixture of 10 ml of *acetate buffer pH 6.0* and 100 ml of *water* treated in the same manner and as the standard solution a mixture of 2 ml of *aluminium standard solution (2 ppm Al)*, 10 ml of *acetate buffer pH 6.0* and 90 ml of *water* treated in the same manner. Measure the fluorescence of the test solution and of the standard solution (2.4.5), using an excitation wavelength of 392 nm and a secondary filter with a transmission band centered at 518 nm, or a monochromator set to transmit at this wavelength, and setting the instrument to zero with the blank solution in each case. The fluorescence of the test solution is not more than that of the standard solution.

Sodium Chloride intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 5.0 Endotoxin Unit per g of sodium chloride.

Storage. Store protected from light.

Labelling. The label states whether or not the material is suitable for use in the manufacture of parenteral preparations or for the preparation of dialysis solutions.

Sodium Chloride and Dextrose Injection

Sodium Chloride and Dextrose Intravenous Infusion; Sodium Chloride and Glucose Injection; Sodium Chloride and Glucose Intravenous Infusion

Sodium Chloride and Dextrose Injection is a sterile solution of Sodium Chloride and Dextrose in Water for Injections.

Sodium Chloride and Dextrose Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amounts of sodium chloride, NaCl, and dextrose, $C_6H_{12}O_6$.

Usual strengths. Injections containing the following amounts of Sodium Chloride, NaCl and Dextrose, $C_6H_{12}O_6$.

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Per cent w/v of sodium chloride (NaCl)	Per cent w/v of dextrose ($C_6H_{12}O_6$)	Per cent w/v of sodium chloride (NaCl)	Per cent w/v of dextrose ($C_6H_{12}O_6$)
0.11	5		
0.18	5	0.45	5
0.2	5	0.45	10
0.225	5	0.9	2.5
0.3	5	0.9	5
0.33	5	0.9	10
0.45	2.5	0.9	25

Description. A clear, colourless or faintly straw-coloured solution.

Identification

A. To 1 ml add 0.05 ml of *potassium cupri-tartrate solution*; the solution remains blue and clear. Heat to boiling, a copious red precipitate is formed.

B. It gives reaction (B) of sodium salts and reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 3.5 to 6.5.

5-Hydroxymethylfurfural and related substances. Dilute a volume containing 1.0 g of dextrose, $C_6H_{12}O_6$, to 500 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 284 nm (2.4.7); absorbance at about 284 nm, not more than 0.25.

Bacterial endotoxins (2.2.3). Not more than 10 Endotoxin Units per g of dextrose.

Other tests. Comply with the tests stated under Parenteral Preparations (Intravenous Infusions).

Assay. *For sodium chloride* — Titrate a measured volume containing 0.1 g of Sodium Chloride with *0.1 M silver nitrate* using *potassium chromate solution* as indicator.

1 ml of *0.1 M silver nitrate* is equivalent to 0.005844 g of NaCl.

For dextrose — To a measured volume containing 2 to 5 g of anhydrous dextrose, $C_6H_{12}O_6$, add 0.2 ml of *5 M ammonia* and sufficient *water* to produce 100.0 ml. Mix well, allow to stand for 30 minutes and measure the optical rotation in a 2-dm tube (2.4.22). The observed rotation in degrees multiplied by 0.9477 represents the weight, in g, of dextrose, $C_6H_{12}O_6$, in the volume of the injection taken for assay.

Storage. Store in single dose containers. On keeping, small solid particles may separate from a glass container.

Labelling. The label states (1) the strength as the percentages w/v of Sodium Chloride and Dextrose; (2) that a solution containing visible particles must not be used.

When the preparation is intended for intravenous infusion, the label states the approximate concentrations, in millimoles per litre, of the sodium and chloride ions and the number of grams per litre of dextrose, $C_6H_{12}O_6$.

Sodium Chloride and Fructose Injection

Sodium Chloride and Fructose Intravenous Infusion; Sodium Chloride and Fructose Infusion; Fructose and Sodium Chloride Injection.

Sodium Chloride and Fructose Injection is a sterile solution of Sodium Chloride and Fructose in Water for Injections.

Sodium Chloride and Fructose Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amounts of sodium chloride, NaCl, and fructose, $C_6H_{12}O_6$. It contains no antimicrobial agent.

Usual strengths. Injections containing the following amounts of Sodium Chloride, NaCl, and Fructose, $C_6H_{12}O_6$.

Per cent w/v of Sodium Chloride (NaCl)	Per cent w/v of Fructose ($C_6H_{12}O_6$)	Per cent w/v of Sodium Chloride (NaCl)	Per cent w/v of Fructose ($C_6H_{12}O_6$)
0.11	5	0.45	2.5
0.18	5	0.45	10
0.225	5	0.9	2.5
0.33	5	0.9	5
0.45	5	0.9	10

Description. A clear, colourless or faintly straw-coloured solution.

Identification

A. To 1 ml add 0.05 ml of *potassium cupri-tartrate solution*; the solution remains blue and clear. Heat to boiling, a copious red precipitate is formed.

B. The solution prepared in the Assay is laevo-rotatory.

C. It gives reaction (B) of sodium salts and reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 3.0 to 6.0.

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5-Hydroxymethylfurfural and related substances. Dilute a volume containing 1.0 g of Fructose to 500.0 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 284 nm (2.4.7); absorbance at about 284 nm, not more than 0.50.

Heavy metals (2.3.13). Evaporate a volume containing 4.0 g of Fructose to 10 ml and add 2 ml of *dilute acetic acid* and sufficient *water* to produce 25 ml. The solution complies with the limit test for heavy metals, Method A (5 ppm).

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *For sodium chloride* — Titrate a measured volume containing about 0.1 g of Sodium Chloride with *0.1 M silver nitrate* using *potassium chromate solution* as indicator.

1 ml of *0.1 M silver nitrate* is equivalent to 0.005844 g of NaCl.

For fructose — To a measured volume containing about 5.0 g of Fructose, add 0.2 ml of *6 M ammonia* and sufficient *water* to produce 100.0 ml. Mix well, allow to stand for 30 minutes and measure the optical rotation in a 2-dm tube (2.4.22). The observed rotation in degrees multiplied by 0.5427 represents the weight, in g, of fructose, $C_6H_{12}O_6$, in the volume taken for assay.

Storage. Store in single dose containers. On keeping, small solid particles may separate from glass containers.

Labelling. The label states (1) the strength as the percentages w/v of Sodium Chloride and Fructose; (2) when the preparation is intended for intravenous infusion, the approximate concentrations, in millimoles per litre, of the sodium and chloride ions and the number of grams per litre of fructose; (3) that a solution containing visible particles should not be used.

Compound Sodium Chloride and Dextrose Injection

Compound Sodium Chloride and Dextrose Intravenous Infusion

Compound Sodium Chloride and Dextrose Injection is a sterile solution containing 0.86 per cent w/v of Sodium Chloride, 0.03 per cent w/v of Potassium Chloride, 0.033 per cent w/v of Calcium Chloride and 5 per cent w/v of Dextrose in Water for Injections.

Compound Sodium Chloride and Dextrose Injection contains not less than 0.82 per cent and not more than 0.90 per cent w/v of sodium chloride, NaCl, not less than 0.0285 per cent and not more than 0.0315 per cent w/v of potassium chloride, KCl, not less than 0.030 per cent and not more than 0.036 per cent w/v of calcium chloride, $CaCl_2,2H_2O$, and not less than 0.523 per cent and not more than 0.580 per cent w/v of chloride, Cl, and not less than 4.75 per cent and not more than 5.25 per cent w/v of dextrose, $C_6H_{12}O_6$. It contains no antimicrobial agent.

Category. Fluid and electrolyte replenisher.

Description. A clear, colourless or faintly straw-coloured solution.

Identification

A. To 1 ml add 0.05 ml of *potassium cupri-tartrate solution*; the solution remains blue and clear. Heat to boiling, a copious red precipitate is formed.

B. It gives reaction (B) of sodium salts and reaction (A) of chlorides (2.3.1).

C. After evaporation to one half of its original volume, the solution gives reaction (A) of potassium salts and reaction (B) of calcium salts (2.3.1).

Tests

pH (2.4.24). 3.5 to 6.5.

5-Hydroxymethylfurfural and related substances. Dilute a volume containing 1.0 g of Dextrose to 500.0 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 284 nm; absorbance at about 284 nm, not more than 0.25 (2.4.7).

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *For sodium chloride* — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 589 nm and using *sodium solution FP* or *sodium solution AAS* respectively, suitably diluted with *water* for the standard solutions.

1 g of Na is equivalent to 2.542 g of NaCl.

For potassium chloride — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 767 nm and using *potassium solution FP* or *potassium solution AAS* respectively, suitably diluted with *water* for the standard solutions.

1 g of K is equivalent to 1.907 g of KCl.

For calcium chloride — To 50.0 ml add 5.0 ml of *0.01 M magnesium sulphate* and 5 ml of *ammonia buffer pH*

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10.9 and titrate with *0.01 M disodium edetate* using *eriochrome black T mixture* as indicator. Carry out a blank titration.

1 ml of *0.01 M disodium edetate* is equivalent to 0.00147 g of $CaCl_2, 2H_2O$.

For total chloride — To 20.0 ml add 30 ml of *water*, 50.0 ml of *0.1 M silver nitrate* and 2 ml of *nitric acid*. Filter, wash the precipitate with *water* slightly acidified with *nitric acid* and titrate the excess of silver nitrate with *0.1 M ammonium thiocyanate* using *ferric ammonium sulphate solution* as indicator until a reddish yellow colour is produced. Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of total Chloride, calculated as Cl.

For dextrose — To a measured volume containing 2 g to 5 g of Dextrose, add 0.2 ml of *5 M ammonia* and sufficient *water* to produce 100.0 ml. Mix well, allow to stand for 30 minutes and measure the optical rotation in a 2-dm tube (2.4.22). The observed rotation in degrees multiplied by 0.9477 represents the weight, in g, of dextrose, $C_6H_{12}O_6$, in the volume taken for assay.

Storage. Store in single dose containers of glass or plastic at a temperature not exceeding 30°. On keeping, small solid particles may separate from the solution in glass containers.

Labelling. The label states (1) the strength as the percentages w/v of Sodium Chloride, Potassium Chloride, Calcium Chloride and Dextrose; (2) that the injection contains, in millimoles per litre, the following approximate amounts of the ions sodium, 147.5, potassium, 4, calcium, 4.5, and Chloride, 156; (3) the total osmolar concentration in mOsmol per litre; (4) that the injection should not be used if it contains visible particles.

Sodium Chloride Hypertonic Injection

Hypertonic Saline

Sodium Chloride Hypertonic Injection is a sterile 1.6 per cent w/v solution of Sodium Chloride in Water for Injections. It contains no antimicrobial agent.

Sodium Chloride Hypertonic Injection contains not less than 1.52 per cent w/v and not more than 1.68 per cent w/v of sodium chloride, NaCl.

Description. A clear, colourless solution.

Identification

It gives the reactions of sodium salts and reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 5.0 to 7.5.

Heavy metals (2.3.13). Evaporate 40 ml to about 20 ml, add 2 ml of *dilute acetic acid* and dilute to 25 ml with *water*. The solution complies with the limit test for heavy metals, Method A (0.5 ppm).

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Intravenous Infusions).

Assay. To a measured volume containing about 0.16 g of Sodium Chloride in a glass-stoppered flask add 50 ml of *water* and 50.0 ml of *0.1 M silver nitrate*, 5 ml of *2 M nitric acid* and 2 ml of *dibutyl phthalate*. Shake well and titrate with *0.1 M ammonium thiocyanate* using 2 ml of *ferric ammonium sulphate solution* as indicator, until the colour becomes reddish yellow. Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.005844 g of NaCl.

Storage. Store in single dose containers of glass or plastic. On keeping, small solid particles may separate from a glass container.

Labelling. The label states (1) the strength as the percentage w/v of Sodium Chloride; (2) that a solution containing visible solid particles must not be used.

When the preparation is intended for intravenous infusion, the label states that the injection contains approximately 270 millimoles each of sodium and chloride ions per litre.

Sodium Chloride Injection

Sodium Chloride Intravenous Infusion

Sodium Chloride Injection is a sterile 0.9 per cent w/v solution of Sodium Chloride in Water for Injections. It contains no antimicrobial agent.

Sodium Chloride Injection contains not less than 0.85 per cent w/v and not more than 0.95 per cent w/v of sodium Chloride, NaCl.

Description. A clear, colourless solution.

Identification

It gives the reactions of sodium salts and reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 4.5 to 7.0.

Heavy metals (2.3.13). Evaporate 67 ml to about 20 ml, add 2 ml of *dilute acetic acid* and dilute to 25 ml with *water*. The solution complies with the limit test for heavy metals, Method A (0.3 ppm).

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Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Intravenous Infusions).

Assay. Titrate a measured volume containing about 0.2 g of sodium chloride with *0.1 M silver nitrate* using *potassium chromate solution* as indicator.

1 ml of *0.1 M silver nitrate* is equivalent to 0.005844 g of NaCl.

Storage. Store in single dose containers of glass or plastic. On keeping, small solid particles may separate from a glass container.

Labelling. The label states (1) the strength as the percentage w/v of Sodium Chloride; (2) that a solution containing visible solid particles must not be used.

When the preparation is intended for intravenous infusion, the label states that the injection contains approximately 150 millimoles each of sodium and chloride ions per litre.

Compound Sodium Chloride Injection

Ringer's Injection

Compound Sodium Chloride Injection is a sterile solution containing 0.86 per cent w/v of Sodium Chloride, 0.03 per cent w/v of Potassium Chloride and 0.033 per cent w/v of Calcium Chloride in Water for Injections. It contains no antimicrobial agent.

Compound Sodium Chloride Injection contains not less than 0.82 per cent w/v and not more than 0.90 per cent w/v of sodium chloride, NaCl, not less than 0.0285 per cent w/v and not more than 0.0315 per cent w/v of potassium chloride, KCl, and not less than 0.030 per cent w/v and not more than 0.036 per cent w/v of calcium chloride, $CaCl_2, 2H_2O$.

Description. A clear, colourless solution.

Identification

It gives reaction (B) of sodium salts and reaction (A) of chlorides (2.3.1). When concentrated to one half of its original volume, it gives reaction (A) of potassium salts and reaction (B) of calcium salts (2.3.1).

Tests

pH (2.4.24). 5.0 to 7.5.

Heavy metals (2.3.13). Evaporate 67 ml to about 20 ml, add 2 ml of *dilute acetic acid* and dilute to 25 ml with *water*. The solution complies with the limit test for heavy metals, Method A (0.3 ppm).

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Intravenous Infusions).

Assay. *For sodium chloride* — Dilute appropriately with water and determine by Method A for flame photometry (2.4.4), measuring at 589 nm or by Method A for atomic absorption spectrophotometry (2.4.2), using sodium solution FP, suitably diluted with water for the standard solutions.

1 g of Na is equivalent to 2.54 g of NaCl.

For potassium Chloride — Dilute appropriately with *water* and determine by Method A for flame photometry (2.4.4), measuring at 767 nm or by Method A for atomic absorption spectrophotometry (2.4.2), using *potassium solution FP*, suitably diluted with water for the standard solutions.

1 g of K is equivalent to 1.907 g of KCl.

For calcium chloride — To 50.0 ml add 5.0 ml of *0.01 M magnesium sulphate* and 5 ml of *ammonia buffer pH 10.9* and titrate with *0.01 M disodium edetate* using *mordant black II mixture* as indicator. Carry out a blank titration.

1 ml of *0.01 M disodium edetate* is equivalent to 0.00147 g of $CaCl_2, 2H_2O$.

Storage. Store in single dose containers of glass or plastic. On keeping, small solid particles may separate from a glass container.

Labelling. The label states (1) the strength as the percentages w/v of Sodium Chloride, Potassium Chloride and Calcium Chloride; (2) that a solution containing visible solid particles must not be used.

When the preparation is intended for intravenous infusion, the label states that the Injection contains in millimoles per litre, the following approximate amounts of ions; sodium, 147.5; potassium, 4; calcium, 2.25 and chloride, 156.

Compound Sodium Chloride Solution

Ringer's Solution

Compound Sodium Chloride Solution is a solution containing 0.86 per cent w/v of Sodium Chloride, 0.03 per cent w/v of Potassium Chloride and 0.033 per cent w/v of Calcium Chloride in Purified Water. The solution may be clarified by filtration.

Compound Sodium Chloride Solution contains not less than 0.82 per cent w/v and not more than 0.90 per cent of sodium chloride, NaCl, not less than 0.025 per cent and not more than 0.035 per cent w/v of potassium chloride, KCl, and not less than 0.030 per cent and not more than 0.036 per cent w/v of calcium chloride, $CaCl_2, 2H_2O$.

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Category. Irrigation solution (for external use).

Description. A clear, colourless solution.

Identification

It gives reaction (B) of sodium salts and reaction (A) of chlorides (2.3.1). When concentrated to one half of its original volume, it gives reaction (A) of potassium salts and reaction (B) of calcium salts (2.3.1).

Tests

pH (2.4.24). 5.0 to 7.5.

Heavy metals (2.3.13). Evaporate 67 ml to about 20 ml, add 2 ml of *dilute acetic acid* and dilute to 25 ml with *water*. The solution complies with the limit test for heavy metals, Method A (0.3 ppm).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *For sodium chloride* — Dilute appropriately with water and determine by Method A for flame photometry (2.4.4), measuring at 589 nm or by Method A for atomic absorption spectrophotometry (2.4.2), using *sodium solution FP*, suitably diluted with water for the standard solutions.

1 g of Na is equivalent to 2.54 g of NaCl.

For potassium chloride — Dilute appropriately with water and determine by Method A for flame photometry (2.4.4), measuring at 767 nm or by Method A for atomic absorption spectrophotometry (2.4.2), using *potassium solution FP*, suitably diluted with water for the standard solutions.

1 g of K is equivalent to 1.907 g of KCl.

For calcium Chloride — To 50.0 ml add 5.0 ml of *0.01 M magnesium sulphate* and 5 ml of *ammonia buffer pH 10.9* and titrate with *0.01 M disodium edetate* using *mordant black II mixture* as indicator. Carry out a blank titration.

1 ml of *0.01 M disodium edetate* is equivalent to 0.00147 g of $CaCl_2, 2H_2O$.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store protected from light.

Labelling. The label states the strength as the percentages w/v of Sodium Chloride, Potassium Chloride and Calcium Chloride. If the contents of the container are sterile, the label states (1) Sterile Compound Sodium Chloride Solution; (2) that the solution is not intended for injection.

Sodium Chloride Irrigation Solution

Sodium Chloride Irrigation Solution is a sterile solution containing 0.9 per cent w/v of Sodium Chloride in Water for Injections.

Sodium Chloride Irrigation Solution contains not less than 0.85 per cent w/v and not more than 0.95 per cent w/v of sodium Chloride, NaCl. It contains no antimicrobial agent.

Description. A clear, colourless solution.

Identification

It gives the reactions of sodium salts and reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 4.5 to 7.0.

Heavy metals (2.3.13). Evaporate 67 ml to about 20 ml, add 2 ml of *dilute acetic acid* and dilute to 25 ml with *water*. The solution complies with limit test for heavy metals, Method A (0.3 ppm).

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Titrate a measured volume containing about 0.135 g of Sodium Chloride with *0.1 M silver nitrate* using *potassium chromate solution* as indicator.

1 ml of *0.1 M silver nitrate* is equivalent to 0.005844 g of NaCl.

Storage. Store in single dose containers. The container may be designed to empty rapidly and may contain a volume of more than 1 litre.

Labelling. The label states (1) the strength as the percentage w/v of Sodium Chloride; (2) that the solution should not be used if it contains visible particles; (3) 'For Irrigation only' and 'Not for Injection'; (4) that once the container is opened, the unused portion should be discarded.

Sodium Citrate Eye Drops

Sodium Citrate Eye Drops are a sterile solution of Sodium Citrate in Purified Water.

Sodium Citrate Eye Drops contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sodium citrate, $C_6H_5Na_3O_7,2H_2O$.

Usual strength. 10 per cent w/v.

Description. A colourless solution.

Identification

It gives reaction (A) of sodium salt and reactions of citrates (2.3.1).

Tests

pH (2.4.24). 7.0 to 7.5.

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Other tests. Comply with the tests stated under Eye Drops.

Assay. Evaporate a volume containing about 0.15 g of Sodium Citrate to dryness and dissolve the residue in *anhydrous glacial acetic acid*, warming to about 50°. Allow to cool. Titrate with *0.1 M perchloric acid*, using 0.25 ml of *1-naphthlolbenzein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.009803 g of $C_6H_5Na_3O_7,2H_2O$.

Storage. Store protected from light.

Sodium Citrate Irrigation Solution

Sodium Citrate Irrigation Solution is a sterile solution of sodium citrate in water for injections containing anhydrous citric acid or citric acid monohydrate.

Sodium Citrate Irrigation Solution contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sodium citrate, $C_6H_5Na_3O_7,2H_2O$.

Usual strength. 3 per cent w/v.

Description. A colourless solution.

Identification

It gives reactions of sodium salt and citrates (2.3.1).

Tests

pH (2.4.24). 6.0 to 7.5.

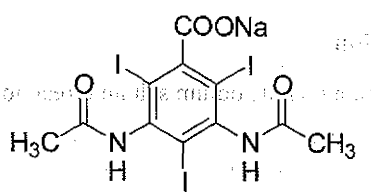
Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Evaporate a volume containing 0.15 g of Sodium Citrate to dryness and dissolve the residue in *anhydrous glacial acetic acid*, warming to about 50° and allow to cool. Titrate with *0.1 M perchloric acid*, using 0.25 ml of *1-naphthlol-benzein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.009803 g of $C_6H_5Na_3O_7,2H_2O$.

Sodium Diatrizoate

Diatrizoate Sodium



$C_{11}H_8I_3N_2NaO_4$ Mol. Wt. 635.9

Sodium Diatrizoate is sodium 3,5-diacetamido-2,4,6-triiodo-benzoate.

Sodium Diatrizoate contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{11}H_8I_3N_2NaO_4$, calculated on the anhydrous basis.

Category. Urographic radio-opaque substance.

Description. A white powder.

Identification

Tests A and D may be omitted if tests B, C, E and F are carried out. Tests B, C and F may be omitted if tests A, D and E are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sodium diatrizoate IPRS* or with the reference spectrum of sodium diatrizoate.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 20 volumes of *chloroform*, 10 volumes of *methanol* and 2 volumes of *strong ammonia solution*.

Test solution. Dissolve 0.1 g of the substance under examination in 100 ml of 0.08 per cent w/v solution of *sodium hydroxide* in *methanol*.

Reference solution. A 0.1 per cent w/v of *sodium diatrizoate IPRS* in 0.08 per cent w/v solution of *sodium hydroxide* in *methanol*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

C. Heat 0.5 g in a crucible; violet vapours of iodine are evolved.

D. To 20 mg add 5 ml of *1 M sodium hydroxide* and boil gently under a reflux condenser for 10 minutes. Cool, add 5 ml of *2 M hydrochloric acid* and cool in ice for 5 minutes. Add 4 ml of a 1 per cent w/v solution of *sodium nitrite*, cool in ice for 5 minutes, add 0.3 g of *sulphamic acid*, shake gently until effervescence ceases and add 2 ml of a 0.4 per cent w/v solution of *N-(1-naphthyl) ethylenediamine dihydrochloride*; an orange-red colour is produced.

E. Heat 0.5 g with 1 ml of *sulphuric acid* on a water-bath until a pale violet solution is produced, add 2 ml of *ethanol (95 per cent)* and heat again; odour of ethyl acetate is produced.

F. It gives the reactions of sodium salts (2.3.1).

Tests

pH (2.4.24). 7.5 to 9.5, determined in a 50 per cent w/v solution.

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Free amine. Place 1.0 g in a 50-ml glass-stoppered volumetric flask, add 5 ml of *water*, 10 ml of *0.1 M sodium hydroxide* and 25 ml of *dimethyl sulphoxide*. Stopper the flask, mix the contents gently and cool in ice, protected from light. After 5 minutes, slowly add 2 ml of *hydrochloric acid*, mix and allow to stand for 5 minutes. Add 2 ml of a 2 per cent w/v solution of *sodium nitrite*, mix and allow to stand for 5 minutes. Add 1 ml of an 8 per cent w/v solution of *sulphamic acid*, mix and allow to stand for 5 minutes. Add 2 ml of a 0.1 per cent w/v solution of *N-(1-naphthyl)ethylenediamine dihydrochloride* in a 70 per cent w/v solution of *1,2-propanediol* and mix. Remove the flask from the ice and allow to stand in water at 25° ± 2° for 10 minutes, with occasional shaking. Add sufficient *dimethyl sulphoxide* to produce 50 ml and mix. Within 5 minutes, measure the absorbance of the resulting solution at the maximum at about 470 nm (2.4.7), using as the blank a solution prepared by treating 5 ml of *water* in the same manner; absorbance, not more than 0.40, calculated on the anhydrous basis.

Free iodine and iodide. Dissolve 2.0 g in 24 ml of *water* taken in a 50-ml glass-stoppered centrifuge tube. Add 5 ml of *toluene* and 5 ml of *1 M sulphuric acid*, shake well and centrifuge; no red colour appears in the toluene layer. To the mixture add 1 ml of a 2 per cent w/v solution of *sodium nitrite*, shake and centrifuge; any red colour in the toluene layer is not more intense than that produced in a solution prepared in the same manner using 2.0 ml of a 0.025 per cent w/v solution of *potassium iodide* and 22 ml of *water* (220 ppm).

Heavy metals. Dissolve 1.0 g in 20.0 ml of *water* and 5 ml of *1 M sodium hydroxide*, transfer the solution to a 50-ml Nessler cylinder, dilute with *water* to 40 ml and mix. Add 10 ml of *sodium sulphide solution*, shake and allow to stand for 5 minutes (20 ppm), the colour of the solution when viewed downward over a white surface is not more intense than that produced by treating 2.0 ml of *lead standard solution (10 ppm Pb)* in the same manner in place of the substance under examination.

Water (2.3.43). 4.0 to 7.0 per cent, determined on 0.4 g.

Assay. Weigh 0.4 g in a glass-stoppered conical flask, add 12 ml of *5 M sodium hydroxide* and 1 g of *zinc powder* and boil under a reflux condenser for 30 minutes. Cool, rinse the condenser with 30 ml of *water*, filter through cotton and wash the flask and filter with two quantities, each of 20 ml, of *water*. To the combined filtrate and washings add 80 ml of *hydrochloric acid*, cool and titrate with *0.05 M potassium iodate* until the dark brown colour becomes pale brown. Add 5 ml of *chloroform* and continue the titration, shaking well after each addition, until the chloroform becomes colourless.

1 ml of *0.05 M potassium iodate* is equivalent to 0.02120 g of $C_{11}H_8I_3N_2NaO_4$.

Storage. Store protected from light.

Sodium Diatrizoate Injection

Diatrizoate Sodium Injection

Sodium Diatrizoate Injection is a sterile solution of Sodium Diatrizoate in Water for Injections. It may contain small amounts of suitable buffers, stabilisers and antimicrobial agents but the preparation intended for intravenous administration contains no antimicrobial preservative.

Sodium Diatrizoate Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sodium diatrizoate, $C_{11}H_8I_3N_2NaO_4$.

Usual strengths. 25 per cent w/v; 40 per cent w/v.

Identification

Evaporate a volume of the injection containing 1 g of Sodium Diatrizoate to dryness. The residue complies with following tests.

A. Heat 0.5 g of residue in a crucible; violet vapours of iodine are evolved.

B. To 20 mg of the residue add 5 ml of *1 M sodium hydroxide* and boil gently under a reflux condenser for 10 minutes. Cool, add 5 ml of *2 M hydrochloric acid* and cool in ice for 5 minutes. Add 4 ml of a 1 per cent w/v solution of *sodium nitrite*, cool in ice for 5 minutes, add 0.3 g of *sulphamic acid*, shake gently until effervescence ceases and add 2 ml of a 0.4 per cent w/v solution of *N-(1-naphthyl) ethylenediamine dihydrochloride*; an orange-red colour is produced.

C. Heat 0.5 g of residue with 1 ml of *sulphuric acid* on a water-bath until a pale violet solution is produced, add 2 ml of *ethanol (95 per cent)* and heat again; odour of ethyl acetate is produced.

Tests

pH (2.4.24). 6.6 to 7.6.

Free amine. To a volume containing 1.0 g of Sodium Diatrizoate in a 50-ml glass-stoppered volumetric flask, add 5 ml of *water*, 10 ml of *0.1 M sodium hydroxide* and 25 ml of *dimethyl sulphoxide*. Stopper the flask, mix the contents gently and cool in ice, protected from light. After 5 minutes, slowly add 2 ml of *hydrochloric acid*, mix and allow to stand for 5 minutes. Add 2 ml of a 2 per cent w/v solution of *sodium nitrite*, mix and allow to stand for 5 minutes. Add 1 ml of a 8 per cent w/v solution of *sulphamic acid*, mix and allow to stand for 5 minutes. Add 2 ml of a 0.1 per cent w/v solution of *N-(1-naphthyl)ethylenediamine dihydrochloride* in a 70 per cent w/v solution of *1,2-propanediol* and mix. Remove the flask from the ice and allow to stand in water at 25° ± 2° for 10 minutes, with occasional shaking. Add sufficient *dimethyl sulphoxide* to produce 50 ml and mix. Within 5 minutes, measure

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the absorbance of the resulting solution at the maximum at about 470 nm (2.4.7), using as the blank a solution prepared by treating 5 ml of *water* in the same manner; absorbance, not more than 0.40, calculated on the anhydrous basis.

Free iodine and iodide. To a volume containing 2.0 g of Sodium Diatrizoate in 24 ml of *water* taken in a 50-ml glass-stoppered centrifuge tube add 5 ml of *toluene* and 5 ml of *1 M sulphuric acid*, shake well and centrifuge; no red colour appears in the toluene layer. To the mixture add 1 ml of a 2 per cent w/v solution of *sodium nitrite*, shake and centrifuge; any red colour in the toluene layer is not more intense than that produced in a solution prepared in the same manner using 2.0 ml of a 0.025 per cent w/v solution of *potassium iodide* and 22 ml of *water* (220 ppm).

Bacterial endotoxins (2.2.3). Not more than 5.6 Endotoxin Units per ml for injections containing 20.0 per cent of sodium diatrizoate; not more than 1.3 Endotoxin Units per ml for injections containing 25.0 per cent of sodium diatrizoate; and not more than 5.0 Endotoxin Units per ml for injections containing 50.0 per cent of sodium diatrizoate.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Weigh 0.5 g in a glass-stoppered conical flask, add 12 ml of *5 M sodium hydroxide* and 1 g of *zinc powder* and boil under a reflux condenser for 30 minutes. Cool, rinse the condenser with 30 ml of *water*, filter through cotton and wash the flask and filter with two quantities, each of 20 ml, of *water*. To the combined filtrate and washings add 80 ml of *hydrochloric acid*, cool and titrate with *0.05 M potassium iodate* until the dark brown colour becomes pale brown. Add 5 ml of *chloroform* and continue the titration, shaking well after each addition, until the chloroform becomes colourless.

1 ml of *0.05 M potassium iodate* is equivalent to 0.02120 g of $C_{11}H_8I_3N_2NaO_4$.

Storage. Store protected from light.

Labelling. The label states (1) the concentration of the active ingredient; (2) whether the contents are intended for intravenous injection and, if so, that the unused portion remaining in the container after use must be discarded.

Sodium Fluoride

NaF Mol. Wt. 42.0

Sodium Fluoride contains not less than 98.5 per cent and not more than 100.5 per cent of NaF, calculated on the dried basis.

Category. Preventive for dental caries.

Description. A white powder or colourless crystals.

Identification

A. Dissolve 2.5 g in sufficient *carbon dioxide-free water* without heating to produce 100 ml (solution A). To 2 ml of solution A add 0.5 ml of *calcium chloride solution*; a gelatinous white precipitate is produced which dissolves on adding 5 ml of *ferric chloride solution*.

B. Add about 4 mg to a mixture of 0.1 ml of *alizarin red S solution* and 0.1 ml of *zirconyl nitrate solution* and mix; the colour changes to yellow.

C. It gives reaction (A) of sodium salts (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. Dissolve 2.5 g of *potassium nitrate* in 40 ml of solution A, dilute to 50 ml with *carbon dioxide-free water*, cool to 0° and add 0.2 ml of *dilute phenolphthalein solution*. If the solution is colourless, not more than 1.0 ml of *0.1 M sodium hydroxide* is required to produce a red colour that persists for not less than 15 seconds. If the solution is red, not more than 0.25 ml of *0.1 M hydrochloric acid* is required to change the colour of the solution. Reserve the neutralised solution for the test for Fluorosilicate.

Chlorides (2.3.12). 40 ml of solution A complies with the limit test for chlorides (250 ppm).

Fluorosilicate. Heat to boiling the solution reserved in the test for Acidity or alkalinity and titrate while hot with *0.1 M sodium hydroxide* until a red colour is produced. Not more than 1.5 ml of *0.1 M sodium hydroxide* is required.

Sulphates (2.3.17). Dissolve 0.25 g in 10 ml of a saturated solution of *boric acid* in *distilled water* and add 5 ml of *distilled water* and 0.6 ml of *7 M hydrochloric acid*. The solution complies with the limit test for sulphates (200 ppm). Prepare the standard by mixing together 0.6 ml of *7 M hydrochloric acid*, 5 ml of *sulphate standard solution (10 ppm SO_4)* and 10 ml of a saturated solution of *boric acid* in *distilled water*.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 130° for 3 hours.

Assay. Weigh 80 mg, add a mixture of 5 ml of *acetic anhydride* and 20 ml of *anhydrous glacial acetic acid* and heat to dissolve. Cool, add 20 ml of *dioxan*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator, until a green colour is produced. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.004199 g of NaF.

Storage. Store protected from moisture.

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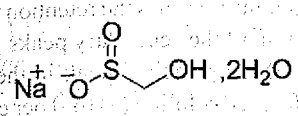
Sodium Formaldehyde Sulphoxylate



$CH_3NaO_3S,2H_2O$ Mol Wt. 154.1

Sodium Formaldehyde Sulphoxylate is monosodium hydroxymethane sulphinate dihydrate. It may contain a suitable stabilising agent such as sodium carbonate.

Sodium Formaldehyde Sulphoxylate contains an amount of CH_3NaO_3S equivalent to not less than 45.0 per cent and not more than 55.0 per cent of SO_2, calculated on the dried basis.

Category. Pharmaceutical aid (antioxidant).

Description. White crystals or hard white masses.

Identification

A. Dissolve about 4 g in 10 ml of *water* in a test-tube and add 1 ml of *ammoniacal silver nitrate solution*; metallic silver is produced either as a finely divided grey precipitate or as a bright metallic mirror on the inner surface of the tube.

B. Add about 50 mg to a solution of 40 mg of *salicylic acid* in 5 ml of *sulphuric acid* and warm very gently; a permanent deep red colour develops.

Tests

Appearance of solution. A 5.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and colourless (2.4.1).

Alkalinity. Dissolve 1.0 g in 50 ml of *water* and add 0.15 ml of *dilute phenolphthalein solution*; not more than 3.5 ml of *0.05 M sulphuric acid* is required to change the colour of the solution.

pH (2.4.24). 9.5 to 10.5, determined in a 2.0 per cent w/v solution in *carbon dioxide-free water*.

Iron (2.3.14). Ignite 1.0 g, initially at a low temperature until thoroughly charred and finally at about 600°, preferably in a muffle furnace, until all the carbon has been burnt off. Cool, dissolve the residue in 2 ml of *hydrochloric acid* and dilute to 50 ml with *water*. Add about 50 mg of *ammonium persulphate* and 5 ml of *ammonium thiocyanate solution*, mix and transfer to a Nessler cylinder. The red colour of the solution is not more intense than that of 1.0 ml of *iron standard solution (10 ppm)* treated in the same manner.

Sulphides. Dissolve 6 g in 14 ml of *water* in a test-tube and wet a strip of *lead acetate paper* in the clear solution; no discolouration is evident within 5 minutes.

Sodium sulphite. Not more than 5.0 per cent, calculated as Na_2SO_3, determined by the following method. Transfer 4.0 ml of the solution obtained in the Assay to a flask, add 2 ml of *formaldehyde solution* and titrate with *0.05 M iodine* that is used for the Assay, adding *starch solution* towards the end of the titration as indicator.

Calculate the percentage of Na_2SO_3 from the expression $78.775(V_2-V_1)(M/W)$,

where V_1 and V_2 are the volumes, in ml, of 0.05 M iodine consumed in this test and in the Assay respectively, M is the exact molarity of 0.05 M iodine solution and W is the weight, in g, of the substance under examination taken for the Assay.

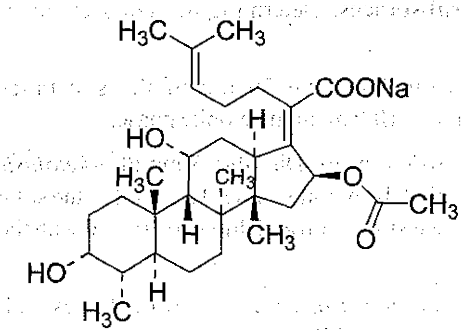
Loss on drying (2.4.19). Not more than 27.0 per cent, determined on 0.5 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 1.0 g in 25 ml of *water*, add sufficient *water* to produce 50.0 ml and mix. To 4.0 ml of the solution add 100 ml of *water* and titrate with *0.05 M iodine* using 3 ml of *starch solution*, added towards the end of the titration, as indicator.

1 ml of *0.05 M iodine* is equivalent to 0.001602 g of SO_2.

Storage. Store protected from light and moisture.

Sodium Fusidate



$C_{31}H_{47}NaO_6$ Mol. Wt. 538.7

Sodium Fusidate is sodium (17*Z*)-16β-acetoxy-3α,11α-dihydroxyfusida-17(20),24-diene-21-oate, produced by the growth of certain strains of *Fusidium coccineum* or by any other means.

Sodium Fusidate contains not less than 97.5 per cent and not more than 101.0 per cent of $C_{31}H_{47}NaO_6$, calculated on the anhydrous basis.

Category. Antibacterial.

Description. A white or almost white, crystalline powder; slightly hygroscopic.

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Identification

A. Dissolve 0.1 g in 5 ml of *water*, add 5 ml of *chloroform* and 0.1 ml of a 10 per cent w/w solution of *phosphoric acid*, shake vigorously for 1 minute, allow to separate and filter the lower layer through absorbent cotton covered with *anhydrous sodium sulphate*. Repeat the extraction with two quantities, each of 5 ml, of *chloroform*, evaporate the combined extracts at a pressure of 2 kPa, dry the residue over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa for 2 hours and dissolve in 1 ml of *chloroform IR*.

On the residue determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *fusidic acid IPRS* or with the reference spectrum of fusidic acid.

B. Ignite 1 g. The residue gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. A 15.0 per cent w/v solution in *carbon dioxide-free water* is not more intensely coloured than reference solution BS6 (2.4.1).

pH (2.4.24). 7.5 to 9.0, determined in a 1.25 per cent w/v solution.

Specific optical rotation (2.4.22). + 5.0° to + 8.0°, determined at 20° by dissolving 1.5 g in 25 ml of *water*, adding 0.1 ml of *5 M ammonia* and diluting to 50 ml with *water*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 10.0 ml of the mobile phase.

Reference solution (a). Dissolve 5 mg of *3-ketofusidic acid IPRS* in 5 ml of the mobile phase. To 1.0 ml of the solution add 0.2 ml of the test solution and dilute to 20.0 ml with the mobile phase.

Reference solution (b). Dilute 20 µl of the test solution to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 12.5 cm x 5 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 10 volumes of *methanol*, 20 volumes of 1.0 per cent w/v solution of *orthophosphoric acid*, 20 volumes of *water* and 50 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 235 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to 3-ketofusidic acid and sodium fusidate is not less than 2.5.

Inject reference solution (a), (b) and the test solution. The signal-to-noise ratio of the principal peak is not less than 3. Run the chromatogram 3.5 times the retention time of principal peak and the sum of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

Water (2.3.43). Not more than 2.0 per cent, determined on 0.5 g.

Assay. Dissolve 0.2 g in a mixture of 15 ml of *water* and 20 ml of *ethanol (95 per cent)*. Titrate with *0.1 M hydrochloric acid* to pH 4.1, stirring continuously, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M hydrochloric acid* is equivalent to 0.05387 g of $C_{31}H_{47}NaO_6$.

Storage. Store protected from light and moisture.

Sodium Hydroxide

Caustic Soda

NaOH Mol. Wt. 40.0

Sodium Hydroxide contains not less than 97.0 per cent and not more than 100.5 per cent of total alkali, calculated as NaOH.

Category. Pharmaceutical aid (alkalising agent).

Description. White, crystalline masses supplied as sticks, pellets or slabs; deliquescent. Readily absorbs carbon dioxide.

CAUTION — Great care should be exercised in handling Sodium Hydroxide as it rapidly destroys tissues.

Identification

A. Carefully dissolve 5.0 g in 12 ml of *distilled water*, add 17 ml of *7 M hydrochloric acid*, adjusted to pH 7.0 with *1 M hydrochloric acid* and add sufficient *distilled water* to produce 50 ml (solution A). 2 ml of solution A gives reaction (A) of sodium salts (2.3.1).

B. pH of a 0.01 per cent w/v solution, not less than 11.0 (2.4.24).

Tests

Appearance of solution. A 10.0 per cent w/v solution is clear (2.4.1), and colourless (2.4.1).

Arsenic (2.3.10). Dissolve 2.5 g in 50 ml of *water*, add 16 ml of *brominated hydrochloric acid AsT*, and remove the excess of bromine with a few drops of *stannous chloride solution AsT*.

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The resulting solution complies with the limit test for arsenic (4 ppm).

Heavy metals (2.3.13). Dissolve 1.0 g in 5 ml of *water* and 10 ml of *3 M hydrochloric acid*; heat to boiling, cool and dilute to 25 ml with *water*. The solution complies with the limit test for heavy metals, Method A (20 ppm).

Iron (2.3.14). 20 ml of solution A complies with the limit test for iron (20 ppm).

Carbonates. Not more than 2.0 per cent, calculated as Na_2CO_3, determined in the Assay.

Chlorides (2.3.12). Dissolve 2.0 g in 5 ml of *water*; acidify with about 8 ml of *nitric acid* and dilute to 20 ml with *water*. The solution, without the addition of *dilute nitric acid*, complies with the limit test for chlorides (125 ppm).

Sulphates (2.3.17). Dissolve 2.0 g in 6 ml of *distilled water*, adjusted to pH 7 with *hydrochloric acid* and dilute to 20 ml with *distilled water*. The resulting solution complies with the limit test for sulphates (75 ppm).

Potassium. Acidify 2.5 ml of solution A with *acetic acid* and add 0.15 ml of *sodium cobaltinitrite solution*; no precipitate is formed.

Assay. Dissolve 2.0 g in about 80 ml of *carbon dioxide-free water*, add 0.3 ml of *phenolphthalein solution* and titrate with *1 M hydrochloric acid*. Add 0.3 ml of *methyl orange solution* and continue the titration with *1 M hydrochloric acid*.

1 ml of *1 M hydrochloric acid* used in the second part of the titration is equivalent to 0.1060 g of Na_2CO_3.

1 ml of *1 M hydrochloric acid* used in the combined titrations is equivalent to 0.0400 g of total alkali, calculated as NaOH.

Storage. Store protected from moisture, in non-metallic containers.

Sodium Lactate Injection

Sodium Lactate Intravenous Infusion

Sodium Lactate Injection is a sterile solution containing 1.85 per cent w/v of sodium lactate in Water for Injections. It is prepared from Lactic Acid with the aid of Sodium Hydroxide and sufficient Dilute Hydrochloric Acid to adjust the pH of the solution.

Sodium Lactate Injection contains not less than 1.75 per cent and not more than 1.95 per cent w/v of sodium lactate, $C_3H_5NaO_3$.

Category. Fluid and electrolyte replenisher.

Description. A clear, colourless solution.

Identification

A. When warmed with *potassium permanganate*, gives acetaldehyde, recognisable by its odour.

B. The residue on evaporation, when moistened with *hydrochloric acid* and introduced on a platinum wire into a flame, imparts a yellow colour to the flame.

C. Carry out reaction C of calcium salts (2.3.1); no white precipitate is produced.

Tests

pH (2.4.24). 5.0 to 7.0.

Bacterial endotoxins (2.2.3). Not more than 2.0 Endotoxin Units per millimole.

Other tests. Comply with the tests stated under Parenteral Preparations (Intravenous Infusions).

Assay. Measure 10 ml, evaporate to dryness in a platinum dish and ignite very gently until completely carbonised. Boil the residue with 25.0 ml of *0.05 M sulphuric acid*, filter and wash thoroughly with hot *water*. Titrate the excess of acid in the combined filtrate and washings with *0.1 M sodium hydroxide* using *methyl orange solution* as indicator. Carry out a blank titration.

1 ml of *0.05 M sulphuric acid* is equivalent to 0.01121 g of $C_3H_5NaO_3$.

Storage. Store in single dose containers of glass or plastic. On keeping, small solid particles may separate from the solution in glass containers.

Labelling. The label states (1) that the Injection is one-sixth molar and contains, in one litre, approximately 167 millimoles each of sodium ions and of bicarbonate ions (as lactate); (2) that the injection should not be used if the solution contains visible solid particles.

Compound Sodium Lactate and Dextrose Injection

Compound Sodium Lactate with Dextrose Intravenous Infusion; Ringer-Lactate Solution with Dextrose for Injection; Hartmann's Solution with Dextrose for Injection.

Compound Sodium Lactate and Dextrose Injection is a sterile solution containing 0.24 per cent v/v of Lactic Acid (equivalent to 0.32 per cent w/v of sodium lactate) with 0.6 per cent w/v of Sodium Chloride, 0.04 per cent w/v of Potassium Chloride, 0.027 per cent w/v of Calcium Chloride and 5 per cent w/v of Dextrose in Water for Injections.

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Compound Sodium Lactate and Dextrose Injection contains not less than 0.27 per cent and not more than 0.32 per cent w/v of sodium, Na, not less than 0.019 per cent and not more than 0.022 per cent w/v of potassium, K, not less than 0.37 per cent and not more than 0.42 per cent w/v of total chloride, Cl, not less than 0.025 per cent and not more than 0.029 per cent w/v of calcium chloride, $CaCl_2,2H_2O$, and not less than 0.23 per cent and not more than 0.28 per cent w/v of lactate, calculated as $C_3H_6O_3$, and not less than 4.50 per cent and not more than 5.25 per cent w/v of dextrose, $C_6H_{12}O_6$. It contains no antimicrobial agent.

Category. Fluid and electrolyte replenisher.

Description. A clear, colourless or faintly straw-coloured solution.

Identification

A. To 1 ml add 0.05 ml of *potassium cupri-tartrate solution*; the solution remains blue and clear. Heat to boiling, a copious red precipitate is formed.

B. When warmed with *potassium permanganate* gives acetaldehyde, recognisable by its odour.

C. The residue on evaporation, when moistened with *hydrochloric acid* and introduced on a platinum wire into a flame imparts a yellow colour to the flame. When viewed through a suitable blue glass, the flame is tinged reddish purple.

D. It gives reaction (C) of calcium salts and reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 4.0 to 6.5.

5-Hydroxymethylfurfural and Related substances. Dilute a volume containing 1.0 g of Dextrose to 500.0 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 284 nm; absorbance at about 284 nm, not more than 0.25 (2.4.7).

Heavy metals (2.3.13). Evaporate a volume containing 4 g of dextrose to 10 ml and add 2 ml of *dilute acetic acid* and sufficient *water* to produce 25 ml. The solution complies with the limit test for heavy metals, Method A (5 ppm).

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *For sodium* — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 589 nm and using *sodium solution FP* or *sodium solution AAS* respectively, suitably diluted with water for the standard solutions.

For potassium — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 767 nm and using *potassium solution FP* or *potassium solution AAS* respectively, suitably diluted with *water* for the standard solutions.

For total chlorides — To 20.0 ml add 30 ml of *water*, 50.0 ml of *0.1 M silver nitrate* and 2 ml of *nitric acid*. Filter, wash the precipitate with *water* slightly acidified with *nitric acid* and titrate the excess of silver nitrate with *0.1 M ammonium thiocyanate* using *ferric ammonium sulphate solution* as indicator until a reddish yellow colour is produced. Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of total chloride, calculated as Cl.

For calcium chloride — To 50.0 ml add 5.0 ml of *0.01 M magnesium sulphate* and 5 ml of *ammonia buffer pH 10.9* and titrate with *0.01 M disodium edetate* using *eriochrome black T mixture* as indicator. Carry out a blank titration.

1 ml of *0.01 M disodium edetate* is equivalent to 0.00147 g of $CaCl_2,2H_2O$.

For lactate — Determine by liquid chromatography (2.4.14).

Test solution. The preparation under examination.

Reference solution. A 0.28 per cent w/v solution of *lithium lactate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 20 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 90 volumes of *water* and 10 volumes of a 2 per cent v/v solution of *octylamine* in *acetonitrile*, the pH of which is adjusted to 7.0 with a 10 per cent v/v solution of *phosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Inject separately the reference solution and the test solution and measure the responses for the major peak.

Calculate the content of $C_3H_6O_3$, in the injection.

For dextrose — To a measured volume containing 2 g to 5 g of Dextrose, add 0.2 ml of *5 M ammonia* and sufficient *water* to produce 100.0 ml. Mix well, allow to stand for 30 minutes and measure the optical rotation in a 2-dm tube (2.4.22). The observed rotation in degrees multiplied by 0.9477 represents the weight, in g, of dextrose, $C_6H_{12}O_6$, in the volume taken for assay.

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Storage. Store in single dose containers of glass or plastic at a temperature not exceeding 30°. On keeping, small particles may separate from the solution in glass containers.

Labelling. The label states (1) that the injection contains, in millimoles per litre, the following approximate amounts of the ions: sodium, 131; potassium, 5; calcium, 2; bicarbonate (as lactate), 29 and Chloride, 111; (2) the total osmolar concentration in mOsmol per litre; (3) that the injection should not be used if it contains visible particles.

Half Strength Compound Sodium Lactate and Dextrose Injection

Half Strength Compound Sodium Lactate with Dextrose Intravenous Infusion; Half Strength Ringer-Lactate Solution with Dextrose Injection

Half Strength Compound Sodium Lactate and Dextrose Injection is a sterile solution containing 0.12 per cent v/v of Lactic Acid (equivalent to 0.16 per cent w/v of sodium lactate) with 0.3 per cent w/v of Sodium Chloride, 0.02 per cent w/v of Potassium Chloride, 0.0135 per cent w/v of Calcium Chloride and 5 per cent w/v of Dextrose in Water for Injections.

Compound Sodium Lactate and Dextrose Injection contains not less than 0.135 per cent and not more than 0.16 per cent w/v of sodium, Na, not less than 0.0095 per cent and not more than 0.011 per cent w/v of potassium, K, not less than 0.185 per cent and not more than 0.210 per cent w/v of total chloride, Cl, not less than 0.0125 per cent and not more than 0.0145 per cent w/v of calcium chloride, $CaCl_2,2H_2O$, and not less than 0.115 per cent and not more than 0.140 per cent w/v of lactate, calculated as $C_3H_6O_3$, and not less than 4.5 per cent and not more than 5.25 per cent w/v of dextrose, $C_6H_{12}O_6$. It contains no antimicrobial agent.

Category. Fluid and electrolyte replenisher.

Description. A clear, colourless or faintly straw-coloured solution.

Identification

A. To 1 ml add 0.05 ml of *potassium cupri-tartrate solution*; the solution remains blue and clear. Heat to boiling, a copious red precipitate is formed.

B. When warmed with *potassium permanganate* gives acetaldehyde, recognisable by its odour.

C. The residue on evaporation, when moistened with *hydrochloric acid* and introduced on a platinum wire into a flame imparts a yellow colour to the flame. When viewed through a suitable blue glass, the flame is tinged reddish purple.

D. It gives reaction (C) of calcium salts and reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 4.0 to 6.5.

5-Hydroxymethylfurfural and Related substances. Dilute a volume containing 1.0 g of Dextrose to 500.0 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 284 nm; absorbance at about 284 nm, not more than 0.25 (2.4.7).

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *For sodium* — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 589 nm and using *sodium solution FP* or *sodium solution AAS* respectively, suitably diluted with *water* for the standard solutions.

For potassium — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 767 nm and using *potassium solution FP* or *potassium solution AAS* respectively, suitably diluted with *water* for the standard solutions.

For total chlorides — To 20.0 ml add 30 ml of *water*, 50.0 ml of *0.1 M silver nitrate* and 2 ml of *nitric acid*. Filter, wash the precipitate with *water* slightly acidified with *nitric acid* and titrate the excess of silver nitrate with *0.1 M ammonium thiocyanate* using *ferric ammonium sulphate solution* as indicator until a reddish yellow colour is produced. Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of total chloride, calculated as Cl.

For calcium chloride — To 50.0 ml add 5.0 ml of *0.01 M magnesium sulphate* and 5 ml of *ammonia buffer pH 10.9* and titrate with *0.01 M disodium edetate* using *eriochrome black T mixture* as indicator. Carry out a blank titration.

1 ml of *0.01 M disodium edetate* is equivalent to 0.00147 g of $CaCl_2,2H_2O$.

For lactate — Determine by liquid chromatography (2.4.14).

Test solution. The preparation under examination.

Reference solution. A 0.28 per cent w/v solution of *lithium lactate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 20 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 90 volumes of *water* and 10 volumes of a 2 per cent v/v solution of *octylamine* in

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acetonitrile, the pH of which is adjusted to 7.0 with a 10 per cent v/v solution of *phosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Inject separately the reference solution and the test solution and measure the responses for the major peak.

Calculate the content of $C_3H_6O_3$ in the injection.

For dextrose — To a measured volume containing 2 g to 5 g of Dextrose, add 0.2 ml of *5 M ammonia* and sufficient *water* to produce 100.0 ml. Mix well, allow to stand for 30 minutes and measure the optical rotation in a 2-dm tube (2.4.22). The observed rotation in degrees multiplied by 0.9477 represents the weight, in g, of dextrose, $C_6H_{12}O_6$, in the volume taken for assay.

Storage. Store in single dose containers of glass or plastic at a temperature not exceeding 30°. On keeping, small particles may separate from the solution in glass containers.

Labelling. The label states (1) that the injection contains, in millimoles per litre, the following approximate amounts of the ions. sodium, 65.5, potassium, 2.5, calcium, 1, bicarbonate (as lactate), 14.5, and chloride, 55.5; (2) the total osmolar concentration in mOsmol per litre; (3) that the injection should not be used if it contains visible particles.

Modified Compound Sodium Lactate and Dextrose Injection

Modified Compound Sodium Lactate with Dextrose Intravenous Infusion; Modified Lactated Ringer's and Dextrose Injection.

Modified Compound Sodium Lactate and Dextrose Injection is a sterile solution containing 0.048 per cent v/v of Lactic Acid (equivalent to 0.064 per cent w/v of sodium lactate) with 0.12 per cent w/v of Sodium Chloride, 0.008 per cent w/v of Potassium Chloride, 0.0054 per cent w/v of Calcium Chloride and 5 per cent w/v of Dextrose in Water for Injections.

Modified Compound Sodium Lactate and Dextrose Injection contains not less than 0.054 per cent and not more than 0.064 per cent w/v of sodium, Na, not less than 0.0038 per cent and not more than 0.0044 per cent w/v of potassium, K, not less than 0.074 per cent and not more than 0.084 per cent w/v of total chloride, Cl, not less than 0.005 per cent and not more than 0.0058 per cent w/v of calcium chloride, $CaCl_2,2H_2O$, and not less than 0.046 per cent and not more than 0.056 per cent w/v of lactate, calculated as $C_3H_6O_3$, and not less than 4.5 per cent and not more than 5.25 per cent w/v of dextrose, $C_6H_{12}O_6$. It contains no antimicrobial agent.

Category. Fluid and electrolyte replenisher.

Description. A clear, colourless or faintly straw-coloured solution.

Identification

A. To 1 ml add 0.05 ml of *potassium cupri-tartrate solution*; the solution remains blue and clear. Heat to boiling, a copious red precipitate is formed.

B. When warmed with *potassium permanganate* gives acetaldehyde, recognisable by its odour.

C. The residue on evaporation, when moistened with hydrochloric acid and introduced on a platinum wire into a flame imparts a yellow colour to the flame. When viewed through a suitable blue glass, the flame is tinged reddish purple.

D. It gives reaction (C) of calcium salts and reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 4.0 to 6.5.

5-Hydroxymethylfurfural and Related substances. Dilute a volume containing 1.0 g of Dextrose to 500.0 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 284 nm; absorbance at about 284 nm (2.4.7), not more than 0.25.

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *For sodium* — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 589 nm and using *sodium solution FP* or *sodium solution AAS* respectively, suitably diluted with *water* for the standard solutions.

For potassium — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 767 nm and using *potassium solution FP* or *potassium solution AAS* respectively, suitably diluted with *water* for the standard solutions.

For total chlorides — To 20.0 ml add 30 ml of *water*, 50.0 ml of *0.1 M silver nitrate* and 2 ml of *nitric acid*. Filter, wash the precipitate with *water* slightly acidified with *nitric acid* and titrate the excess of silver nitrate with *0.1 M ammonium thiocyanate* using *ferric ammonium sulphate solution* as indicator until a reddish yellow colour is produced. Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of total chloride, calculated as Cl.

For calcium chloride — Evaporate 100.0 ml on a water-bath to 50 ml, add to this solution 5.0 ml of *0.01 M magnesium sulphate* and 5 ml of *ammonia buffer pH 10.9* and titrate with *0.01 M disodium edetate* using *eriochrome black T mixture* as indicator. Carry out a blank titration.

1 ml of *0.01 M disodium edetate* is equivalent to 0.00147 g of $CaCl_2,2H_2O$.

For lactate — Determine by liquid chromatography (2.4.14).

Test solution. The preparation under examination.

Reference solution. A 0.28 per cent w/v solution of *lithium lactate IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 20 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 90 volumes of *water* and 10 volumes of a 2 per cent v/v solution of *octylamine* in *acetonitrile*, the pH of which is adjusted to 7.0 with a 10 per cent v/v solution of *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Inject separately the reference solution and the test solution and measure the responses for the major peak.

Calculate the content of $C_3H_6O_3$ in the injection.

For dextrose — To a measured volume containing 2 g to 5 g of Dextrose, add 0.2 ml of *5 M ammonia* and sufficient *water* to produce 100.0 ml. Mix well, allow to stand for 30 minutes and determine the optical rotation in a 2-dm tube (2.4.22). The observed rotation in degrees multiplied by 0.9477 represents the weight, in g, of dextrose, $C_6H_{12}O_6$, in the volume taken for assay.

Storage. Store in single dose containers of glass or plastic at a temperature not exceeding 30°. On keeping, small particles may separate from the solution in glass containers.

Labelling. The label states (1) that the injection contains, in millimoles per litre, the following approximate amounts of the ions. sodium, 26.2, potassium, 1, calcium, 0.4, bicarbonate (as lactate), 5.8, and chloride, 22.2; (2) the total osmolar concentration in mOsmol per litre; (3) that the injection should not be used if it contains visible particles.

Compound Sodium Lactate Injection

Compound Sodium Lactate Intravenous Infusion; Ringer-Lactate Solution for Injection; Hartmann's Solution for Injection

Compound Sodium Lactate Injection is a sterile solution containing 0.24 per cent v/v of Lactic Acid (equivalent to 0.32 per cent w/v of sodium lactate) with 0.6 per cent w/v of Sodium Chloride, 0.04 per cent w/v of Potassium Chloride and 0.027 per cent w/v of Calcium Chloride in Water for Injections.

Compound Sodium Lactate Injection contains not less than 0.27 per cent and not more than 0.32 per cent w/v of sodium, Na, not less than 0.019 per cent and not more than 0.022 per cent w/v of potassium, K, not less than 0.37 per cent and not more than 0.42 per cent w/v of total chloride, Cl, not less than 0.025 per cent and not more than 0.029 per cent w/v of calcium chloride, $CaCl_2,2H_2O$, and not less than 0.23 per cent and not more than 0.28 per cent w/v of lactate, calculated as $C_3H_6O_3$.

Category. Fluid and electrolyte replenisher.

Description. A clear, colourless solution.

Identification

A. When warmed with potassium permanganate gives acetaldehyde, recognisable by its odour.

B. The residue on evaporation, when moistened with hydrochloric acid and introduced on a platinum wire into a flame, imparts a yellow colour to the flame. When viewed through a suitable blue glass, the flame is tinged with reddish purple.

C. It gives reaction (C) of calcium salts (2.3.1).

Tests

pH (2.4.24). 5.0 to 7.0.

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Intravenous Infusions).

Assay. *For sodium* — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by atomic absorption spectrophotometry (2.4.2), measuring at 589 nm and using *sodium solution FP* or *sodium solution AAS*, suitably diluted for the standard solutions.

For potassium — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by atomic absorption spectrophotometry (2.4.2), measuring at 767 nm and using *potassium solution FP* or *potassium solution AAS*, suitably diluted for the standard solutions.

For total chlorides — To 20 ml add 30 ml of *water*, 50.0 ml of *0.1 M silver nitrate* and 2 ml of nitric acid, filter, wash the precipitate with water slightly acidified with nitric acid and titrate the excess of silver nitrate with *0.1 M ammonium thiocyanate* using ferric ammonium sulphate solution as indicator. Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of total chloride, calculated as Cl.

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For calcium chloride — To 50 ml add 5.0 ml of *0.01 M magnesium sulphate* and 5 ml of *ammonia buffer pH 10.9* and titrate with *0.01 M disodium edetate* using *mordant black 11 mixture* as indicator. Carry out a blank titration.

1 ml of *0.01 M disodium edetate* is equivalent to 0.00147 g of $CaCl_2, 2H_2O$.

For lactate — Determine by liquid chromatography (2.4.14).

Test solution. Use the preparation under examination.

Reference solution (a). A 0.3 per cent w/v solution of *sodium lactate IPRS* in *water*.

Reference solution (b). A solution containing 0.3 per cent w/v, each of, *anhydrous sodium acetate* and *sodium lactate IPRS* in *water*.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5µm),
- mobile phase: a mixture of 99.8 volumes of *water*, 0.1 volume of *dicyclohexylamine* and 0.1 volume of *formic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to acetate and lactate is not less than 2 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 2.0 per cent and the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_3H_6O_3$ in the injection.

Storage. Store in single dose containers of glass or plastic. On keeping, small solid particles may separate from the solution in glass containers.

Labelling. The label states (1) that the injection contains, in millimoles per litre, the following approximate amounts of the ions: sodium, 131, potassium, 5, calcium, 2, bicarbonate (as lactate), 29 and chloride, 111; (2) that the injection should not be used if the solution contains visible solid particles.

Compound Sodium Lactate Solution for Irrigation

Ringer-Lactate Solution for Irrigation; Hartmann's Solution for Irrigation

Compound Sodium Lactate Solution for Irrigation is a sterile solution containing 0.24 per cent v/v of Lactic Acid (equivalent to 0.32 per cent w/v of sodium lactate) with 0.6 per cent w/v of Sodium Chloride, 0.04 per cent w/v of Potassium Chloride and 0.027 per cent w/v of Calcium Chloride in Water for Injections.

Compound Sodium Lactate Solution for Irrigation contains not less than 0.27 per cent and not more than 0.32 per cent w/v of sodium, Na, not less than 0.019 per cent and not more than 0.022 per cent w/v of potassium, K, not less than 0.37 per cent and not more than 0.42 per cent w/v of total chloride, Cl, not less than 0.025 per cent and not more than 0.029 per cent w/v of calcium chloride, $CaCl_2, 2H_2O$, and not less than 0.23 per cent and not more than 0.28 per cent w/v of lactate, calculated as $C_3H_6O_3$. It contains no antimicrobial agent.

Category. Irrigation fluid.

Description. A clear, colourless solution.

Identification

A. When warmed with *potassium permanganate* gives acetaldehyde, recognisable by its odour.

B. The residue on evaporation, when moistened with *hydrochloric acid* and introduced on a platinum wire into a flame imparts a yellow colour to the flame. When viewed through a suitable blue glass, the flame is tinged reddish purple.

C. It gives reaction (C) of calcium salts and reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 5.0 to 7.0.

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *For sodium* — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 589 nm and using *sodium solution FP* or *sodium solution AAS* respectively, suitably diluted with *water* for the standard solutions.

For potassium — Dilute suitably with *water* and determine by Method A for flame photometry (2.4.4), or by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 767 nm and using *potassium solution FP* or *potassium solution AAS* respectively, suitably diluted with *water* for the standard solutions.

For calcium chloride — To 50.0 ml add 5.0 ml of *0.01 M magnesium sulphate* and 5 ml of *ammonia buffer pH 10.9* and titrate with *0.01 M disodium edetate* using *eriochrome black T mixture* as indicator. Carry out a blank titration.

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1 ml of *0.01 M disodium edetate* is equivalent to 0.00147 g of $CaCl_2, 2H_2O$.

For total chlorides — To 20.0 ml add 30 ml of *water*, 50.0 ml of *0.1 M silver nitrate* and 2 ml of *nitric acid*. Filter, wash the precipitate with *water* slightly acidified with *nitric acid* and titrate the excess of silver nitrate with *0.1 M ammonium thiocyanate* using *ferric ammonium sulphate solution* as indicator until a reddish yellow colour is produced. Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of total chloride, calculated as Cl.

For lactate — Determine by liquid chromatography (2.4.14).

Test solution. The preparation under examination.

Reference solution. A 0.28 per cent w/v solution of *lithium lactate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 20 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 90 volumes of *water* and 10 volumes of a 2 per cent v/v solution of *octylamine* in *acetonitrile*, the pH of which is adjusted to 7.0 with a 10 per cent v/v solution of *phosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Inject separately the reference solution and the test solution and measure the responses for the major peak.

Calculate the content of $C_3H_6O_3$, in the injection.

Storage. Store in single dose containers of glass or plastic. On keeping, small solid particles may separate from the solution in glass containers. The container may be designed to empty rapidly and may contain a volume of more than one litre.

Labelling. The label states (1) that the irrigation solution contains, in millimoles per litre, the following approximate amounts of the ions. sodium,131, potassium, 5, calcium, 2, bicarbonate (as lactate), 29, and chloride, 111; (2) that the solution should not be used if it contains visible particles; (3) 'For irrigation only' and 'Not for injection'; (4) that once the container is opened, the unused portion should be discarded.

Sodium Lauryl Sulphate

Sodium Lauryl Sulphate is a mixture of sodium alkyl sulphates consisting mainly of sodium dodecyl sulphate, $CH_3[CH_2]_{10}CH_2OSO_3Na$.

Sodium Lauryl Sulphate contains not less than 85.0 per cent of sodium alkyl sulphates, calculated as $C_{12}H_{25}NaO_4S$.

Category. Pharmaceutical aid (anionic emulsifying agent).

Description. A white or pale yellow powder or crystals.

Identification

A. A 1 per cent w/v solution, when shaken, produces plenty of foam.

B. Mix 0.1 ml of a 1 per cent w/v solution with 0.1 ml of a 0.1 per cent w/v solution of *methylene blue* and 2 ml of *1 M sulphuric acid*, add 2 ml of *dichloromethane* and shake; the dichloromethane layer is intensely blue.

C. Mix about 10 mg with 10 ml of *ethanol* and heat to boiling on a water-bath, shaking frequently. Filter immediately and evaporate the *ethanol*. Dissolve the residue in 8 ml of *water*, add 3 ml of *2 M hydrochloric acid*, evaporate the solution to half its volume and cool. Filter and to the filtrate add 1 ml of *barium chloride solution*; a white, crystalline precipitate is produced.

D. It gives reaction (B) of sodium salts (2.3.1).

Tests

Alkalinity. Dissolve 1.0 g in 100 ml of *carbon dioxide-free water* and add 0.1 ml of *phenol red solution*. Not more than 0.5 ml of *0.1 M hydrochloric acid* is required to change the colour of the solution.

Non-esterified alcohols. Not more than 4 per cent, determined by the following method. Dissolve 10.0 g in 100 ml of *water*, add 100 ml of *ethanol (95 per cent)* and extract the solution with three quantities, each of 50 ml, of *n-pentane*, adding *sodium chloride*, if necessary, to promote separation of the two layers. Wash the combined organic layers with three quantities, each of 50 ml, of *water*. Dry the organic solution over *anhydrous sodium sulphate*, filter and evaporate on a water-bath until the odour of pentane is no longer detectable. Heat the residue at 105° for 15 minutes, cool and weigh.

Sodium Chloride and Sodium Sulphate. Not more than a total of 8.0 per cent, determined by the following methods.

For sodium chloride — Dissolve 5.0 g in 50 ml of *water*, add *2 M nitric acid* dropwise until the solution is neutral to *litmus paper*, add 2 ml of *potassium chromate solution* and titrate with *0.1 M silver nitrate*.

1 ml of *0.1 M silver nitrate* is equivalent to 0.005844 g of NaCl.

For sodium sulphate — Dissolve 0.5 g in 20 ml of *water*, warming gently if necessary, and add 1 ml of a 0.05 per cent w/v solution of *dithizone* in *acetone*. If the solution is red, add *1 M nitric acid*, dropwise, until it becomes bluish green. Add 2 ml of *dichloroacetic acid solution* and 80 ml of *acetone* and titrate with *0.01 M lead nitrate* until a permanent orange-red colour is obtained.

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1 ml of *0.01 M lead nitrate* is equivalent to 0.001420 g of Na_2SO_4.

Assay. Weigh 1.15 g, dissolve in sufficient *water* to produce 1000.0 ml, warming if necessary. To 20.0 ml add 15 ml of *chloroform*, 10 ml of *dilute sulphuric acid* and 1 ml of *dimethyl yellow-oracet blue B solution* and titrate with *0.004 M benzethonium chloride*, shaking vigorously and allowing the layers to separate after each addition, until the chloroform layer acquires a permanent clear green colour.

1 ml of *0.004 M benzethonium chloride* is equivalent to 0.001154 g of sodium alkyl sulphates, calculated as $C_{12}H_{25}NaO_4S$.

Storage. Store protected from moisture.

Sodium Metabisulphite

Sodium Pyrosulphite; Sodium Disulphite

$Na_2S_2O_5$ Mol. Wt. 190.1

Sodium Metabisulphite may be prepared by saturating a solution of Sodium Hydroxide with sulphur dioxide and allowing crystallisation to occur.

Sodium Metabisulphite contains not less than 95.0 per cent and not more than 100.5 per cent of $Na_2S_2O_5$.

Category. Pharmaceutical aid (antioxidant).

Description. Colourless, prismatic crystals or a white or creamy white powder.

Identification

A. A 5 per cent w/v solution in *carbon dioxide free water* (solution A) gives reaction (A) of sodium salts (2.3.1).

B. To 0.4 ml of *iodinated potassium iodide solution*, add 8 ml of *water* and 1 ml of solution A, diluted 1 to 10 in *water*. The solution is colourless and gives reaction (A) of sulphates (2.3.1).

Tests

pH (2.4.24). 3.5 to 5.0, determined in solution A.

Arsenic (2.3.10). Mix 2.5 g in a porcelain dish with 10 ml of *water*, 1.25 g of *potassium chlorate* and 16 ml of *hydrochloric acid* and heat to expel chlorine, remove the last traces of chlorine with a few drops of *stannous chloride solution AsT* and add 35 ml of *water*. The resulting solution complies with the limit test for arsenic (4 ppm).

Heavy metals (2.3.13). Dissolve 1.0 g in 10 ml of *water*, add 5 ml of *hydrochloric acid* and evaporate to dryness on a water-bath. Dissolve the residue in 25 ml of *water* containing 2 ml of *dilute acetic acid*. The solution complies with the limit test for heavy metals, Method A (20 ppm).

Iron. To 0.5 g add 2 ml of *hydrochloric acid* and evaporate on a water-bath to dryness. Dissolve the residue in 2 ml of *hydrochloric acid* and 20 ml of *water* and add a few drops of *bromine solution*, cool, dilute with *water* to 25 ml, then add 50 mg of *ammonium persulphate* and 5 ml of *ammonium thiocyanate solution*. Any colour produced is not more intense than that obtained by adding 5 ml of *ammonium thiocyanate solution* to a mixture of 1 ml of *iron standard solution (20 ppm Fe)*, 2 ml of *hydrochloric acid*, 50 mg of *ammonium persulphate* and sufficient *water* to produce 25 ml (40 ppm).

Thiosulphate. Dissolve 1.0 g in 10 ml of *2 M hydrochloric acid* and heat on a water-bath for 10 minutes; not more than a faint opalescence is produced.

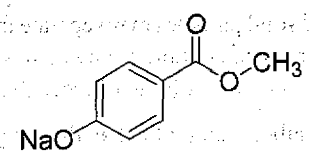
Assay. Dissolve 0.1 g in 50.0 ml *0.05 M iodine*, add 1 ml of *hydrochloric acid* and titrate the excess of iodine with *0.1 M sodium thiosulphate* using *starch solution*, added towards the end of the titration, as indicator.

1 ml of *0.05 M iodine* is equivalent to 0.004753 g of $Na_2S_2O_5$.

Storage. Store protected from light and moisture. On exposure to air and moisture it is slowly oxidised to sulphate with disintegration of the crystals.

Sodium Methylparaben

Sodium Methyl Hydroxybenzoate



$C_8H_7NaO_3$ Mol. Wt. 174.1

Sodium Methylparaben is the sodium salt of methyl 4-hydroxybenzoate.

Sodium Methylparaben contains not less than 95.0 per cent and not more than 102.0 per cent of $C_8H_7NaO_3$, calculated on the anhydrous basis.

Category. Pharmaceutical aid (antimicrobial preservative).

Description. A white, crystalline powder; hygroscopic.

Identification

A. Dissolve 0.5 g in 5 ml of *water* and acidify to *litmus paper* with *hydrochloric acid*; a white precipitate is produced. Wash the precipitate with *water* and dry.

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Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *methylparaben IPRS*.

B. A 5 per cent w/v solution gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution in *water* is clear (2.4.1).

pH (2.4.24). 9.5 to 10.5, determined in a 0.1 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 2.5 ml of *methanol* and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of *4-hydroxybenzoic acid* and 5 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 50 mg of *methylparaben IPRS* in 2.5 ml of *methanol* and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of the test solution to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of 0.68 per cent w/v solution of *potassium dihydrogen phosphate*, and 65 volumes of *methanol*,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 272 nm,
- injection volume: 10 µl.

The relative retention time with reference to methyparaben for 4-hydroxybenzoic acid is about 0.6.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to methylparaben and 4-hydroxybenzoic acid is not less than 2.0.

Inject reference solution (c) and the test solution. Run the chromatogram five times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any peak corresponding to 4-hydroxybenzoic acid multiplied by 1.4 is not more than the six times the area of the principal peak in the chromatogram obtained with reference solution (c) (3.0 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent) and the sum of areas of all the secondary peaks other than 4-hydroxybenzoic acid is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent). Ignore the peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Chlorides (2.3.12). Dissolve 1.0 g in 20 ml of *water*, add 0.4 ml of *nitric acid* and filter. 15 ml of the filtrate complies with the limit test for chlorides (330 ppm).

Sulphates (2.3.17). Dissolve 0.5 g in 40 ml of *water*, add 3.5 ml of *2 M hydrochloric acid*, dilute to 60 ml with *water* and filter. 15 ml of the filtrate complies with the limit test for sulphates (0.12 per cent).

Water (2.3.43). Not more than 5.0 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances.

Inject reference solution (b) and the test solution.

Calculate the content of $C_8H_7NaO_3$, multiplying the content of methylparaben by a correction factor of 1.145.

Storage. Store protected from moisture.

Sodium Nitrite

$NaNO_2$ Mol. Wt. 69.0

Sodium Nitrite is nitrous acid, sodium salt.

Sodium Nitrite contains not less than 97.0 per cent and not more than 101.0 per cent of $NaNO_2$, calculated on the dried basis.

Identification

A 10 per cent w/v solution complies with the tests of sodium salts (2.3.1) and nitrite (2.3.1).

Tests

Heavy metals (2.3.13). Dissolve 1 g in 6 ml of *3 M hydrochloric acid*, and evaporate on a steam-bath to dryness. Reduce the residue to a coarse powder, and continue heating on the steam-bath until the odor of hydrochloric acid no longer is perceptible. Dissolve the residue in 23 ml of *water*, and add 2 ml of *1 M acetic acid*. The solution complies with the limit test for heavy metals, method A (20 ppm).

Loss on drying (2.4.19). Not more than 0.25 per cent, drying it over silica gel for 4 hours.

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Assay. Dissolve 1 g in 250.0 ml of *water*. Pipette 25 ml of the solution into a mixture of 50.0 ml of *0.02 M potassium permanganate*, 50 ml of *water* and 20 ml of *dilute sulphuric acid*. When adding the sodium nitrite solution, immerse the tip of the pipette beneath the surface of the permanganate mixture. Stopper the flask, allow it to stand for 15 minutes with occasional shaking and add 3 g of *potassium iodide* and titrate the liberated iodine with *0.1 M sodium thiosulphate* using starch solution, added towards the end of titration as an indicator. Carry out a blank titration.

1 ml of *0.02 M potassium permanganate* is equivalent to 0.00345 g of $NaNO_2$.

Storage. Store protected from moisture, at a temperature between 15° to 30°.

Sodium Nitrite Injection

Sodium Nitrite Injection is a sterile solution of Sodium Nitrite in Water for Injections.

Sodium Nitrite Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of $NaNO_2$.

Usual strength. 30 mg per ml.

Identification

The injection complies with the tests of sodium salts (2.3.1) and nitrite (2.3.1).

Tests

pH (2.4.24). 7.0 to 9.0.

Bacterial endotoxins (2.2.3). Not more than 0.33 Endotoxin Unit per mg of sodium nitrite.

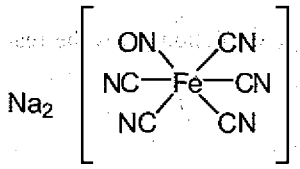
Other tests. Comply with the tests stated under Injections.

Assay. Pipette a volume of injection containing 90 mg of Sodium Nitrite into a mixture of 50.0 ml of *0.02 M potassium permanganate*, 50 ml of *water* and 20 ml of *dilute sulphuric acid*, immersing the tip of the pipette beneath the surface of the permanganate mixture during the addition. Stopper the flask, allow it to stand for 15 minutes with occasional shaking and add 3 g of *potassium iodide* and titrate the liberated iodine with *0.1 M sodium thiosulphate* using starch solution added towards the end of titration as an indicator. Carry out a blank titration.

1 ml of *0.02 M potassium permanganate* is equivalent to 0.00345 g of $NaNO_2$.

Storage. Preserve in single-dose containers, preferably of Type I glass.

Sodium Nitroprusside



$Na_2[Fe(CN)_5NO],2H_2O$ Mol. Wt. 298.0

Sodium Nitroprusside is sodium pentacyanonitrosylferrate.

Sodium Nitroprusside contains not less than 99.0 per cent and not more than 100.5 per cent of sodium pentacyanonitrosylferrate (III)-$Na_2[Fe(CN)_5NO]$, calculated on the anhydrous basis.

Category. Vasodilator.

Description. A reddish-brown powder or crystals.

Identification

A. When examined in the range 350 nm to 600 nm (2.4.7), a 0.7 per cent w/v solution in *water* shows an absorption maximum at 395 nm, a shoulder at 510 nm and a minima at 370 nm. The specific absorbance at the maximum is 0.65 to 0.80.

B. Dissolve 20 mg in 2 ml of *water* and add 0.1 ml of *sodium sulphide solution*; a deep violet-red colour is produced.

C. Dissolve 50 mg in 1 ml of *water* and acidify the solution by the addition of *hydrochloric acid*. Place a drop of the solution in an oxidising flame; a persistent yellow colour is produced.

Tests

Insoluble matter. Dissolve 10 g without heating in 50 ml of *water*. Allow to stand for 30 minutes and filter through a sintered-glass filter. Wash the filter with cold *water* until the filtrate is colourless. Dry the residue on the filter at 105°. The residue weighs not more than 1 mg (100 ppm).

Chlorides (2.3.12). In a metallic crucible (nickel) mix 1.0 g with 8 ml of a 20 per cent w/v solution of *sodium hydroxide*. Heat slowly and evaporate carefully to dryness over a small flame, then heat to a dull red colour for 30 minutes. Allow to cool and transfer the solid residue with three successive portions, each of 8 ml of *dilute sulphuric acid*. Filter the *sulphuric acid* extracts on a filter paper and collect the filtrates. Render the filtrate acid to litmus paper by adding, if necessary, a few drops of *dilute sulphuric acid*. Wash the crucible and the filter paper with three successive portions of 10 ml of *water*, add the washings to the main sulphuric acid solution and dilute to 60 ml with *water*. 15 ml of the solution complies with the limit test for chlorides (200 ppm), using 2 ml of *chloride standard solution (25 ppm Cl)*.

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Ferricyanides. Dissolve 1.25 g in 50.0 ml of *acetate buffer solution pH 4.6*. Use three 50-ml volumetric flasks (A, B, C). To flask B, add 1.0 ml of *ferricyanide standard solution* (50 ppm $Fe(CN)_6$). To flasks A and B, add 1 ml of a 0.5 per cent w/v solution of *ferrous ammonium sulphate*. To the three flasks, add 10.0 ml of the solution of the substance under examination. Dilute the contents of each flask to 50.0 ml with *water*. Allow to stand for 30 minutes. The absorbance of the solution in flask A measured at 720 nm (2.4.7) using the solution in flask C as the compensation liquid is not more than the absorbance of the solution in flask B measured at 720 nm using the solution in flask A as the compensation liquid (200 ppm).

Ferrocyanides. Dissolve 4.0 g in 100.0 ml of *water*. Use three 50-ml volumetric flasks (A, B, C). To flask B, add 2.0 ml of *ferrocyanide standard solution* (100 ppm $Fe(CN)_6$). To flasks A and B, add 1 ml of *ferric chloride solution*. To the three flasks, add 25.0 ml of the solution of the substance under examination. Dilute the contents of each flask to 50.0 ml with *water*. Allow to stand for 30 minutes. The absorbance of the solution in flask A measured at 695 nm (2.4.7) using the solution in flask C as the compensation liquid is not more than the absorbance of the solution in flask B measured at 695 nm using the solution in flask A as the compensation liquid (200 ppm).

Sulphates (2.3.17). Not more than 100 ppm.

Test solution. Dissolve 3.6 g in 120 ml of *water*, add with mixing 4 ml of sulphate standard solution (10 ppm SO_4) and 20 ml of a 25 per cent w/v solution of *cupric chloride* and dilute to 150.0 ml with *water*. Allow to stand for 16 hours and filter or centrifuge until a clear light-blue solution is obtained.

Reference solution. To 40 ml of sulphate standard solution (10 ppm SO_4), add 80 ml of *water* and 12 ml to 13 ml of a 25 per cent w/v solution of *cupric chloride*, dilute to 150.0 ml with *water*. The volume of cupric chloride solution added is such that the colour of the final solution matches that of the test solution.

Allow the solutions to stand. Filter both solutions, discarding the first 25 ml of filtrate. To 100 ml of each filtrate, add 0.5 ml of *acetic acid*. Mix and add 2 ml of a 25 per cent w/v solution of *barium chloride* and mix again. The test solution is not more opalescent than the reference solution.

Water (2.3.43). 9.0 to 15.0 per cent, determined on 0.25 g.

Assay. Dissolve 0.25 g in 100 ml of *water* and add 0.1 ml of *dilute sulphuric acid*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (2.4.25), using silver-mercurous sulphate electrode system.

1 ml of *0.1 M silver nitrate* is equivalent to 0.0131 g of $Na_2[Fe(CN)_5NO]$.

Storage. Store protected from light.

Sodium Nitroprusside Injection

Sodium Nitroprusside Injection is a sterile material obtained from a solution of Sodium Nitroprusside by freeze drying. It may contain excipients. It is filled in a sealed container.

The injection is constituted immediately before use by dissolving Sodium Nitroprusside Injection in the requisite amount of Glucose Intravenous Infusion (50 g per litre) and diluting the resulting solution with 250 to 500 times its volume of Glucose Intravenous Infusion (50 g per litre).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Sodium Nitroprusside Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of sodium nitroprusside dihydrate, $Na_2Fe(CN)_5NO, 2H_2O$.

Usual strength. 50 mg per vial.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Identification

A. When examined in the range 350 nm to 600 nm (2.4.7), a 1.6 per cent w/v solution exhibits a maximum at 395 nm and the absorbance at 395 nm is about 1.1.

B. Dissolve 20 mg in 2 ml of *water* and add 0.1 ml of *sodium sulphide solution*; a deep purple colour is produced.

C. It gives the reactions of sodium salts (2.3.1).

Tests

Bacterial endotoxins (2.2.3). Not more than 0.05 Endotoxin Unit per µg of sodium nitroprusside.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

NOTE— Protect the solutions from light.

Test solution. Dissolve the contents of a sealed container in 5 ml of *water* and dilute the resulting solution with *water* to produce a solution containing the equivalent of 0.15 per cent w/v of Sodium nitroprusside dihydrate.

Reference solution (a). A 0.15 per cent w/v solution of *sodium nitroprusside IPRS* in *water*.

Reference solution (b). A solution containing 0.015 per cent w/v each of *sodium nitroprusside IPRS* and *potassium hexacyanoferrate (III)* in *water*.

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Chromatographic system

- a stainless steel column 30 cm x 3.9 mm packed with phenyl groups chemically bonded to porous silica (10 µm) (Such as µBondapak),
- mobile phase: a mixture of 6 volumes of *methanol* and 9 volumes of a solution containing 0.34 per cent w/v of *tetrabutylammonium hydrogen sulphate* and 0.284 per cent w/v of *anhydrous disodium hydrogen orthophosphate*, adjusted to pH 6.0 with *orthophosphoric acid*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks corresponding to ferricyanide and nitroprusside is not less than 3.0.

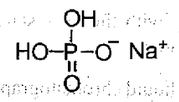
Inject reference solution (a) and the test solution.

Calculate the content of $Na_2Fe(CN)_5NO,2H_2O$ in the injection.

Labelling. The label states (1) the content of Sodium Nitroprusside in terms of the equivalent of sodium nitroprusside dihydrate contained in it; (2) that the contents of the sealed container should be dissolved in, and immediately diluted with, the stated volume of Glucose Intravenous Infusion (50 g per litre); (3) that Sodium Nitroprusside Intravenous Infusion must be used immediately after preparation; (4) that highly coloured solutions must not be used; (5) that the diluted solution must be protected from light during infusion.

Monobasic Sodium Phosphate

Sodium Dihydrogen Phosphate



NaH_2PO_4,xH_2O

Monosodium phosphate anhydrous	Mol. Wt.120.0
Monosodium phosphate monohydrate	Mol. Wt.138.0
Monosodium phosphate dihydrate	Mol. Wt.156.0

Monobasic Sodium Phosphate contains (one or two molecules of water of hydration or it is anhydrous) not less than 98.0 per cent and not more than 103.0 per cent of NaH_2PO_4, calculated on the anhydrous basis.

Category. Pharmaceutical aid.

Description. A white powder or colourless crystals.

Identification

A. A 5.0 per cent w/v solution in *carbon dioxide free water* (solution A) gives the reactions of phosphates (2.3.1).

B. Solution A gives reactions of sodium salt (2.3.1).

Tests

pH (2.4.24). 4.1 to 4.5, in a solution containing the equivalent of 1.0 g of NaH_2PO_4,H_2O in 20 ml of *water*.

Insoluble substances. Dissolve a portion equivalent to 1.0 g of NaH_2PO_4,H_2O in 100.0 ml of hot *water*, filter through a tarred crucible, wash the insoluble residue with hot *water* and dry at 105° for 2 hours. The weight of the residue is not more than 0.2 per cent.

Chlorides (2.3.12). To a portion equivalent to 1.78 g of NaH_2PO_4,H_2O, add 10 ml of *nitric acid* and 5 ml of *water*. The solution complies with the limit test for chlorides (140 ppm).

Sulphates (2.3.17). Dissolve a portion equivalent to 0.1 g of NaH_2PO_4,H_2O shows no more sulphate than corresponds to 0.3 ml of *0.01 M sulphuric acid* (0.15 per cent).

Aluminium, calcium and related elements. A portion equivalent to 1.0 g of NaH_2PO_4,H_2O in 10 ml of *water* does not become turbid when rendered slightly alkaline to litmus paper with *6 M ammonium hydroxide*.

Arsenic (2.3.10). Dissolve a portion equivalent to 1.25 g of NaH_2PO_4,H_2O in 35 ml of *water* and add 10 ml of *sannated hydrochloric acid AsT*. The resulting solution complies with limit test for arsenic (8 ppm).

Heavy metals (2.3.13). Dissolve a portion equivalent to 1.0 g of NaH_2PO_4,H_2O in 20 ml of *water*, and add 1 ml of *3 M hydrochloric acid* and dilute to 25 ml with *water*, the solution complies with the limit test for heavey metals, Method A (20 ppm).

Water (2.3.43). Not more than 2.0 per cent (anhydrous form), 10.0 to 15.0 per cent (monohydrate), 18.0 to 26.5 per cent (dihydrate), using Method 1.

Assay. Dissolve 2.5 g in 10 ml of *cold water*, add 20 ml of cold, saturated solution of *sodium chloride* then add *phenolphthalein* as indicator and titrate with *1 M sodium hydroxide* keeping the tempearture of the solution 10° to 15° during the entire titration. Carry out a blank titration.

1 ml of *1 M sodium hydroxide* is equivalent to 0.12 g of NaH_2PO_4.

Storage. Store protected from moisture.

Sodium Phosphate

Disodium Hydrogen Phosphate; Disodium Hydrogen Phosphate Dodecahydrate

$Na_2HPO_4,12H_2O$ Mol. Wt. 358.1

Sodium phosphate contains not less than 98.5 per cent and not more than 102.5 per cent of $Na_2HPO_4,12H_2O$.

Category. Cathartic; pharmaceutical aid (buffering agent).

Description. Colourless, transparent crystals; very efflorescent.

Identification

A 10.0 per cent w/v solution in *distilled water* (solution A) gives the reactions of sodium salts and of phosphates (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). Dissolve 2.0 g in 10 ml of *water*, adding 4 ml of *1 M acetic acid* and diluting to 25 ml with *water*. The solution complies with the limit test for heavy metals, Method A (10 ppm).

Iron (2.3.14). 20 ml of solution A complies with the limit test for iron (20 ppm).

Chlorides (2.3.12). To 10 ml of solution A add 10 ml of *2 M nitric acid* and dilute to 20 ml with *water*. The resulting solution complies with the limit test for chlorides (250 ppm).

Sulphates (2.3.17). To 2.5 ml of solution A add 2 ml of *2 M hydrochloric acid* and dilute to 15 ml with *distilled water*. The resulting solution complies with the limit test for sulphates (600 ppm).

Reducing substances. To 5 ml of solution A add 5 ml of *1 M sulphuric acid* and 0.25 ml of *0.02 M potassium permanganate* and heat on a water-bath for 5 minutes; the red colour is not completely discharged.

Sodium dihydrogen phosphate. The value of the expression $(n_2 - 25)/(25 - n_1)$,

where n_1 and n_2 are the titres of *1 M sodium hydroxide* obtained in the Assay, does not exceed 0.025.

Loss on drying (2.4.19). 57.0 to 61.0 per cent, determined on 1.0 g by drying in an oven at 130° for 4 hours.

Assay. Weigh 4.0 g (w), dissolve in 25 ml of *water*, add 25.0 ml of *1 M hydrochloric acid* and titrate potentiometrically with *1 M sodium hydroxide* to the first inflection point ($n_1$ml). Continue the titration until the second inflection of the curve is reached; the total volume of sodium hydroxide required is ($n_2$ml). Carry out a blank titration ($n_3$ml).

Calculate the content of $Na_2HPO_4,12H_2O$ from the expression

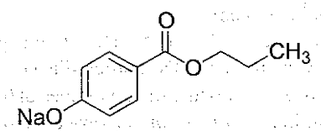
$$\frac{3581(n_3 - n_1)}{w \times 100}$$

where d is the percentage water content.

Storage. Store protected from moisture.

Sodium Propylparaben

Sodium Propyl Hydroxybenzoate



$C_{10}H_{11}NaO_3$ Mol. Wt. 202.2

Sodium Propylparaben is the sodium salt of propyl 4- hydroxybenzoate.

Sodium Propylparaben contains not less than 94.0 per cent and not more than 102.0 per cent of $C_{10}H_{11}NaO_3$, calculated on the anhydrous basis.

Category. Pharmaceutical aid (antimicrobial preservative).

Description. A white, crystalline powder; hygroscopic.

Identification

A. Dissolve 0.5 g in 5 ml of *water* and acidify to litmus paper with *hydrochloric acid*; a white precipitate is produced. Wash the precipitate with *water* and dry.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *propylparaben IPRS*.

B. A 5 per cent w/v solution gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution in *water* is clear (2.4.1).

pH (2.4.24). 9.5 to 10.5, determined in a 0.1 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

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Test solution. Dissolve 50 mg of the substance under examination in 2.5 ml of *methanol* and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of *4-hydroxybenzoic acid*, 5 mg of *ethyl parahydroxybenzoate IPRS (propylparaben impurity C IPRS)* and 5 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 50 mg of *propylparaben IPRS* in 2.5 ml of *methanol* and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of the test solution to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of 0.68 per cent w/v solution of *potassium dihydrogen phosphate*, and 65 volumes of *methanol*,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 272 nm,
- injection volume: 10 µl.

The relative retention time with reference to propylparaben for 4-hydroxybenzoic acid is about 0.3, for propylparaben impurity C is about 0.7.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to propylparaben and propylparaben impurity C is not less than 3.0.

Inject reference solution (c) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any peak corresponding to 4-hydroxybenzoic acid multiplied by 1.4 is not more than 8 times the area the principal peak in the chromatogram obtained with reference solution (c) (4.0 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent) and the sum of areas of all the secondary peaks other than 4-hydroxybenzoic acid is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent). Ignore the peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Chlorides (2.3.12). Dissolve 1.0 g in 20 ml of *water*, add 0.4 ml of *nitric acid* and filter. 15 ml of the filtrate complies with the limit test for chlorides (330 ppm).

Sulphates (2.3.17). Dissolve 0.5 g in 40 ml of *water*, add 3.5 ml of *2 M hydrochloric acid*, dilute to 60 ml with *water* and filter. 15 ml of the filtrate complies with the limit test for sulphates (0.12 per cent).

Water (2.3.43). Not more than 5.0 per cent, determined on 1.0 g.

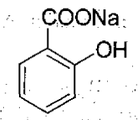
Assay. Determine by liquid chromatography (2.4.14), as described under Related substances.

Inject reference solution (b) and the test solution.

Calculate the content of $C_{10}H_{11}NaO_3$, multiplying the content of propylparaben by a correction factor of 1.122.

Storage. Store protected from moisture.

Sodium Salicylate



$C_7H_5NaO_3$ Mol. Wt. 160.1

Sodium Salicylate is sodium 2-hydroxybenzoate.

Sodium Salicylate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_7H_5NaO_3$, calculated on the dried basis.

Category. Anti-inflammatory; analgesic.

Description. Colourless, small crystals or shiny flakes or a white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Test B may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sodium salicylate IPRS* or with the reference spectrum of sodium salicylate.

B. A 10.0 per cent w/v solution in *carbon dioxide-free water* prepared from *distilled water* (solution A) gives the reactions of salicylates (2.3.1).

C. It gives reaction (B) of sodium salts (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and not more intensely coloured than reference solution BYS6 (2.4.1).

Acidity. To 20 ml of solution A add 0.1 ml of *phenol red solution*, the solution is yellow. Titrate with *0.01 M sodium hydroxide*

to a reddish violet colour; not more than 2.0 ml of *0.01 M sodium hydroxide* is required.

Arsenic (2.3.10). Mix 5.0 g with 10 ml of *bromine solution* and evaporate to dryness on a water-bath. Ignite gently, cool, dissolve the residue, ignoring any carbon, in 50 ml of *water* and 14 ml of *brominated hydrochloric acid AsT* and remove the excess of bromine with 2 ml of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). Dissolve 2.0 g in 46 ml of *water*, add with constant stirring 4 ml of *dilute hydrochloric acid*, filtering and using 25 ml of the filtrate. The solution complies with the limit test for heavy metals, Method A (20 ppm).

Chlorides (2.3.12). To 25 ml of solution A add 15 ml of *water* and 10 ml of *2 M nitric acid* and filter. 25 ml of the filtrate complies with the limit test for chlorides (200 ppm).

Sulphates (2.3.17). 2.5 ml of solution A diluted to 15 ml with *distilled water* complies with the limit test for sulphates (600 ppm).

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh 0.15 g, dissolve in 30 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01601 g of $C_7H_5NaO_3$.

Storage. Store protected from light and moisture.

Sodium Starch Glycolate (Type A)

Sodium Starch Glycolate (Type A) is the sodium salt of a cross linked partly O- carboxymethylated starch.

Sodium Starch Glycolate (Type A) contains not less than 2.8 per cent and not more than 4.2 per cent of sodium, Na, calculated on the material washed with Ethanol (95 per cent) and dried as described under Assay.

Category. Pharmaceutical aid (tablet disintegrant).

Description. A white or almost white, fine, free-flowing powder, very hygroscopic.

Identification

A. pH (see Test).

B. Shake 4.0 g of the substance under examination with 20 ml of *carbon-dioxide-free water*, without heating. This has the appearance of a gel. Add 100 ml of *carbon-dioxide-free water* and shake. A suspension forms that settles after standing.

C. To an acidified solution, add *iodinatedpotassium iodide solution*; a blue or violet colour produced.

D. Solution A (see Test) gives reaction (a) of sodium salts (2.3.1).

Tests

Solution A. Dissolve 2.5 g of substance under examination in 2 ml of a 50 per cent w/v solution of *sulphuric acid*. Heat on a water-bath, then cautiously over a naked flame, raising the temperature progressively, then incinerate in a muffle furnace at 600 ± 25°. Continue heating until all black particles have disappeared. Allow to cool, add a few drops of *dilute sulphuric acid*, heat and incinerate as above. Allow to cool, add a few drops of *ammonium carbonate solution*, evaporate to dryness and incinerate. Allow to cool and dissolve the residue in 50 ml of *water*.

Appearance of solution. Centrifuge the suspension obtained in identification test B at 2500 rpm for 10 minutes. The supernatant liquid is clear (2.4.1) and colourless (2.4.1).

pH (2.4.24). 5.5 to 7.5, determined in a 3.3 per cent w/v solution in *carbon-dioxide-free water*.

Heavy metals (2.3.13). To 4.0 g in a silica or platinum dish, add 2 ml of a 50 per cent w/v solution of *sulphuric acid;* heat in a water-bath and then cautiously over a flame at about 600°. Continue heating until all black particles have disappeared, allow to cool, add 0.1 ml of *1 M sulphuric acid*, heat to ignition once again and allow to cool. Add 0.1 ml of *2 M ammonium carbonate*, evaporate to dryness and cautiously ignite. To the residue, add 5 ml of *hydrochloric acid*, evaporate to dryness on a water-bath and dissolve the residue in 100 ml of *water*. 25 ml of a solution complies with the limit test for heavy metals, Method A (20 ppm).

Iron (2.3.14). 10 ml of solution A complies with the limit test for iron (20 ppm).

Sodium chloride. Not more than 7.0 per cent.

Weigh 0.5 g, suspend in a mixture of 100 ml of *water* add 1 ml of *nitric acid* and mix. Titrate with *0.1 M silver nitrate*, determining the end point potentiometrically (2.4.25), using a silver indicator electrode. Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.005844 g of NaCl.

Sodium glycolate.

Test solution. To 0.2 g add 5 ml of *glacial acetic acid*, mix well and add 5 ml of *water*; stir until dissolution is complete (about 10 minutes). Add 50 ml of *acetone* and 1 g of *sodium chloride*. Filter through a fast filter paper impregnated with *acetone*, and filter with *acetone*. Combine the filtrate and washing and dilute to 100.0 ml with *acetone*. Allow to stand for 24 hours without shaking. Use the clear supernatant.

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Reference solution. Dissolve 0.31 g of *glycolic acid* previously dried under vacuum over *phosphorus pentoxide* at room temperature overnight with *water* and dilute to 500 ml with *water*. Take 5.0 ml of the solution, add 5 ml of *acetic acid* and allow to stand for about 30 minutes. Add 50 ml of *acetic acid* and allow to stand for 30 minutes. Add 50 ml of *acetone* and 1 g of *sodium chloride*, filter through a fast filter paper impregnated with *acetone*. Combine the filtrate and washing and dilute to 100.0 ml with *acetone*. Allow to stand for 24 hours without shaking. Use the clear supernatant.

Transfer 2.0 ml of the test solution to an open flask, heat on a water-bath for 20 minutes, cool to room temperature, add 20.0 ml of *2,7-dihydroxynaphthalene solution*. Shake and heat on water-bath for 20 minutes. Cool under running water, transfer to a volumetric flask and diluted to 25.0 ml with *sulphuric acid*, maintaining the flask under running water. Within 10 minutes, measure the absorbance at 540 nm using water as blank. The absorbance of the solution prepared with the test solution is not more than the solution prepared at the same time and in the same manner using 2.0 ml of the reference solution (2.0 per cent).

Settling volume. To 70 ml of *water* in a100 ml graduated cylinder, add 1 g of the substance under examination (dried substance), stir with a glass rod to avoid lumps and dilute to 100.0 ml with *water* and stir againuntil the substance is homogeneously distributed. Allow to stand for 2 hours. The volume of the sediment is less than 45.0 ml.

Microbial contamination (2.2.9). 1.0 g is free from *Escherichia coli* and 10.0 g is free from *Salmonella*.

Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 1.0 g by drying in an oven at 130° for 1.5 hours.

Assay. Weigh 1.0 g, add 20 ml of *ethanol (80 per cent)*, shake for 10 minutes and filter. Repeat the extraction three times, or until a test for chloride ions is negative using *silver nitrate solution*. Dry the residue at 105° to constant weight. Weigh 0.7 g of the residue, add 80 ml of *anhydrous glacial acetic acid*, heat under a reflux condenser for 2 hours, cool. Titrate with *0.1 M perchloricacid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.0023 g of Na.

Storage. Store protected from light and moisture.

Sodium Starch Glycolate (Type B)

Sodium Starch Glycolate (Type B) is the sodium salt of a cross linked partly O-carboxymethylated starch.

Sodium Starch Glycolate (Type B) contains not less than 2.0 per cent and not more than 3.4 per cent of sodium, Na, calculated on the material washed with *ethanol (80 per cent)* and dried as described under Assay.

Category. Pharmaceutical aid (tablet disintegrant).

Description. A white or almost white, fine, free-flowing powder, very hygroscopic.

Identification

A. pH (see Test)

B. Shake 4.0 g of the substance under examination with 20 ml of *carbon-dioxide-free water*, without heating. This has the appearance of a gel. Add 100 ml of *carbon-dioxide-free water* and shake. A suspension forms that settles after standing.

C. To an acidified solution, add *iodinated potassium iodide solution*; a blue or violet colour produced.

D. Solution A (see Test) gives reaction (a) of sodium salts (2.3.1).

Tests

Solution A. Dissolve 2.5 g of substance under examination in 2 ml of a 50 per cent w/v solution of *sulphuric acid*. Heat on a water-bath, then cautiously over a naked flame, raising the temperature progressively, then incinerate in a muffle furnace at 600 ± 25°. Continue heating until all black particles have disappeared. Allow to cool, add a few drops of *dilute sulphuric acid*, heat and incinerate as above. Allow to cool, add a few drops of *ammonium carbonate solution*, evaporate to dryness and incinerate. Allow to cool and dissolve the residue in 50 ml of *water*.

Appearance of solution. Centrifuge the suspension obtained in identification test B at 2500 rpm for 10 minutes. The supernatant liquid is clear (2.4.1) and colourless (2.4.1).

pH (2.4.24). 3.0 to 5.0, determined in a 3.3 per cent w/v solution in *carbon-dioxide-free water*.

Heavy metals (2.3.13). To 4.0 g in a silica or platinum dish, add 2 ml of a 50 per cent w/v solution of *sulphuric acid*; heat in a water-bath and then cautiously over a flame at about 600°. Continue heating until all black particles have disappeared, allow to cool, add 0.1 ml of *1 M sulphuric acid*, heat to ignition once again and allow to cool. Add 0.1 ml of *2M ammonium carbonate*, evaporate to dryness and cautiously ignite. To the residue, add 5 ml of *hydrochloric acid*, evaporate to dryness on a water-bath and dissolve the residue in 100 ml of *water*. 25 ml of a solution complies with the limit test for heavy metals, Method A (20 ppm).

Iron (2.3.14). 10 ml of solution A, complies with the limit test for iron (20 ppm).

Sodium Chloride. Not more than 7.0 per cent.

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Weigh 0.5 g, suspend in a mixture of 100 ml of *water* add 1 ml of *nitric acid* and mix. Titrate with *0.1 M silver nitrate*, determining the end point potentiometrically (2.4.25), using a silver indicator electrode. Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.005844 g of NaCl.

Sodium glycolate.

Test solution. To 0.2 g add 5 ml of *glacial acetic acid*, mix well and add 5 ml of *water*, stirring until dissolution is complete (about 10 minutes). Add 50 ml of *acetone* and 1 g of *sodium chloride*. Filter through a fast filter paper impregnated with *acetone*, and filter with *acetone*. Combine the filtrate, washing and dilute to 100.0 ml with *acetone*. Allow to stand for 24 hours without shaking. Use the clear supernatant.

Reference solution. Dissolve 0.31 g of *glycolic acid* previously dried under vacuum over *phosphorus pentoxide* at room temperature overnight with *water* and dilute to 500 ml with *water*. Take 5.0 ml of the solution, add 5 ml of *acetic acid* and allow to stand for about 30 minutes. Add 50 ml of *acetone* and 1 g of *sodium chloride*, filter through a fast filter paper impregnated with *acetone*. Combine the filtrate and washing and dilute to 100.0 ml with *acetone*. Allow to stand for 24 hours without shaking. Use the clear supernatant.

Transfer 2.0 ml of the test solution to an open flask, heat on a water-bath for 20 minutes, cool to room temperature, add 20.0 ml of *2,7-dihydroxynaphthalene solution*. Shake and heat on water-bath for 20 minutes. Cool under running water, transfer to a volumetric flask and diluted to 25.0 ml with *sulphuric acid*, maintaining the flask under running water. Within 10 minutes, measure the absorbance at 540 nm using water as blank. The absorbance of the solution prepared with the test solution is not more than the solution prepared at the same time and in the same manner using 2.0 ml of the reference solution (2.0 per cent).

Settling volume. To 70 ml of *water* in a 100 ml graduated cylinder, add 1 g of the substance under examination (dried substance), stir with a glass rod to avoid lumps and dilute to 100.0 ml with *water* and stir again until the substance is homogeneously distributed. Allow to stand for 2 hours. The volume of the sediment is less than 45.0 ml.

Microbial contamination (2.2.9). 1.0 g is free from *Escherichia coli* and 10.0 g is free from *Salmonellae*.

Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 1.0 g by drying in an oven at 130° for 1.5 hours.

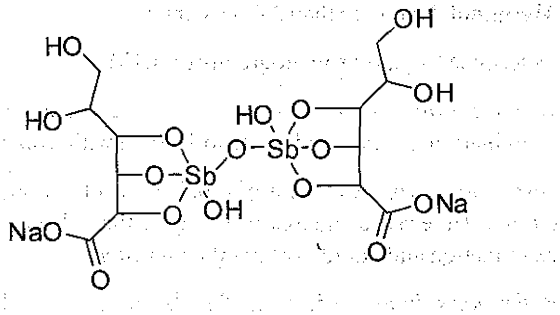
Assay. Weigh 1.0 g, add 20 ml of *ethanol (80 per cent)*, shake for 10 minutes and filter. Repeat the extraction three times, or until a test for chloride ions is negative using *silver nitrate solution*. Dry the residue at 105° to constant weight. Weigh 0.7 g of the residue, add 80 ml of *anhydrous glacial acetic acid*, heat under a reflux condenser for 2 hours, cool. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.0023 g of sodium.

Storage. Store protected from light and moisture.

Sodium Stibogluconate

Sodium Antimony Gluconate



Sodium Stibogluconate is mainly the disodium salt of μ-oxy-bis[gluconato(3)-O^2,O^3,O^4-hydroxoantimony].

The method of manufacture is such as to ensure consistently controlled reaction stoichiometry in order to yield sodium stibogluconate that is satisfactory with regard to intrinsic toxicity.

Sodium Stibogluconate contains not less than 30.0 per cent and not more than 34.0 per cent of pentavalent antimony, calculated on the dried and methanol-free basis.

Category. Antiprotozoal.

Description. A colourless, mostly amorphous powder.

Identification

A. An aqueous solution is *dextro-rotatory*.

B. Pass *hydrogen sulphide* into a 5 per cent w/v solution for several minutes; an orange precipitate is produced.

C. When heated, it chars without melting, emitting an odour of burnt sugar and leaving a residue which gives the reactions of antimony compounds and the reactions of sodium salts (2.3.1).

Tests

pH (2.4.24). 5.0 to 5.6, determined in the solution obtained in the test for Stability of solution.

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Stability of solution. Heat a solution containing 10.0 per cent w/v of pentavalent antimony in an autoclave at 115.5° and at a pressure of 70 kPa for 30 minutes. The resulting solution is colourless or almost colourless.

Trivalent antimony. Dissolve 2.0 g in 30 ml of *water*, add 15 ml of *hydrochloric acid* and titrate with *0.00833 M potassium bromate* using *methyl orange solution* as indicator. Not more than 1.3 ml of *0.00833 M potassium bromate* is required.

Chlorides. Dissolve 2.5 g in 50 ml of *water* and add 2 ml of *2 M nitric acid* and 75 ml of *acetate buffer pH 5.0*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (2.4.25). Not more than 3.0 ml of *0.1 M silver nitrate* is required.

Methanol. Not more than 2.0 per cent w/w.

Determine by gas chromatography (2.4.13).

Test solution (a). Dissolve 0.5 g of the substance under examination in *water* and dilute to 10.0 ml with *water*.

Test solution (b). Add 5 ml of a 0.2 per cent v/v solution of *ethanol* (internal standard) to 0.5 g of the substance under examination and dilute to 10.0 ml with *water*.

Reference solution. Add 1 ml of a 1.0 per cent v/v solution of *methanol* to 5 ml of a 0.2 per cent v/v solution of the internal standard and dilute to 10.0 ml with *water*.

Chromatographic system
- a glass column 1.5 m x 4 mm, packed with porous polymer beads (80 to 100 mesh) (Such as Porapak Q),
- temperature:
  column: 130°,
  inlet port 180° and detector at 180°,
- flame ionization detector,
- flow rate: 40 ml per minute using nitrogen as carrier gas,
- injection volume: 2 µl.

Inject the reference solution, test solution (a) and (b).

Calculate the content of methanol taking 0.792 g as its weight per ml (2.4.29) at 20°.

Undue toxicity. Dissolve a suitable quantity of the substance under examination in *water for injections* to give a solution containing 28 mg of pentavalent antimony per ml. Inject intravenously 0.3 ml of the solution into each of 10 mice that have been deprived of food for not less than 17 hours. After injections allow the mice access to food and water. None of the mice dies within 24 hours. If one of the mice dies within 24 hours, repeat the test. None of the second group of mice dies within 24 hours.

Loss on drying (2.4.19). Not more than 15.0 per cent, determined on 0.25 g by drying in an oven at 130° at a pressure not exceeding 0.7 kPa.

Assay. Dissolve 0.16 g in 30 ml of *hydrochloric acid*, add 70 ml of *phosphoric acid* and stir carefully until completely mixed. Titrate with *0.05 M ferrous ammonium sulphate* prepared using *sulphuric acid (1 per cent)*, determining the end-point potentiometrically (2.4.25), using a platinum electrode and a silver-silver chloride reference electrode.

1 ml of *0.05 M ferrous ammonium sulphate* is equivalent to 0.003044 g of pentavalent antimony.

Storage. Store protected from moisture.

Sodium Stibogluconate Injection

Sodium Antimony Gluconate Injection

Sodium Stibogluconate Injection is a sterile solution of Sodium Stibogluconate in Water for Injections. Injection in multiple dose containers must not contain phenol as preservative.

Sodium Stibogluconate Injection contains not less than 9.5 per cent w/v and not more than 10.5 per cent w/v of pentavalent antimony, Sb.

Usual strength. The equivalent of 100 mg of pentavalent antimony per ml.

Description. A clear, colourless or faintly straw-coloured solution.

Identification

A. It is *dextro*-rotatory.

B. Dilute 2 ml to 10 ml with *water* and pass hydrogen sulphide through the solution; no immediate precipitate is produced. Continue to pass hydrogen sulphide through the solution for several minutes; an orange precipitate is produced.

C. The residue obtained by evaporation to dryness, after incineration, gives the reactions of antimony compounds and the reactions of sodium salts (2.3.1).

Tests

pH (2.4.24). 5.0 to 5.6.

Undue toxicity. Dilute a suitable volume of the injection under examination with sufficient *water for injections* to give a solution containing the equivalent of 28 mg of pentavalent antimony per ml. Inject intravenously 0.3 ml of the solution into each of 10 mice that have been deprived of food for not less than 17 hours. After injections allow the mice access to food and water. None of the mice dies within 24 hours. If one of the mice dies within 24 hours, repeat the test. None of the second group of mice dies within 24 hours.

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Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. To 1.0 ml, measured, add 30 ml of *hydrochloric acid* and 70 ml of *phosphoric acid* and mix. Titrate with *0.05 M ferrous ammonium sulphate* prepared using *sulphuric acid (1 per cent)*, determining the end-point potentiometrically (2.4.25), using a platinum electrode and a silver-silver chloride reference electrode.

1 ml of *0.05 M ferrous ammonium sulphate* is equivalent to 0.003044 g of pentavalent antimony.

Labelling. The label states the strength in terms of the equivalent amount of pentavalent antimony per ml.

Sodium Thiosulphate

Sodium Hyposulphite

$Na_2S_2O_3,5H_2O$ Mol. Wt. 248.2

Sodium Thiosulphate contains not less than 99.0 per cent and not more than 101.0 per cent of $Na_2S_2O_3,5H_2O$.

Category. Antidote to cyanide poisoning.

Description. Colourless large crystals or a coarse, crystalline powder; deliquescent in moist air and effloresces in dry air at temperature above 33°. It dissolves in its water of crystallisation at about 49°.

Identification

A. To 0.5 ml of a 10.0 per cent w/v solution in *carbon dioxide-free water* (solution A) add 0.5 ml of *water* and 2 ml of *0.1 M silver nitrate*; a white precipitate is produced which quickly becomes yellowish and finally black.

B. To a portion of solution A add a few drops of *iodine solution*; the colour is discharged.

C. Dilute 2.5 ml of solution A to 5 ml with *water* and add 1 ml of *hydrochloric acid*; a gas is evolved which turns starch-iodate paper blue and a precipitate of sulphur is produced.

D. 1 ml of solution A gives reaction (A) of sodium salts (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

pH (2.4.24). 6.0 to 8.4, determined in solution A.

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). Dissolve 1.0 g in 10 ml of *water*. Slowly add 5 ml of *dilute hydrochloric acid* and evaporate the mixture to dryness on a water-bath. Gently boil the residue with 15 ml of *water* for 2 minutes and filter. Heat the filtrate to boiling, add sufficient *bromine solution* to the hot filtrate to produce a clear solution and add a slight excess of *bromine solution*. Boil the solution to expel the bromine completely, cool to room temperature and add a drop of *phenolphthalein solution* and *sodium hydroxide solution* until a slight pink colour is produced. Add 2 ml of *dilute acetic acid* and dilute with *water* to 25 ml. The solution complies with the limit test for heavy metals, Method A (20 ppm).

Chlorides (2.3.12). To 12.5 ml of solution A add 15 ml of *2 M nitric acid*, boil gently for 3 to 4 minutes, cool and filter. The filtrate complies with the limit test for chlorides (200 ppm).

Sulphides. To 10 ml of solution A add 0.05 ml of a freshly prepared 5 per cent w/v solution of *sodium nitroprusside*; the solution does not become violet.

Sulphates and sulphites (2.3.17). Dilute 2.5 ml of solution A to 10 ml with *distilled water*. To 3 ml of the solution add 2 ml of *iodine solution* and gradually add more *iodine solution* and dilute to 15 ml with *distilled water*. The resulting solution complies with the limit test for sulphates (0.2 per cent).

Assay. Dissolve 0.5 g in 20 ml of *water* and titrate with *0.05 M iodine* using *starch solution*, added towards the end of the titration, as indicator.

1 ml of *0.05 M iodine* is equivalent to 0.02482 g of $Na_2S_2O_3,5H_2O$.

Storage. Store protected from moisture.

Sodium Thiosulphate Injection

Sodium Hyposulphite Injection

Sodium Thiosulphate Injection is a sterile solution of Sodium Thiosulphate in Water for Injections.

Sodium Thiosulphate Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sodium thiosulphate, $Na_2S_2O_3,5H_2O$.

Usual strength. 100 mg per ml.

Description. A clear, colourless solution.

Identification

A. To 0.5 ml of a 10.0 per cent w/v solution in *carbon dioxide-free water* (solution A) add 0.5 ml of *water* and 2 ml of *0.1 M silver nitrate*; a white precipitate is produced which quickly becomes yellowish and finally black.

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B. To a portion of solution A add a few drops of *iodine solution*; the colour is discharged.

C. Dilute 2.5 ml of solution A to 5 ml with *water* and add 1 ml of *hydrochloric acid*; a gas is evolved which turns starch-iodate paper blue and a precipitate of sulphur is produced.

D. 1 ml of solution A gives reaction (A) of sodium salts (2.3.1).

Tests

pH (2.4.24): 7.0 to 9.0.

Bacterial endotoxins (2.2.3). Not more than 0.03 Endotoxin Unit per mg of sodium thiosulphate.

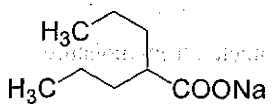
Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. To a measured volume containing about 0.5 g of Sodium Thiosulphate add about 20 ml of *water* and titrate with *0.05 M iodine*, using 3 ml of *starch solution*, added towards the end of the titration, as indicator.

1 ml of *0.05 M iodine* is equivalent to 0.02482 g of $Na_2S_2O_3,5H_2O$.

Storage. Store in single dose containers.

Sodium Valproate



$C_8H_{15}NaO_2$ Mol. Wt. 166.2

Sodium Valproate is sodium 2-propylpentanoate.

Sodium Valproate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_8H_{15}NaO_2$, calculated on the dried basis.

Category. Anticonvulsant.

Description. A white or almost white, crystalline powder; hygroscopic.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sodium valproate IPRS* or with the reference spectrum of sodium valproate.

B. Dissolve 1.25 g in 20 ml of *distilled water* in a separating funnel, add 5 ml of *2 M nitric acid*, shake and allow the mixture to stand for 12 hours; the lower layer (solution A) gives reactions of sodium salts (2.3.1).

Tests

Appearance of solution. A 20.0 per cent w/v solution is not more opalescent than opalescence standard OS2 (2.4.1), and is not more intensely coloured than reference solution YS6 (2.4.1).

Acidity or alkalinity. To 10 ml of a 10.0 per cent w/v solution in *carbon dioxide-free water* add 0.1 ml of *dilute phenolphthalein solution*; not more than 0.75 ml of either *0.1 M sodium hydroxide* or *0.1 M hydrochloric acid* is required to change the colour of the solution.

Related substances. Determine by gas chromatography (2.3.13).

Test solution. Dissolve 0.5 g of the substance under examination in 10 ml of *water*. Add 5 ml of *dilute sulphuric acid* and shake with 3 quantities, each of 20 ml, of *heptane*. Dilute the combined upper layers to 100.0 ml with *heptane*.

Reference solution (a). Dissolve 5 mg of *valproic acid for system suitability IPRS* (containing impurity K) in 1.0 ml of *heptane*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *heptane*.

Chromatographic system

- a capillary column 30 m x 0.53 mm, wide bore fused silica, coated with macrogol 20 000 and 2-nitroterephthalate (film thickness 0.5 µm),
- temperature:

column	time (in minutes)	temperature (°)
	0 - 5	80
	5 - 15	80 - 150
	15 – 28.3	150 – 190
	28.3 – 30	190

  inlet port and detector at 220°,
- flow rate: 8 ml per minute, using helium as the carrier gas.

The relative retention time with reference to valproic acid (retention time = about 17 min) for valproic acid impurity K is about 0.97.

Inject reference solution (a). The test is not valid unless the resolution between valproic acid and impurity K is not less than 2.0.

Inject 1 µl of reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of peak corresponding to valproic acid impurity K is not more than 0.15 times the area of the principal peak in the

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chromatogram obtained with reference solution (0.15 per cent), the area of any secondary peak is not more than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (0.05 per cent) and the sum of the areas of all the other secondary peaks is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (0.2 per cent). Ignore any peak with an area less than 0.03 times the area of the principal peak in the chromatogram obtained with reference solution (0.03 per cent).

Chlorides (2.3.12). Dissolve 2.5 g in 40 ml of *water* in a separating funnel, add 10 ml of *dilute nitric acid* and shake. Allow the mixture to stand for 12 hours. 25 ml of lower layer complies with the limit test for chlorides (200 ppm).

Sulphates (2.3.17). 15 ml of solution A complies with the limit test for sulphates (200 ppm).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B. Use 2 ml of *lead standard solution (10 ppm)* to prepare the standard (20 ppm).

Loss on drying (2.4.19). Not more than 2.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh 0.15 g and dissolve in 25 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01662 g of $C_8H_{15}NaO_2$.

Storage. Store protected from moisture.

Sodium Valproate Injection

Sodium Valproate Injection is a sterile aqueous solution of sodium valproate, formed from the interaction of valproic acid and sodium hydroxide, in Water for Injections, and one or more suitable buffering or sequestering agents.

Sodium Valproate Injection contains sodium valproate equivalent to not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of valproic acid, $C_8H_{16}O_2$. It contains no antimicrobial agents.

Usual strength. 20 mg per ml.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

B. Gives the reaction of sodium salts (2.3.1).

Tests

pH (2.4.24). 7.0 to 9.0.

Bacterial endotoxins (2.2.3). Not more than 23.0 Endotoxin Units per ml.

Sterility (2.2.11). Complies with the test for sterility.

Other tests. Complies with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 55 volumes of *water* and 45 volumes of *acetonitrile*.

Test solution. Dilute a volume of the injection containing 50 mg of valproic acid to 100.0 ml with *water*.

Reference solution (a). A 0.05 per cent w/v solution of *valproic acid IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.05 per cent w/v of *valproic acid IPRS* and 0.005 per cent w/v of *valproic acid related compound B* ((2*RS*)-2-(1-methylethyl) pentanoic acid) *IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5µm),
- mobile phase: a mixture of 55 volumes of a buffer solution, prepared by dissolving 3.5 gm of *sodium dihydrogenphosphate* in 1000 ml of *water*, adjusted to pH 3.5 with *dilute phosphoric acid* and 45 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Run the chromatogram at least 1.6 times the retention time of the valproic acid. The relative retention time with reference to valproic acid for valproic acid related compound B is about 0.9.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to valproic acid and valproic acid related compound B is not less than 2.0 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_8H_{16}O_2$ in the injection.

Storage. Store at a temperature not exceeding 30° and in single dose containers, preferably of Type 1 glass.

Labelling. The label states the amount of any buffering or sequestering agents used.

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Sodium Valproate Oral Solution

Sodium Valproate Elixir

Sodium Valproate Oral Solution is a solution of Sodium Valproate in a suitable flavoured vehicle.

Sodium Valproate Oral Solution contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sodium valproate, $C_8H_{15}NaO_2$.

Usual strength. 200 mg in 5 ml.

Identification

A. Shake a quantity containing about 0.25 g of Sodium Valproate with two quantities, each of 25 ml, of *chloroform* and discard the chloroform extracts. Add 10 ml of a saturated solution of *sodium chloride* and 10 ml of *2 M hydrochloric acid*, mix and shake with 25 ml of *chloroform*. Wash the chloroform layer with 5 ml of *water*, shake with *anhydrous sodium sulphate*, filter and evaporate to dryness.

On the residue determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *valproic acid IPRS* or with the reference spectrum of valproic acid.

B. Shake a quantity containing about 0.25 g of Sodium Valproate with a mixture of 10 ml of a saturated solution of *sodium chloride*, 10 ml of *2 M hydrochloric acid* and 25 ml of *chloroform*. Evaporate the chloroform layer to dryness, dissolve the residue in 2 ml of *water*, make just alkaline with *2 M sodium hydroxide* and add 0.5 ml of a 10 per cent w/v solution of *cobalt nitrate*; a purple precipitate is produced which is soluble in *dichloromethane*.

Tests

Related substances. Determine by gas chromatography (2.4.13).

Test solution (a). Mix a quantity of the oral solution containing 0.50 g of Sodium Valproate with 10 ml of *water*, acidify with *2 M sulphuric acid* and shake with three quantities, each of 20 ml, of *dichloromethane*. Wash the combined dichloromethane extracts with 10 ml of *water*, shake with *anhydrous sodium sulphate*, filter and evaporate the filtrate to a volume of about 10 ml at a temperature not exceeding 30° using a rotary evaporator.

Test solution (b). Mix a quantity of the oral solution containing 0.5 g of Sodium Valproate with 10 ml of a 0.02 per cent w/v solution of *octanoic acid* (internal standard) in *0.1 M sodium hydroxide* and continue as described for test solution (a) beginning at the words "acidify with *2 M sulphuric acid*...".

Reference solution. A 0.02 per cent w/v solution of the internal standard in *dichloromethane*.

Chromatographic system

- a glass column 1.5 m x 4 mm, packed with *acid-washed, silanised diatomaceous support* (80 to 180 mesh) coated with 15 per cent w/w of free fatty acid phase (Such as Supelco FFAP 2-1063) and *1 per cent w/w of phosphoric acid*,
- temperature: column: 170°, inlet port at 190° and detector at 250°.

Inject 1 µl of the reference solution, test solution (a) and (b). In the chromatogram obtained with test solution (b), the sum of the areas of all the secondary peaks is not more than the area of peak in the chromatogram obtained with the reference solution (0.4 per cent).

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Weigh a quantity containing about 0.15 g of Sodium Valproate, add 50 ml of *water*, mix thoroughly and add 10 ml of saturated solution of *sodium chloride*, 10 ml of *2 M hydrochloric acid* and 40 ml of a mixture of 2 volumes of *ether* and 1 volume of *light petroleum (40° to 60°)*, shake, allow to separate and reserve the ether layer. Shake the aqueous layer with a further 40 ml of the *ether-light petroleum mixture* and discard the aqueous layer. Shake each of the ether extracts with the same three quantities, each of 10 ml, of a saturated solution of *sodium chloride*, combine the extracts and evaporate to a volume of about 1 ml at a temperature not exceeding 30°. Add 50 ml of *ethanol (95 per cent)*, previously neutralised with *0.01 M sodium hydroxide* using *dilute phenolphthalein solution* as indicator, and titrate with *0.1 M sodium hydroxide*.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01662 g of $C_8H_{15}NaO_2$.

Determine the weight per ml of the preparation (2.4.29) and calculate the content of $C_8H_{15}NaO_2$ weight in volume.

Sodium Valproate Tablets

Sodium Valproate Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sodium valproate, $C_8H_{15}NaO_2$.

Usual strengths. 100 mg; 200 mg.

Identification

A. Shake a quantity of the powdered tablets containing about 0.5 g of Sodium Valproate with 10 ml of *water* and centrifuge. Acidify 5 ml of the supernatant liquid with *2 M sulphuric acid*, shake with 25 ml of *chloroform* and wash the chloroform layer with 5 ml of *water*. Dry by shaking with *anhydrous sodium sulphate*, filter and evaporate the chloroform.

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On the residue determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *valproic acid IPRS* or with the reference spectrum of valproic acid.

B. Shake a quantity of the powdered tablets containing about 0.25 g of Sodium Valproate with 3 ml of *water* and centrifuge. To 2 ml of the supernatant liquid add 0.5 ml of a 10 per cent w/v solution of *cobalt nitrate*; a purple precipitate is produced which is soluble in *dichloromethane*.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium: 900 ml of *phosphate buffer* pH 6.8,

Speed and time: 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *sodium valproate IPRS* with the dissolution medium to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Chromatographic system

- a stainless steel column 30 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as µ bondapak C18),
- mobile phase: a mixture of 45 volumes of a buffer solution prepared by dissolving 0.32 g *potassium dihydrogen orthophosphate* in 100 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 55 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 50 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{15}NaO_2$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_8H_{15}NaO_2$.

Related substances. Determine by gas chromatography (2.4.13).

Test solution. Shake a quantity of the powdered tablets containing 0.5 g of Sodium Valproate with 10 ml of *water*, acidify with *2M sulphuric acid* and shake with three quantities, each of 20 ml of *heptane*. Wash the combined *heptane* extracts with 10 ml of *water*, shake with *anhydrous sodium sulphate*, filter and dilute to 100.0 ml with *heptane*.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *heptane*. Dilute 1.0 ml of the solution to 10.0 ml with *heptane*.

Reference solution (b). A 0.5 per cent w/v solution of *valproic acid for system suitability IPRS* in *heptane*.

Chromatographic system

- a capillary column 30 m x 0.53 mm, bonded with a 0.5 µm layer of macrogol 20,000 2-nitroterephthalate (Such as DB-FFAP),
- temperature:

column	time (in min)	temperature (°)
	0 - 5	80
	5 - 15	80 - 150
	15-28.3	150 - 190
	28.3 - 30	190
	30 - 40	190 - 80

- inlet port at 200° and detector at 220°,
- flame ionization detector,
- flow rate: 8 ml per minute using nitrogen as carrier gas,
- injection volume: 1 µl.

Inject reference solutions (b). The test is not valid unless the resolution between the peaks due to valproic acid impurity K and valproic acid is not less than 2.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by gas chromatography (2.4.13).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.5 g of Sodium Valproate with 10 ml of *water*, acidify with *2M sulphuric acid* and shake with three quantities, each of 20 ml of *heptane*. Wash the combined *heptane* extracts with 10 ml of *water*, shake with *anhydrous sodium sulphate*, filter and dilute to 100.0 ml with *heptane*. Dilute 1.0 ml of the solution to 10.0 ml with *heptane*.

Reference solution (a). Weigh a quantity of 0.5 g of *sodium valproate IPRS*, disperse in 10 ml of *water*, acidify with *2 M sulphuric acid* and shake with three quantities, each of 20 ml of *heptane*. Wash the combined *heptane* extracts with 10 ml of *water*, shake with *anhydrous sodium sulphate*, filter and dilute to 100.0 ml with *heptane*. Dilute 1.0 ml of the solution to 10.0 ml with *heptane*.

Reference solution (b). A 0.5 per cent w/v solution of *valproic acid for system suitability IPRS* in *heptane*.

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Use chromatographic system as described under Related substances.

Inject reference solution (b). The test is not valid unless the resolution between the peak due to valproic acid impurity K and valproic acid is not less than 2.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_8H_{15}NaO_2$ in the tablets.

Sodium Valproate Gastro-resistant Tablets

Sodium Valproate Gastro-resistant Tablets contain Sodium Valproate. They are made gastro-resistant by enteric-coating or by other means.

Sodium Valproate Gastro-resistant Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sodium valproate, $C_8H_{15}NaO_2$.

Identification

Shake a quantity of the powdered tablets containing 0.5 g of Sodium Valproate with 10 ml of *water* and centrifuge. Acidify 5 ml of the supernatant liquid with *2 M sulphuric acid*, shake with 25 ml of *dichloromethane* and wash the *dichloromethane* layer with 5 ml of *water*. Dry by shaking with *anhydrous sodium sulphate*, filter and evaporate to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sodium valporate IPRS* treated in the same manner or with the reference spectrum of valproic acid.

Tests

Dissolution (2.5.2).

A. Apparatus No. 1 (Basket),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 100 rpm and 120 minutes.
Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium to obtain a solution containing 0.001 per cent w/v of Sodium Valproate.

Reference solution. Dissolve a quantity of *sodium valproate IPRS* in the dissolution medium and dilute to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 30 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 45 volumes of a buffer solution prepared by dissolving 0.32 g of *potassium dihydrogen orthophosphate* in 100 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 55 volumes of *acetronitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 50 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{15}NaO_2$ in the medium.

Complies with the acceptance criteria given in Acid stage.

B. Apparatus No. 1 (Basket),
Medium. Replace *0.1 M hydrochloric acid* with 900 ml of *phosphate buffer pH 6.8*,
Speed and time. 100 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14), using the chromatographic system as described in Method A.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium to obtain a solution containing 0.002 per cent w/v of Sodium Valproate.

Reference solution. Dissolve a quantity of *sodium valproate IPRS* in the dissolution medium and dilute to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{15}NaO_2$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_8H_{15}NaO_2$.

Related substances. Determine by gas chromatography (2.4.13).

Test solution. Shake a quantity of the powdered tablets containing 0.5 g of Sodium Valproate with 10 ml of *water*, acidify with *2 M sulphuric acid* and shake with three quantities, each of 20 ml of *heptane*. Wash the combined *heptane* extracts with 10 ml of *water*, shake with *anhydrous sodium sulphate*, filter and dilute to 100.0 ml with *heptane*.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *heptane*. Dilute 1.0 ml of the solution to 10.0 ml with *heptane*.

Reference solution (b). A 0.5 per cent w/v solution of *valproic acid for system suitability IPRS* in *heptane*.

Chromatographic system
- a capillary column 30 m x 0.53 mm, bonded with a 0.5 µm layer of macrogol 20,000 2-nitroterephthalate (Such as DB-FFAP),

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– temperature:

column	time (in min)	temperature (°)
	0 - 5	80
	5 - 15	80 - 150
	15-28.3	150 - 190
	28.3 - 30	190
	30 - 40	190 - 80

– inlet port at 200° and detector at 220°,
– flame ionization detector,
– flow rate: 8 ml per minute using nitrogen as carrier gas,
– injection volume: 1 µl.

Inject reference solutions (b). The test is not valid unless the resolution between the peaks due to valproic acid impurity K and valproic acid is not less than 2.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to valproic acid impurity K is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of areas of all peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by gas chromatography (2.4.13).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.5 g of Sodium Valproate with 10 ml of *water,* acidify with *2 M sulphuric acid* and shake with three quantities, each of 20 ml of *heptane.* Wash the combined *heptane* extracts with 10 ml of *water,* shake with *anhydrous sodium sulphate,* filter and dilute to 100.0 ml with *heptane.* Dilute 1.0 ml of the solution to 10.0 ml with *heptane.*

Reference solution (a). Weigh a quantity of 0.5 g of *sodium valproate IPRS,* disperse in 10 ml of *water,* acidify with *2 M sulphuric acid* and shake with three quantities, each of 20 ml of *heptane.* Wash the combined *heptane* extracts with 10 ml of *water,* shake with *anhydrous sodium sulphate,* filter and dilute to 100.0 ml with *heptane.* Dilute 1.0 ml of the solution to 10.0 ml with *heptane.*

Reference solution (b). A 0.5 per cent w/v solution of *valproic acid for system suitability IPRS* in *heptane.*

Use chromatographic system as described under Related substances.

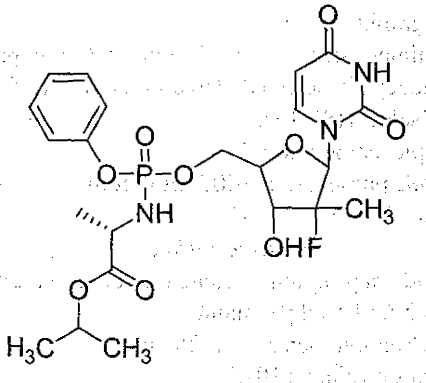
Inject reference solution (b). The test is not valid unless the resolution between the peak due to valproic acid impurity K and valproic acid is not less than 2.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_8H_{15}NaO_2$ in the tablets.

Labelling. The label states that the tablets should be swallowed whole and not chewed.

Sofosbuvir



$C_{22}H_{29}FN_3O_9P$ Mol. Wt. 529.5

Sofosbuvir is propan-2-yl (2S)-2-[[[(2R,3R,4R,5R)-5-(2,4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyloxolan-2-yl]methoxy-phenoxyphosphoryl]amino]propanoate.

Sofosbuvir contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{22}H_{29}FN_3O_9P$, calculated on the anhydrous basis.

Category. Antiretroviral.

Description. A white to off-white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sofosbuvir IPRS* or with the reference spectrum of sofosbuvir.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Buffer solution. A 0.01M ammonium acetate buffer prepared by dissolving 0.77 g of *ammonium acetate* in 1000 ml of *water,* adjusted to pH 4.0 with *dilute acetic acid.*

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Solvent mixture. 60 volumes of the buffer solution and 40 volumes of *methanol.*

Test solution. Dissolve 48 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution (a). A 0.048 per cent w/v solution of *sofosbuvir IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Reference solution (c). A solution containing 0.048 per cent w/v of *sofosbuvir IPRS* and 0.000048 per cent w/v of *sofosbuvir Rp isomer IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm) (Such as Xselect HSS T3),
- sample temperature: 5°,
- mobile phase: A. a 0.05 per cent v/v solution of *orthophosphoric acid* in *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	98	2
27.4	21	79
28	98	2
34	98	2

Name	Relative retention time	Correction factor
Fluoro uridine impurity[1]	0.38	0.5
Ethyl analog[2]	0.93	—
Sofosbuvir Rp isomer[3]	0.98	—
Sofosbuvir	1.00	—
Chloro analog[4]	1.05	—
Pentafluoro phenol[5*]	1.13	—
Phosphoramidate sofosbuvir[6]	1.41	1.5
Phosphoramidate intermediate[7]	1.57	—

*This impurity is to be disregarded as controlled under Content of Pentafluoro phenol and Phosphoramidate intermediate test.

[1]2'-deoxy-2'-fluoro-2'-methyluridine,

[2](S)-2[(S)-[[(2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihyd-2*H*-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyltetrahydro-2-furanyl]methoxy](phenoxy) phosphorylamino]propanoic acid-1-ethyl ester,

[3]propan-2-yl(2*S*)-2-{[(*R*)-{[(2*R*,3*R*,4*R*,5*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl]methoxy}(phenoxy)phosphoryl]amino}propanoate,

[4]propan-2-yl(2*S*)-2-{[(*S*)-{[(2*R*,3*R*,4*R*,5*R*)-4-chloro-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-3-hydroxy-4-methyltetrahydrofuran-2-yl]methoxy}(phenoxy)phosphoryl]amino}propanoate,

[5]2,3,4,5,6-pentofluorophenol,

[6]propan-2-yl(2*S*)-2-{[{[(2*R*,3*R*,4*R*,5*R*)-2-[3*S*,5*S*)-5,8-dimethyl-3,6-oxido-6-oxo-3-phenoxy-2,7-dioxa-4-aza-3λ^5-phosphanon-1-yl]-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-4-fluoro-4-methyltetrahydrofuran-3-yl]oxy}(phenoxy)phosphoryl]amino}propanoate,

[7]*N*-[(*S*)-(2,3,4,5,6-pentafluorophenoxy)phenoxyphosphinyl]-*L*-Alanine-1-methylethyl ester.

Inject reference solution (b) and (c). The test is not valid unless the resolution between the peaks due to sofosbuvir Rp isomer and sofosbuvir is not less than 1.5 in the chromatogram obtained with reference solution (c), the column efficiency is not less than 100000 theoretical plates, the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution the area of any peak corresponding to fluoro uridine impurity, Rp isomer, chloro analog and phosphoramidate sofosbuvir, each of, is not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent), the area of any peak corresponding to ethyl analog is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent) and the sum of areas of all peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with the reference solution (b) (1.0 per cent). Ignore the peaks due to pentafluoro phenol and phosphoramidate intermediate and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Content of Pentafluoro phenol and Phosphoramidate intermediate. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *acetonitrile* and *water.*

Test solution. Dissolve 20 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution. A solution containing 0.0003 per cent w/v, each of, *pentafluoro phenol IPRS* and *phosphoramidate intermediate IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 10 cm x 2.1 mm, packed with octadecylsilane bonded to porous silica (1.7 µm) (Such as Acquity UPLC BEH C18),

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- column temperature: 45°,
- sample temperature: 5°,
- mobile phase: A. a 0.05 per cent v/v solution of *orthophosphoric acid* in *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 0.4 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 2 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	65	35
8	30	70
10	30	70
10.1	65	35
12	65	35

The relative retention time with reference to phosphoramidate intermediate for pentafluoro phenol is about 0.38.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 1.8 for pentafluoro phenol and the relative standard deviation for replicate injections is not more than 5.0 per cent for both the peaks.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to pentafluoro phenol and phosphoramidate intermediate, each of, is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.15 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 100000 theoretical plates, the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{22}H_{29}FN_3O_9P$.

Storage. Store protected from moisture, at a temperature not exceeding 30°

Sofosbuvir Tablets

Sofosbuvir Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sofosbuvir, $C_{22}H_{29}FN_3O_9P$.

Usual strength. 400 mg.

Identification

In the Assay, the principal peak in chromatogram obtained with the test solution corresponds to the peak in chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* and 0.9 g of *sodium hydroxide* in 950 ml of *water,* adjusted to pH 6.8 and dilute to 1000 ml with *water*,
Speed and time. 75 rpm and 15 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted, if necessary with the dissolution medium at about 262 nm (2.4.7). Calculate the content of sofosbuvir, $C_{22}H_{29}FN_3O_9P$ in the medium from the absorbance obtained from a solution of known concentration of *sofosbuvir IPRS* in the dissolution medium.

Q. Not less than 80 per cent of the stated amount of $C_{22}H_{29}FN_3O_9P$.

Related substances. Determine by liquid chromatography (2.4.14).

Buffer solution. Dissolve 0.77 g of *ammonium acetate* in 1000 ml of *water,* adjusted to pH 4.0 with *dilute acetic acid.*

Solvent mixture. 60 volumes of the buffer solution and 40 volumes of *methanol.*

Test solution. Transfer 10 intact tablets to a 1000-ml volumetric flask, add about 600 ml of the buffer solution continuously stir flask vigorously using magnetic stirrer, till tablets are completely disintegrated, add 300 ml of *methanol* and stir vigorously for 60 minutes, dilute to volume with *methanol,* mix and filter. Centrifuge at 5000 rpm for 10 minutes. Dilute 3.0 ml of the clear supernatant to 25.0 ml with the solvent mixture.

Reference solution (a). A 0.00024 per cent w/v solution of *sofosbuvir IPRS* in the solvent mixture.

Reference solution (b). Dissolve 0.5 mg of *sofosbuvir Rp isomer IPRS* in 3.0 ml of *methanol* and dilute to 10.0 ml with the solvent mixture.

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Reference solution (c). Dissolve 48 mg of *sofosbuvir IPRS* in 70 ml of the solvent mixture, add 1.0 ml of reference solution (b) and dilute to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (3.5 µm) (Such as X Select HSS T3),
- sample temperature: 5°,
- mobile phase: A. a 0.05 per cent v/v solution of *orthophosphoric acid* in *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	98	2
2.5	98	2
27.4	21	79
28	98	2
35	98	2

Name	Relative retention time	Correction factor
Fluoro uridine phosphate impurity[3]	0.21	0.93
Fluoro uridine impurity[1]	0.39	0.5
Uridine alanine phosphate impurity[2]	0.45	1.61
Uridine phenyl phosphate impurity[4]	0.61	1.11
Uridine isopropyl alanine phosphate impurity[5]	0.65	0.9
Phenol impurity	0.72	2.17
Ethyl analog*	0.93	—
Sofosbuvir Rp isomer*	0.98	—
Sofosbuvir (Retention time is about 16.5 minutes)	1.0	
Chloro analog impurity*	1.05	—
Penta fluoro phenyl impurity*	1.13	—
Phosphoramidate sofosbuvir impurity*	1.41	—
Phosphoramidate intermediate impurity*	1.57	—

*Process impurity include for identification only and not included in the calculation of total degradation products.

[1]2′-deoxy-2′-fluoro-2′-methyluridine,

[2](2*S*)-2{[{[(2*R*,3*R*,4*R*,5*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl]methoxy}(hydroxyl)phosphoryl]amino}propanoic acid,

[3]2′-deoxy-2′-fluoro-2′-methyluridine 5′-(dihydrogen phosphate),

[4]2′-deoxy-2-fluoro-5-O-[hydroxyl(phenoxy)phosphoryl]-2′-methyluridine,

[5]propan-2-yl(2*S*)-2-{[{[(2*R*,3*R*,4*R*,5*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-4-fluoro-3hydroxy-4-methyltetrahydrofuran-2-yl]methoxy}(hydroxy)phosphoryl]amino}propanoate.

Inject reference solution (a) and (c). The test is not valid unless the resolution between the peaks due to sofosbuvir Rp isomer and sofosbuvir is not less than 1.5 in the chromatogram obtained with reference solution (c), the column efficiency is not less than 100000 theoretical plates, the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 10.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to fluoro uridine impurity, uridine alanine phosphate impurity, phenol impurity, fluoro uridine phosphate impurity, uridine phenyl phosphate impurity and uridine isopropyl alanine phosphate impurity, each of, is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of areas of all peak is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modifications.

Reference solution. A 0.0048 per cent w/v solution of *sofosbuvir IPRS* in the solvent mixture.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 100000 theoretical plates, the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{29}FN_3O_9P$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

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Sofosbuvir and Daclatasvir Tablets

Sofosbuvir and Daclatasvir Dihydrochloride Tablets

Sofosbuvir and Daclatasvir Tablets contain daclatasvir dihydrochloride equivalent to not less than 95.0 per cent and not more than 105.0 per cent of the stated amounts of daclatasvir, $C_{40}H_{50}N_8O_6$ and sofosbuvir, $C_{22}H_{29}FN_3O_9P$.

Usual strength. Sofosbuvir, 400 mg and Daclatasvir, 60 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with test solution (a) and test solution (b) correspond to the principal peaks in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 1000 ml of *phosphate buffer pH 6.8* with 0.75 per cent w/v *polyoxyethylene glycol dodecyl ether (Brij 35)* solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* and 0.9 g *sodium hydroxide* in 1000 ml of *water*, adjusted to pH 6.8 with *0.2 M sodium hydroxide*. Add 7.5 g of *polyoxyethylene glycol dodecyl ether* and sonicate to dissolve and mix,

Speed and time. 75 rpm and 15 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Solvent mixture. 70 volumes of *water* and 30 volumes of *methanol*.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution (a). A 0.132 per cent w/v solution of *daclatasvir dihydrochloride IPRS* in *methanol*.

Reference solution (b). Dissolve 40 mg of *sofosbuvir IPRS* in 70 ml of the solvent mixture, add 5.0 ml of reference solution (a) and dilute to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm) (Such as Inertsil ODS 3V),
- sample temperature: 10°,
- mobile phase: A. a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in 1000 ml of *water*. Add 2.0 ml of *triethylamine* and mix, adjusted to pH 2.5 with *dilute orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 262 nm for sofosbuvir and 318 nm for daclatasvir,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	75	25
10	45	55
15	20	80
17	75	25
22	75	25

The elution order is the daclatasvir followed by sofosbuvir peak.

Inject reference solution (b) at 318 nm and 262 nm. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injection is not more than 2.0 per cent for both the peaks.

Inject reference solution (b) and the test solution at 318 nm and at 262 nm.

Calculate content of $C_{40}H_{50}N_8O_6$ and $C_{22}H_{29}FN_3O_9P$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{40}H_{50}N_8O_6$ and $C_{22}H_{29}FN_3O_9P$.

Related substances.

For Daclatasvir —

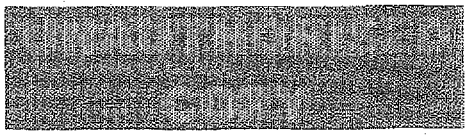
Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing 60 mg of Daclatasvir in 70 ml of *methanol*, with the aid of ultrasound for 30 minutes with intermittent shaking and dilute to 100.0 ml with *methanol*. Centrifuge the solution at 5000 rpm for 10 minutes. Use the clear supernatant.

Reference solution. A 0.033 per cent w/v solution of *daclatasvir dihydrochloride IPRS* in *methanol*. Dilute 1.0 ml of the solution to 100.0 ml with *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (Such as ACE 5 C18-HL),
- sample temperature: 10°,
- mobile phase: A. a 0.136 per cent w/v of *potassium dihydrogen phosphate* in *water*, add 2.0 ml of *triethylamine* and mix, adjusted to pH 2.5 with *orthophosphoric acid*,
  B. a mixture of 10 volumes of *water* and 90 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.2 ml per minute,



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– spectrophotometer set at 300 nm,
– injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
8	95	5
15	85	15
25	85	15
35	75	25
45	70	30
55	60	40
65	50	50
85	45	55
90	48	52
95	20	80
105	20	80
110	95	5
120	95	5

Name	Relative retention time
Coupled amine hydrochloride impurity*	0.49
Mono impurity*	0.85
Acetyl impurity*	0.87
Daclatasvir (Retention time: about 41minutes)	1.0
SSSR-Diastermer*	1.08
RSSR-Diasteromer*	1.14
Oxazolidine impurity*	1.48

*Process impurity include for identification only and not included in the calculation of total degradation products.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.4 times the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent). Ignore the peak due to sofosbuvir and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

For Sofosbuvir —

Determine by liquid chromatography (2.4.14), as described under Related substances of Daclatasvir with the following modifications.

Solvent mixture. 30 volumes of *water* and 70 volumes of *methanol.*

Test solution. Disperse a quantity of powdered tablets containing 0.4 g of Sofosbuvir in the 75 ml of the solvent mixture, with the aid of ultrasound for 30 minutes with intermittent shaking and dilute to 100.0 ml with the solvent mixture. Centrifuge the solution at 5000 rpm for about 10 minutes. Use the clear supernatant.

Reference solution. A 0.002 per cent w/v solution of *sofosbuvir IPRS* in the solvent mixture.

– sample temperature: 25°,
– spectrophotometer set at 262 nm.

Name	Relative retention time	Correction factor
Fluoro uridine impurity	0.14	0.49
Ethyl analog impurity*	0.91	—
Sofosbuvir Rp isomer*	0.97	—
Sofosbuvir	1.00	—
Chloro analog impurity*	1.06	—
Penta fluoro phenyl impurity*	1.09	—
Disubstitued impurity*	1.53	—
Phosphoramidate intermediate impurity*	1.73	—

*Process impurity include for identification only and not included in the calculation of total degradation products.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to fluoro uridine impurity is not more than 0.4 times the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent) and the sum of areas of all peak is not more than 0.4 times the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent). Ignore any peak due to daclatasvir and with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

The sum of all the impurities (daclatasvir and sofosvuvir impurities) is not more than 1.0 per cent.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 70 volumes of *water* and 30 volumes of *methanol.*

Test solution (a). Weigh and powder 20 tablets. Disperse a quantity of powder containing 0.3 g of Daclatasvir in 20 ml *water,* with the aid of ultrasound for 10 minutes. Add 180 ml of *methanol,* sonicate for 60 minutes with intermittent shaking

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and dilute to 250.0 ml with *methanol*. Centrifuge at 5000 rpm for 10 minutes. Dilute 5.0 ml of clear supernatant to 100.0 ml with the solvent mixture.

Test solution (b). Dilute 10.0 ml of test solution (a) to 25.0 ml with the solvent mixture.

Reference solution. A solution containing 0.066 per cent w/v of *daclatasvir dihydrochloride IPRS* and 0.16 per cent w/v of *sofosbuvir IPRS* in *methanol*. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Use chromatographic system as described under Dissolution.

Inject the reference solution at 318 nm and 262 nm. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injection is not more than 2.0 per cent for both the peaks.

Inject the reference solution and test solution (a) at 318 nm (for daclatasvir).

Inject the reference solution and test solution (b) at 262 nm (for sofosbuvir).

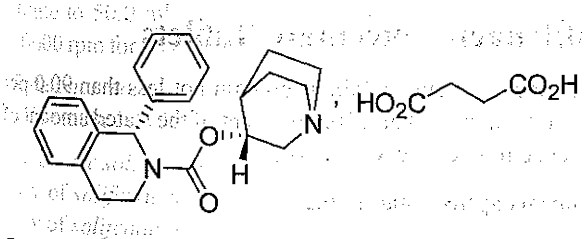
Calculate the content of $C_{40}H_{50}N_8O_6$ and $C_{22}H_{29}FN_3O_9P$ in the tablets.

1 mg of daclatasvir hydrochloride, $C_{40}H_{52}Cl_2N_8O_6$ is equivalent to 0.91 mg of daclatasvir, $C_{40}H_{50}N_8O_6$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labeling. The label states the strength in terms of the equivalent amount of daclatasvir and sofosbuvir.

Solifenacin Succinate



$C_{27}H_{32}N_2O_6$ Mol. Wt. 480.6

Solifenacin Succinate is (3R)-1-Azabicyclo[2.2.2] octan-3-yl (1S)-1-phenyl-3,4-dihydroisoquinoline-2(1*H*)-carboxylate hydrogen butanedioate.

Solifenacin Succinate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{27}H_{32}N_2O_6$, calculated on the dried basis.

Category. Muscarinic M_3 receptor antagonist; anticholinergic.

Description. A white or light yellow powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained from *solifenacin succinate IPRS* or with the reference spectrum of solifenacin succinate.

B. Isomeric purity (see Tests).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in 100.0 ml of the mobile phase A.

Reference solution (a). Dissolve 5 mg of impurity A (*1-phenyl-1;2,3,4-tetrahydroisoquinoline*) in the test solution and dilute to 25.0 ml with the test solution.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A. Dilute 1.0 ml of the solution to 10.0 ml with mobile phase A.

Chromatographic system

- a stainless steel column 10 cm × 2.1 mm, packed with end-capped polar embedded octadecylsilane bonded to amorphous organosilica (1.7 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 55 volumes of a buffer solution prepared by dissolving 0.6 g of *ammonium phosphate* in 1000 ml of *water*, add 1.0 ml of *diethylamine* adjusted to pH 11 with *concentrated ammonia* and 45 volumes of *acetonitrile*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 0.4 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 2.5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
0.5	90	10
10.5	68	32
12.5	68	32
13.0	90	10
15.0	90	10

Name	Relative retention time
Solifenacin Succinate	0.2
Solifenacin impurity A	0.7
Solifenacin (Retention time is about 2 minutes)	1.0

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Inject reference solution (a). The test is not valid unless the resolution between the peak due to impurity A and solifenacin is not less than 4.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of individual impurity is not more than the area of peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 3 times the area of the peak in the chromatogram obtained with reference solution (b) (0.3 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Isomeric purity. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 20.0 ml of the mobile phase.

Reference solution(a). Dissolve the contents of a vial of *solifenacin for system suitability IPRS* (containing impurities F, G and H) in 1.0 ml of the mobile phase.

Reference solution(b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with amylose derivative of silica (5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 0.1 volume of *diethylamine*, 200 volumes of *anhydrous ethanol* and 800 volumes of *heptane*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

Name	Relative retention time
Solifenacin impurity F[1]	0.7
Solifenacin impurity H[2]	0.76
Solifenacin impurity G[3]	0.84
Solifenacin (Retention time is about 17 minutes)	1.0

[1](*3S*)-1-azabicyclo[2.2.2] octan-3-yl(*1R*)-1-phenyl-3,4-dihydroisoquinoline-2(*1H*)-carboxylate,

[2](*3S*)-1-azabicyclo[2.2.2]octan-3yl (*1S*)-1-phenyl-3,4-dihydroisoquinoline-2(*1H*)-carboxylate,

[3](*3R*)-1-azabicyclo[2.2.2]octan-3yl (*1R*)-1-phenyl-3,4-dihydroisoquinoline-2 (*1H*)-carboxylate.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to impurity F and H is not less than 1.5.

Inject reference solution (b) and the test solution. In the chromatogram obtained with test solution, the area of each peak corresponding to impurity F, G and H is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent).

Impurity E. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase: A mixture of 6 volumes of *concentrated ammonia*, 31 volumes of *anhydrous ethanol* and 63 volumes of *toluene*.

Test solution. Dissolve 0.5 g of the substance under examination in 5.0 ml of *methanol*.

Reference solution. Dissolve 5 mg of impurity E[(*3R*)-1-azabicyclo[2.2.2]octan-3-ol (3- quinuclidinol)] in 50.0 ml of *methanol*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise two third of the plate. Dry the plate at 100° to 105° for 1 hour and expose to iodine vapour for 2 hours. Relative retention time with reference to solifenacin (Rf about 0.6) and impurity E is 0.2. Any spot due to impurity E is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.1 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Weigh accurately about 0.4 g and dissolve in 50 ml of *anhydrous acetic acid*. Titrate with *0.1 M perchloric acid*. Determine the end point potentiometrically (2.4.25). Carry out the blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.04806 g of $C_{27}H_{32}N_2O_6$.

Solifenacin Succinate Tablets

Solifenacin Succinate Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of solifenacin succinate, $C_{27}H_{32}N_2O_6$.

Usual strengths. 5 mg ;10 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *water*,

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Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *solifenacin succinate IPRS* in the dissolution medium to obtain a solution of known concentration similar to the expected concentration of the test solution.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as inertsil),
- column temperature: 40°,
- mobile phase: a mixture of 68 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water,* add 1 ml of *triethylamine*, adjusted to pH 3.6 with *orthophosphoric acid* and 32 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{32}N_2O_6$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{27}H_{32}N_2O_6$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 50 mg of Solifenacin Succinate in 30 ml of *methanol* with the aid of ultrasound for 15 minutes and dilute to 50.0 ml with *methanol* .Centrifuge the solution at 10000 rpm for 10 minutes and filter.

Reference solution (a). A 0.001 per cent w/v solution of *solifenacin succinate IPRS* in *methanol.*

Reference solution (b). A solution containing 0.0002 per cent w/v of *solifenacin-N- oxide impurity IPRS* and 0.1 per cent w/v of *solifenacin succinate IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as inertsil),
- column temperature: 10°,
- autosampler temperature: 10°,
- mobile phase: A. a buffer solution prepared by dissolving 2 g of *1-hexane sulphonic acid sodium salt* in 1000 ml of *water* add 2 ml of *triethylamine*, mix and adjust the pH 2.6 with dilute *orthophosphoric acid,*

B. *acetonitrile,*

- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	62	38
15	62	38
25	40	60
50	40	60
52	62	38
60	62	38

Name	Relative retention time	Correction factor
(1S)-1-phenyl-1,2,3,4-tetra hydro isoquinoline (SLF-4)	0.42	0.5
Solifenacin (Retention time is about 11 minutes)	1.0	—
Solifenacin-*N*-oxide	1.24	0.9

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 7500 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (b). The test is not valid unless, the resolution between solifenacin and solifenacin-*N*-oxide peaks is not less than 2.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent) and the sum of areas of all the secondary peaks is not more than the 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Disperse one tablet in suitable volume of the solvent mixture, with the aid of ultrasound and dilute with the solvent mixture to obtain a solution containing 0.005 per cent w/v solution of solifenacin succinate.

Calculate the content of $C_{27}H_{32}N_2O_6$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

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Solvent mixture. Dissolve about 2.9 g of *sodium dihydrogen orthophosphate monohydrate* in 600 ml of *water*. Adjust the pH 6.5 with *10 M sodium hydroxide*, dilute to 750 ml with *water*. To this solution, add 250 ml of *acetonitrile*, mix and filter.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 100 mg of Solifenacin Succinate in 150 ml of the solvent mixture, with the aid of ultrasound for 20 minutes and dilute to 200.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.005 per cent w/v solution of *solifenacin succinate IPRS* in the solvent mixture.

Use Chromatographic system as described under Dissolution.

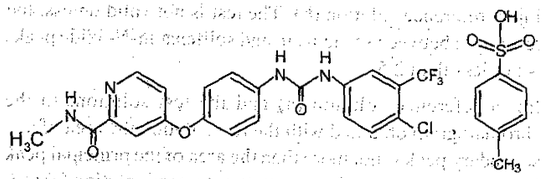
Inject the reference solution. The test is not valid unless the column efficiency is not less than 3500 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{32}N_2O_6$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Sorafenib Tosylate



$C_{21}H_{16}ClF_3N_4O_3.C_7H_8SO_3$ Mol. Wt. 637.0

Sorafenib Tosylate is 4-[4-({[4-chloro-3-(trifluoromethyl) phenyl] carbamoyl} amino) phenoxy] - N-methylpyridine-2-carboxamide.

Sorafenib Tosylate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{21}H_{16}ClF_3N_4O_3.C_7H_8SO_3$, calculated on the dried basis.

Category. Anticancer.

Description. A cream to yellow crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sorafenib tosylate IPRS* or with the reference spectrum of sorafenib tosylate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Paratoluenesulphonic acid. 25.6 per cent to 28.3 per cent.

Dissolve 0.3 g of the substance under examination in 50 ml of *methanol*. Titrate with *0.1 M sodium hydroxide*, determining the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01722 g of paratoluenesulphonic acid.

Related substances. Determine by liquid chromatography (2.4.14).

Solvemt mixture. Equal volumes of mobile phase A and *acetonitrile*.

Test solution. Disssolve 25 mg of the substance under examination in 50.0 ml of the solvent mixture.

Reference solution (a). A solution containing 0. 005 per cent of *sorafenib tosylate IPRS* and 0.0075 per cent w/v each of *sorafenib impurity A IPRS, sorafenib impurity B IPRS* and *sorafenib impurity C IPRS* in the solvent mixture. Further dilute 1.0 ml of the solution to 100.0 ml with the same solvent.

Reference solution (b). A solution containing 0.0005 per cent w/v of *sorafenib tosylate IPRS* and 0.000075 per cent each of *sorafenib impurity A IPRS, sorafenib impurity B IPRS* and *sorafenib impurity C IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (3 µm) (Such as YMC Basic),
- mobile phase: A. dissolve 2.72 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*,
  B. a mixture of 90 volumes of *acetonitrile* and 10 volumes of *tetrahydrofuran*,
- a gradient programme using the conditions given below,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	60	40
5	60	40
10	50	50
20	50	50
25	30	70
35	30	70
37	60	40
45	60	40

Name	Relative retention time
Sorafenib impurity A[1]	0.18
Sorafenib impurity C[2]	0.96
Sorefenib	1.0
Sorafenib impurity B[3]	1.65

[1]4-(2-*N*-methylcarbamoyl)-4-pyridyloxy)aniline,

[2]4-[4-[[2-Chloro-3-(trifluoromethyl)phenyl] carbamoylamino] phenoxy]-*N*-methyl-pyridine-2-caboxamide,4-methylbenzene sulfonic acid,

[3]1, 3-bis (4-Chloro-3-(trifluomethyl)phenyl] urea.

Inject reference solution (a). The test is not valid unless the column efficiency of the principal peak is not less than 5000 theoretical plates, the tailing factor is not more than 2.0 and the resolution between sorafenib impurity C and sorafenib is not less than 1.5.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent) and the sum of areas of all the secondary peaks is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak due to para toluene sulphonic acid (relative retention time about 0.16).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of mobile phase A and *acetonitrile*.

Test solution. Dissolve 25 mg of the substance under examination in 50.0 ml of the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.005 per cent w/v solution of *sorafenib tosylate IPRS* in the solvent mixture.

Use chromatographic system as described under Related substances with spectrophotometer set at 265 nm.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 5000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{16}ClF_3N_4O_3.C_7H_8SO_3$.

Storage. Store protected from light.

Sorafenib Tablets

Sorafenib Tosylate Tablets

Sorafenib Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of sorafenib, $C_{21}H_{16}ClF_3N_4O_3$.

Usual strength. 200 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid* in 1.0 per cent w/v *sodium dodecyl sulphate*,
Speed and time. 75 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *methanol* and *acetonitrile*.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *sorafenib tosylate IPRS* in the solvent mixture and dilute with the dissolution medium to obtain a solution having a known concentration to the expected concentration of test solution.

Use the chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Q. Not less than 75 per cent of the stated amount of $C_{21}H_{16}ClF_3N_4O_3$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *methanol* and *acetonitrile*.

Test solution. Disperse a quantity of powdered tablets containing 100 mg of sorafenib in 200.0 ml of the solvent mixture.

Reference solution. A solution containing 0.0001 per cent w/v of *sorafenib impurity C IPRS (4-[4-[[[[2-Chloro-3-(trifluoromethyl) phenyl]amino]carbonyl]amino]-phenoxy]-N-6-methyl-2-pyridinecaboxamide tosylate IPRS*) and

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0.00005 per cent w/v of *sorafenib tosylate IPRS* equivalent to sorafenib in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (3 µm) (Such as YMC Basic),
- mobile phase: A. dissolve 2.72 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*,
  B. a mixture of 90 volumes of *acetonitrile* and 10 volumes of *tetrahydrofuran*,
- a gradient programme using the conditions given below,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	60	40
5	60	40
10	50	50
20	50	50
25	30	70
35	30	70
37	60	40
45	60	40

Inject the reference solution. The test is not valid unless the resolution between the peaks due sorafenib impurity C and sorafenib is not less than 1.5, the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent) and the sum of areas of all the secondary peaks is not more than 15 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent). Ignore any peak due to para toluene sulphonic acid (relative retention time about 0.16).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *methanol* and *acetonitrile*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 0.2 g of Sorafenib in the solvent mixture and dilute to 250.0 ml with the solvent mixture. Dilute 3.0 ml of the solution to 250.0 ml with the solvent mixture.

Reference solution. A 0.0013 per cent w/v solution of *sorafenib tosylate IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (3 µm) (Such as YMC Basic),
- column temperature: 40°,
- mobile phase: a mixture of 530 volumes of buffer prepared by dissolving 2.72 g of *potassium dihydrogen orthophosphate* in 1000 ml *water*, adjusted to pH 3.0 with *orthophosphoric acid*, 395 volumes of *acetonitrile* and 75 volumes of *tetrahydrofuran*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

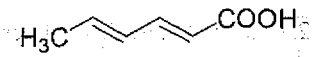
Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{16}ClF_3N_4O_3$ in the tablets.

Storage. Store protected from light.

Labelling. The label states the strength in terms of the equivalent amount of Sorafenib.

Sorbic Acid



$C_6H_8O_2$ Mol. Wt. 112.1

Sorbic Acid is (2*E*,4*E*)-hexa-2,4-dienoic acid.

Sorbic Acid contains not less than 99.0 per cent and not more than 101.0 per cent of $C_6H_8O_2$, calculated on the anhydrous basis.

Category. Pharmaceutical aid (antimicrobial preservative).

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sorbic acid IPRS* or with the reference spectrum of sorbic acid.

B. Dissolve 50 mg in sufficient *water* to produce 250 ml and dilute 2 ml of the solution to 200 ml with *0.1 M hydrochloric acid*.

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When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows an absorption maximum at about 264 nm; absorbance at about 264 nm, 0.43 to 0.51.

C. Dissolve 0.2 g in 2 ml of *ethanol (95 per cent)* and add 0.2 ml of *bromine water*; the solution is decolorised.

Tests

Appearance of solution. A 5.0 per cent w/v solution in *ethanol (95 per cent)* is clear (2.4.1), and colourless (2.4.1).

Heavy metals. 12 ml of 5 per cent w/v solution in *ethanol (95 per cent)* complies with the test for heavy metals, Method D (10 ppm), using 5 ml of *lead standard solution (1ppm Pb)* and 5 ml of *ethanol (95 per cent)*.

Aldehydes. Not more than 0.15 per cent, determined by the following method. Dissolve 1.0 g in a mixture of 50 ml of *2-propanol* and 30 ml of *water*, adjust the pH of the solution to 4.0 with *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* and dilute to 100 ml with *water*. To 10 ml of the resulting solution add 1 ml of *decolorised fuchsin solution* and allow to stand for 30 minutes. Any colour produced is not more intense than that obtained in a solution prepared simultaneously by adding 1 ml of *decolorised fuchsin* solution to a mixture of 1.5 ml of *acetaldehyde standard solution (100 ppm C_2H_4O)*, 4 ml of *2-propanol* and 4.5 ml of *water*.

Sulphated ash (2.3.18). Not more than 0.2 per cent.

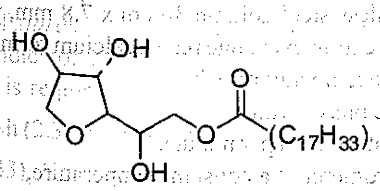
Water (2.3.43). Not more than 1.0 per cent, determined on 2.0 g.

Assay. Weigh 0.2 g, dissolve in 20 ml of *ethanol (95 per cent)* and titrate with *0.1 M sodium hydroxide*, using 0.2 ml of *phenolphthalein solution* as indicator, until a pink colour is produced. Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01121 g of $C_6H_8O_2$.

Storage. Store protected from light and moisture.

Sorbitan Oleate



$C_{24}H_{44}O_6$ Mol. Wt. 428.6

Sorbitan Oleate is mixture usually obtained by esterification of 1 mole of sorbitol and its mono-and di- anhydrides per mole of oleic (*cis*-9-octadecenoic) acid. A suitable antioxidant may be added.

NOTE—When sorbitan mono-oleate is demanded, Sorbitan Oleate shall be supplied.

Category. Pharmaceutical aid.

Description. A brownish-yellow, viscous liquid.

Identification

A. Complies with the test for hydroxyl value (2.3.27).

B. Complies with the test for iodine value (2.3.28).

C. Complies with the test for composition of fatty acids.

D. *Margaric acid.* Not more than 0.2 per cent for oleic acid of vegetable origin and not more than 4.0 per cent for oleic acid of animal origin.

Tests

Relative density (2.4.29). About 0.99.

Acid value (2.3.23). Not more than 8.0, determined on 5.0 g.

Hydroxyl value (2.3.27). 190 to 210.

Saponification value (2.3.37). 145 to 160. Carry out the saponification for 1 hour.

Iodine value (2.3.28). 62 to 76.

Peroxide value (2.3.35). Not more than 10.0.

Composition of fatty acids. Determine Fatty Acid Composition by gas chromatography (2.3.50, Method C).

Composition of the fatty acid fraction of the substance:

- *myristic acid:* Not more than 5.0 per cent;
- *palmitic acid:* Not more than 16.0 per cent;
- *palmitoleic acid:* Not more than 8.0 per cent;
- *stearic acid:* Not more than 6.0 per cent;
- *oleic acid:* 65.0 per cent to 88.0 per cent;
- *linoleic acid:* Not more than 18.0 per cent;
- *linolenic acid:* Not more than 4.0 per cent;
- *fatty acids with chain length greater than C18:* Not more than 4.0 per cent.

Heavy metals (2.3.13). In a silica crucible, mix thoroughly 2.0 g of the substance under examination with 0.5 g of *magnesium oxide*. Ignite to dull redness until a homogeneous white or greyish-white mass is obtained.

If after 30 min of ignition the mixture remains coloured, allow to cool, mix using a fine glass rod and repeat the ignition. If necessary repeat the operation. Heat at 800° for about 1 hour. Take up the residue in 2 quantities, each of 5 ml, of a mixture of equal volumes of *hydrochloric acid* and *water*. Add 0.1 ml of

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phenolphthalein solution and then *concentrated ammonia* until a pink colour is obtained. Cool, add *glacial acetic acid* until the solution is decolourised and add 0.5 ml in excess. Filter if necessary and wash the filter. Dilute to 20 ml with *water*. 12 ml of resultant solution complies with the limit test for heavy metals, Method D (10 ppm). Prepare the standard using 10.0 ml of *lead standard solution (1 ppm Pb)*.

Total ash (2.3.19). Not more than 0.5 per cent, determined on 1.5 g.

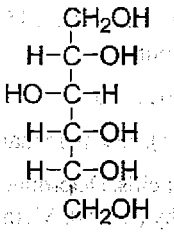
Water (2.3.43). Not more than 1.5 per cent, determined on 1.0 g.

Storage. Store protected from light.

Labelling. The label states the origin of the oleic acid used (animal or vegetable).

Sorbitol

D-Glucitol



$C_6H_{14}O_6$ Mol. Wt. 182.2

Sorbitol is D-glucitol, a hexahydric alcohol related to glucose.

Sorbitol contains not less than 98.0 per cent and not more than 101.0 per cent of $C_6H_{14}O_6$, calculated on the anhydrous basis.

Category. Nutrient (for parenteral administration); pharmaceutical aid (sweetening agent; excipient).

Description. A white, crystalline powder.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

B. Dissolve 50 mg in 3 ml of *water*, add 3 ml of *catechol solution* and pour the mixture into 6 ml of *sulphuric acid*; a pink colour is produced.

C. Dissolve 5 g in 3 ml of *water* with the aid of gentle heat, cool, add 7 ml of *methanol*, 1 ml of *benzaldehyde* and 1 ml of *hydrochloric acid*, mix and shake continuously for 2 hours. Filter, dissolve the crystals in 20 ml of boiling *sodium bicarbonate solution* and allow to crystallise. The residue, after washing rapidly with 5 ml of a mixture of equal volumes of *methanol* and *water* and drying in a current of air, melts at about 175° (2.4.21).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. Dissolve 5.0 g in sufficient *carbon dioxide-free water* prepared from *distilled water* to produce 50 ml (solution A). To 10 ml of solution A add 10 ml of *carbon dioxide-free water*. To 10 ml of the resulting solution add 0.05 ml of *phenolphthalein solution*; not more than 0.2 ml of *0.01 M sodium hydroxide* is required to change the colour of the solution to pink. To a further 10 ml of the solution add 0.05 ml of *methyl red solution*; Not more than 0.3 ml of *0.01 M hydrochloric acid* is required to change the colour of the solution to red.

Specific optical rotation (2.4.22). +4.0° to +7.0°, determined in a solution prepared in the following manner. Dissolve a mixture of 5.0 g of the substance under examination and 6.4 g of *borax* in 40 ml of *water*, allow to stand for 1 hour, shaking occasionally, dilute to 50 ml with *water* and filter, if necessary.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 5.0 g of the substance under examination in 100.0 ml of *water*.

Reference solution (a). Dissolve 0.5 g of *sorbitol IPRS* in 10.0 ml of *water*.

Reference solution (b). Dilute 2.0 ml of the test solution to 100.0 ml with *water*.

Reference solution (c). Dilute 5.0 ml of reference solution (b) to 100.0 ml with *water*.

Reference solution (d). Dissolve 0.5 g each of *sorbitol* and *mannitol (sorbitol impurity A)* in 10.0 ml of *water*.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm, packed with strong cation exchange resin (calcium form) (9 µm),
- column temperature: 85°,
- mobile phase: *water*,
- flow rate: 0.5 ml per minute,
- refractometer at a constant temperature,
- injection volume: 20 µl.

The relative retention time with reference to sorbitol for maltitol (sorbitol impurity C) is about 0.6, for mannitol (sorbitol impurity A) is about 0.8 and for iditol (sorbitol impurity B) is about 1.1.

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Inject reference solution (d). The test is not valid unless the resolution between the peaks due to sorbitol and sorbitol impurity A is not less than 2.0.

Inject reference solutions (b), (c), (d) and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent) and the sum of all the secondary peaks is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method A (10 ppm).

Chlorides (2.3.12). 5.0 g complies with the limit test for chlorides (50 ppm).

Nickel. Dissolve 10.0 g in sufficient *water* to produce 20 ml, add 3 ml of *bromine water* and 2 ml of a 20 per cent w/v solution of *citric acid*, mix and add 10 ml of *6 M ammonia* and 1 ml of a 1 per cent w/v solution of *dimethylglyoxime* in *ethanol (95 per cent)*. Mix, dilute to 50 ml with *water* and allow to stand for 5 minutes; any colour produced is not more intense than that produced by treating in the same manner and at the same time 1.0 ml of *nickel standard solution (10 ppm Ni)* diluted to 20 ml with *water* (1 ppm).

Sulphates (2.3.17). 12 ml of solution A complies with the limit test for sulphates (125 ppm).

Reducing sugars. Dissolve 5.0 g in 3 ml of *water* with the aid of gentle heat, cool, add 20 ml of *cupri-citric solution* and a few glass beads, heat in such a manner that the solution boils in 4 minutes and continue boiling for a further 3 minutes. Cool rapidly and add 100 ml of a 2.4 per cent v/v solution of *glacial acetic acid* followed by 20.0 ml of *0.025 M iodine*. Add, shaking continuously, 25 ml of a 6 per cent v/v solution of *hydrochloric acid* and, when any precipitate has redissolved, titrate the excess iodine with *0.05 M sodium thiosulphate* using 1 ml of *starch solution*, added towards the end of the titration, as indicator. Not less than 12.8 ml of *0.05 M sodium thiosulphate* is required.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.5 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14) as described under test for Related substances with the following modification.

Inject reference solution (a) and the test solution.

Calculate the content of $C_6H_{14}O_6$.

Sorbitol intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins *complies with the following additional requirement.*

Bacterial endotoxins (2.2.3). Not more than 4.0 Endotoxin Units per g for parenteral preparations having a concentration of less than 10 per cent w/v of sorbitol and not more than 2.5 Endotoxin Units per g for parenteral preparations containing 10 per cent w/v or more of sorbitol.

Storage. Store protected from moisture.

Labelling. The label states whether or not the contents are intended for use in the manufacture of parenteral preparations.

Sorbitol Solution (70 Per cent) (Crystallising)

Sorbitol (70 per cent) (Crystallising)

Sorbitol Solution (70 per cent) (Crystallising) is an aqueous solution of hydrogenated, partly hydrolysed starch.

Sorbitol Solution (70 per cent) (Crystallising) contains not less than 68.0 per cent w/w and not more than 72.0 per cent w/w of anhydrous substances and not less than 92.0 per cent w/w and not more than 101.0 per cent w/w of D-glucitol, $C_6H_{14}O_6$, calculated on the anhydrous basis.

Category. Pharmaceutical aid.

Description. A clear, colourless , syrupy liquid, miscible with water.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

B. To 7.0 g, add 40 ml of *water* and 6.4 g of *disodium tetraborate*. Allow to stand for 1 hour, shake occasionally and dilute to 50.0 ml with *water*. The angle of rotation (2.4.22) is 0° to +1.5°.

C. It is clear syrupy liquid at 25°.

Tests

Appearance of solution. A 14.0 per cent w/v solution in *water* is clear (2.4.1) and colourless (2.4.1).

Conductivity (2.4.9). Not more than 10 μS cm^{-1}, measured on undiluted liquid sorbitol (crystallising) while gently stirring with a magnetic stirrer.

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Reducing sugars. Not more than 0.2 per cent, calculated as glucose equivalent.

To 5.0 g, add 6.0 ml of *water*, 20.0 ml of *cupri-citric solution* and a few glass beads. Heat so that boiling begins after 4 minutes and maintain boiling for 3 minutes. Cool rapidly and add 100.0 ml of a 2.4 per cent v/v solution of *glacial acetic acid* and 20.0 ml of *0.025 M iodine*. With continuous shaking, add 25.0 ml of a mixture of 6 volumes of *hydrochloric acid* and 94 volumes of *water* and when the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulphate* using 1 ml of *starch solution*, added towards the end of the titration, as indicator. Not less than 12.8 ml of *0.05 M sodium thiosulphate* is required.

Lead. Not more than 0.5 ppm.

Determine by atomic absorption spectrometry (2.4.2), Method A.

Solvent mixture. Equal volumes of *dilute acetic acid* and *water*.

Test solution. Dissolve 100 g of the substance under examination in 500 ml of the solvent mixture. To 100 ml of the solution add 2.0 ml of 1 per cent w/v solution of *ammonium pyrrolidinedithio-carbamate* and 10.0 ml of *methyl isobutyl ketone*, shake for few seconds protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solution (a). Dissolve 0.5 ml of *lead standard solution (10 ppm Pb)* in 100 ml of the test solution. Add 2.0 ml of a clear 1 per cent w/v solution of *ammonium pyrrolidinedithiocarbamate* and 10.0 ml of *methyl isobutyl ketone*, shake for few seconds and protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solution (b). Dissolve 1.0 ml of *lead standard solution (10 ppm Pb)* in 100 ml of the test solution. Add 2.0 ml of a clear 1 per cent w/v solution of *ammonium pyrrolidinedithiocarbamate* and 10.0 ml of *methyl isobutyl ketone*, shake for few seconds and protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solution (c). Dissolve 1.5 ml of *lead standard solution (10 ppm Pb)* in 100 ml of the test solution. Add 2.0 ml of a clear 1 per cent w/v solution of *ammonium pyrrolidinedithiocarbamate* and 10.0 ml of *methyl isobutyl ketone*, shake for few seconds and protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Set the zero of the instrument using *methyl isobutyl ketone* treated as described for the test solution without the substance under examination. Measure the absorbance at 283.3 nm using a lead hollow-cathode lamp as source of radiation and an air-acetylene flame.

Nickel. Dissolve 10.0 g in sufficient *water* to produce 20 ml, add 3.0 ml of *bromine water* and 2.0 ml of a 20 per cent w/v solution of *citric acid*, mix and add 10.0 ml of *6 M ammonia* and 1.0 ml of a 1.0 per cent w/v solution of *dimethylglyoxime* in *ethanol (95 per cent)*. Mix, dilute to 50.0 ml with *water* and allow to stand for 5 minutes; any colour produced is not more intense than that produced by treating in the same manner and at the same time 1.0 ml of *nickel standard solution (10 ppm Ni)* diluted to 20.0 ml with *water* (1 ppm).

Water (2.3.43): 28.0 per cent to 32.0 per cent, determined on 0.1 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 1.0 g of the substance under examination in 50.0 ml of *water*.

Reference solution (a). Dissolve 65 mg of *sorbitol IPRS* in 5.0 ml of *water*.

Reference solution (b). Dissolve 65 mg of *mannitol* and 65 mg of *sorbitol* in 5.0 ml of *water*.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm, packed with strong cation exchange resin (calcium form) (9 µm),
- column temperature: 85°,
- mobile phase: *water*,
- flow rate: 0.5 ml per minute,
- refractometer set at a constant temperature,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to sorbitol and mannitol is not less than 2.0. The relative retention time with reference to sorbitol (Retention time is about 27 minutes) for mannitol is about 0.8.

Inject reference solution (a) and the test solution. Run the chromatogram for three times the retention time of sorbitol.

Calculate the content of D-sorbitol (D-glucitol), $C_6H_{14}O_6$.

Storage. Store protected from moisture.

Sorbitol Solution (70 Per cent) (Non-Crystallising)

Sorbitol (70 per cent) (Non-crystallising)

Sorbitol Solution (70 per cent) (Non-crystallising) is an aqueous solution of hydrogenated, partly hydrolysed starch.

Sorbitol Solution (70 per cent) (Non-crystallising) contains not less than 68.0 per cent w/w and not more than 72.0 per cent w/w of anhydrous substances and not less than 72.0 per cent w/w and not more than 92.0 per cent w/w of D-glucitol, $C_6H_{14}O_6$, calculated on the anhydrous basis.

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Category. Pharmaceutical aid (sweetening vehicle); humectant.

Description. A clear, colourless or faintly yellow, syrupy liquid.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

B. To 7.0 g, add 40 ml of *water* and 6.4 g of *disodium tetraborate*. Allow to stand for 1 hour, shake occasionally and dilute to 50.0 ml with *water*. The angle of rotation (2.4.22) is +1.5° to +3.5°.

C. It is clear syrupy liquid at 25°.

Tests

Appearance of solution. A 14.0 per cent w/v solution in *water* is clear (2.4.1) and colourless (2.4.1).

Conductivity (2.4.9). Not more than 10 μS cm^{-1}, measured on undiluted liquid sorbitol while gently stirring with a magnetic stirrer.

Reducing sugars. Not more than 0.2 per cent, calculated as glucose equivalent.

To 5.0 g, add 6.0 ml of *water*, 20.0 ml of *cupri-citric solution* and a few glass beads. Heat so that boiling begins after 4 minutes and maintain boiling for 3 minutes. Cool rapidly and add 100.0 ml of a 2.4 per cent v/v solution of *glacial acetic acid* and 20.0 ml of *0.025 M iodine*. With continuous shaking, add 25.0 ml of a mixture of 6 volumes of *hydrochloric acid* and 94 volumes of *water* and when the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulphate* using 1 ml of *starch solution*, added towards the end of the titration, as indicator. Not less than 12.8 ml of *0.05 M sodium thiosulphate* is required.

Reducing sugars after hydrolysis. Not more than 9.3 per cent, calculated as glucose equivalent.

To 6.0 g, add 35 ml of *water*, 40 ml of *1 M hydrochloric acid* and a few glass beads. Reflux for 4 hours. Cool and neutralize with *dilute sodium hydroxide solution*, using 0.2 ml of *bromothymol blue solution* as indicator. Cool and dilute to 100.0 ml with *water*. To 3.0 ml of the solution, add 5.0 ml of *water*, 20 ml of *cupri-citric solution* and a few glass beads. Heat so that boiling begins after 4 minutes and maintain boiling for 3 minutes. Cool rapidly and add 100 ml of a 2.4 per cent v/v solution of *glacial acetic acid* and 20.0 ml of *0.025 M iodine*. With continuous shaking, add 25 ml of a mixture of 6 volumes of *hydrochloric acid* and 94 volumes of *water* and when the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulphate* using 1 ml of *starch solution*, added towards the end of the titration, as indicator. Not less than 8.0 ml of *0.05 M sodium thiosulphate* is required.

Lead. Not more than 0.5 ppm.

Determine by atomic absorption spectrometry (2.4.2).

Solvent mixture. Equal volumes of *dilute acetic acid* and *water*.

Test solution. Dissolve 100 g of the substance under examination in 500 ml of the solvent mixture. To 100 ml of the solution add 2.0 ml of 1 per cent w/v solution of *ammonium pyrrolidinedithio-carbamate* and 10.0 ml of *methyl isobutyl ketone*, shake for few seconds protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solution (a). Add 0.5 ml of *lead standard solution (10 ppm Pb)* in 100 ml of the test solution. Add 2.0 ml of a clear 1 per cent w/v solution of *ammonium pyrrolidinedithio-carbamate* and 10.0 ml of *methyl isobutyl ketone*, shake for few seconds and protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solution (b). Add 1.0 ml of *lead standard solution (10 ppm Pb)* in 100 ml of the test solution. Add 2.0 ml of a clear 1 per cent w/v solution of *ammonium pyrrolidinedithio-carbamate* and 10.0 ml of *methyl isobutyl ketone*, shake for few seconds and protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solution (c). Add 1.5 ml of *lead standard solution (10 ppm Pb)* in 100 ml of the test solution. Add 2.0 ml of a clear 1 per cent w/v solution of *ammonium pyrrolidinedithio-carbamate* and 10.0 ml of *methyl isobutyl ketone*, shake for few seconds and protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Set the zero of the instrument using *methyl isobutyl ketone* treated as described for the test solution without the substance under examination. Measure the absorbance at 283.3 nm using a lead hollow-cathode lamp as source of radiation and an air-acetylene flame.

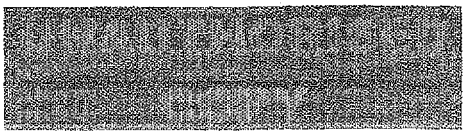
Nickel. Dissolve 10.0 g in sufficient *water* to produce 20 ml, add 3.0 ml of *bromine water* and 2.0 ml of a 20 per cent w/v solution of *citric acid*, mix and add 10.0 ml of *6 M ammonia* and 1.0 ml of a 1.0 per cent w/v solution of *dimethylglyoxime* in *ethanol (95 per cent)*. Mix, dilute to 50.0 ml with *water* and allow to stand for 5 minutes; any colour produced is not more intense than that produced by treating in the same manner and at the same time 1.0 ml of *nickel standard solution (10 ppm Ni)* diluted to 20.0 ml with *water* (1 ppm).

Water (2.3.43). 28.0 per cent to 32.0 per cent, determined on 0.1 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 1.0 g of the substance under examination in 50.0 ml of *water*.

Reference solution (a). Dissolve 55 mg of *sorbitol IPRS* in 5.0 ml of *water*.



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Reference solution (b). Dissolve 55 mg of *mannitol IPRS* and 55 mg of *sorbitol IPRS* in 5.0 ml of *water*.

Chromatographic system
- a stainless steel column 30 cm x 7.8 mm, packed with strong cation exchange resin (calcium form) (9 µm),
- column temperature: 85°,
- mobile phase: *water*,
- flow rate: 0.5 ml per minute,
- refractometer set at a constant temperature,
- injection volume: 20 µl.

The relative retention time with reference to sorbitol (Retention time is about 27 minutes) for mannitol is about 0.8.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to sorbitol and mannitol is not less than 2.0.

Inject reference solution (a) and the test solution. Run the chromatogram for three times the retention time of sorbitol.

Calculate the content of D-sorbitol (D-glucitol), $C_6H_{14}O_6$.

Storage. Store protected from moisture.

Soyabean Oil

Soyabean Oil is a fixed oil obtained from the seeds of Glycine soja Sieb. and Zucc. and Glycine max (L) Merr. (G. hispida (Moench) Maxim.) by extraction and subsequent refining; it may contain a suitable antioxidant.

Category. Pharmaceutical aid.

Description. A clear, pale-yellow coloured liquid, with a bland taste that solidifies at -10° to -16°.

Identification

Complies with the test of Fatty Acid Composition by Gas Chromatography (2.3.50) and exhibits the following composition profile of fatty acids.

Carbon-Chain length	No. of Double bonds	Percentage
<14	0	≤0.1
14	0	≤0.2
16	0	9.0 to 13.0
16	1	≤0.3
18	0	2.5 to 5.0
18	1	17.0 to 30.0
18	2	48.0 to 58.0
18	3	5.0 to 11.0
20	0	≤1.0
20	1	≤1.0
22	0	≤1.0
22	1	≤0.3
24	0	≤0.5

Tests

Weight per ml (2.4.29). 0.916 g to 0.922 g.

Refractive index (2.4.27). 1.471 to 1.475.

Heavy metals (2.3.13). 2.0 g complies with limit test for heavy metals, Method B (10 ppm).

Acid value (2.3.23). Not more than 0.3.

Peroxide value (2.3.35). Not more than 10.0.

Iodine value (2.3.28). 120 to 141.

Saponification value (2.3.37). 180 to 200.

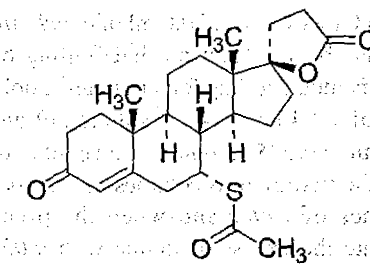
Unsaponifiable matter (2.3.39). Not more than 1.5 per cent.

Water (2.3.43). Not more than 0.1 per cent.

Storage. Store protected from light and moisture.

Labelling. The label states the name and quantity of any added antioxidant.

Spironolactone



$C_{24}H_{32}O_4S$ Mol. Wt. 416

Spironolactone is 7α-acetylthio-3-oxo-17α-pregn-4-ene-21,17β-carbolactone.

Spironolactone contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{24}H_{32}O_4S$, calculated on the dried basis.

Category. Diuretic.

Description. A yellowish white to buff coloured powder. It shows polymorphism (2.5.11).

Identification

Test A may be omitted if tests B and C are carried out. Test B may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *spironolactone IPRS* or with the reference spectrum of spironolactone.

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B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. A mixture of 90 volumes of *acetone* and 10 volumes of *1,2-propanediol*.

Mobile phase. A mixture of 40 volumes of *cyclohexane* and 10 volumes of *toluene*.

Test solution. Dissolve 25 mg of the substance under examination in 10 ml of the solvent mixture.

Reference solution (a). Dissolve 25 mg of *spironolactone IPRS* in 10 ml of the solvent mixture.

Reference solution (b). Mix equal volumes of the test solution and reference solution (a).

Place the dry plate in a tank containing a shallow layer of the solvent mixture, allow the solvent mixture to ascend to the top, remove the plate from the tank and allow the solvent to evaporate. Use within 2 hours, with the flow of the mobile phase in the direction in which the aforementioned treatment was done.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of warm air, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulphuric acid (20 per cent v/v)*. Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

C. Shake about 10 mg with 2 ml of *sulphuric acid (50 per cent)*; an orange solution with an intense yellowish fluorescence is produced. Heat the solution gently; the colour becomes deep red and hydrogen sulphide is evolved which turns *lead acetate paper* black. Add the solution to 10 ml of *water*; a greenish yellow solution is produced which shows opalescence or a precipitate.

Tests

Specific optical rotation (2.4.22). −37.0° to −33.0°, determined in a 1.0 per cent w/v solution in *chloroform*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 62.5 mg of the substance under examination in 2.5 ml of *tetrahydrofuran* and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 25 mg of *canrenone IPRS* in 1.0 ml of *tetrahydrofuran* and dilute to 10.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 1.0 ml of the test solution and 1 ml of reference solution (b) to 100.0 ml with the mobile phase.

Reference solution (e). Dilute 0.5 ml of reference solution (a) to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 8 volumes of *acetonitrile*, 18 volumes of *tetrahydrofuran* and 74 volumes of *water*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 254 nm and 283 nm,
- injection volume: 20 µl.

Inject reference solution (d). The test is not valid unless the resolution between the peaks due to canrenone and spironolactone is not less than 1.4 and the signal-to-noise ratio of the principal peak is not less than 6.

Inject reference solution (a), (c), (d), (e) and the test solution. At 254 nm, run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution, the sum of the areas of all the secondary peaks other than canrenone, is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (e). At 283 nm, the area of the peak due to canrenone is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (1.0 per cent). Calculate the content of canrenone at 283 nm and the content of the other impurities at 254 nm. The sum of areas of all the peaks is not more than 1.0 per cent.

Chromium. Mix 0.2 g with 1 g of *potassium carbonate* and 0.3 g of *potassium nitrate* in a platinum crucible, heat gently until fused and ignite at 600° to 650° until the carbon is removed. Cool, dissolve the residue as completely as possible in 10 ml of *water* with the aid of gentle heat, filter and dilute to 20 ml with *water*. To 10 ml of the solution add 0.5 g of *urea* and just acidify with *sulphuric acid (14 per cent)*. When effervescence ceases add a further 1 ml of *sulphuric acid (14 per cent)*, dilute to 20 ml with *water* and add 0.5 ml of *diphenylcarbazide solution*. Any colour produced is not more intense than that obtained by adding 1 ml of *sulphuric acid (14 per cent)* to 0.5 ml of a freshly prepared 0.00283 per cent w/v solution of *potassium dichromate*, diluting to 20 ml with *water* and adding 0.5 ml of *diphenylcarbazide solution* (50 ppm).

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Free mercapto compounds. Shake 2.0 g with 20 ml of *water* for 1 minute and filter. To 10 ml of the filtrate add 0.05 ml of *0.01 M iodine* and 0.1 ml of *starch solution* and mix; a blue colour develops.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours,

Assay. Weigh 50 mg, dissolve in sufficient *methanol* to produce 250.0 ml, dilute 5.0 ml to 100.0 ml with *methanol* and measure the absorbance of the resulting solution at the maximum at about 238 nm (2.4.7).

Calculate the content of $C_{24}H_{32}O_4S$ taking 470 as the specific absorbance at 238 nm.

Storage. Store protected from light and moisture.

Spironolactone Tablets

Spironolactone Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of spironolactone, $C_{24}H_{32}O_4S$. The tablets may contain added flavouring agents.

Usual strengths. 25 mg; 50 mg; 100 mg.

Identification

A. Extract a quantity of the powdered tablets containing 0.125 g of Spironolactone with two quantities, each of 10 ml, of *chloroform*, filter, evaporate the combined filtrates to dryness and dissolve the residue in 2.5 ml of chloroform.

On the resulting solution determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *spironolactone IPRS* or with the reference spectrum of spironolactone.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. A mixture of 90 volumes of *acetone* and 10 volumes of *1,2-propanediol*.

Mobile phase. A mixture of 40 volumes of *cyclohexane* and 10 volumes of *toluene*.

Test solution. Extract a quantity of the powdered tablets containing 50 mg of Spironolactone with 10 ml of *chloroform*, filter and evaporate the filtrate to dryness. Dissolve 25 mg the residue in 10 ml of the solvent mixture.

Reference solution (a). Dissolve 25 mg of *spironolactone IPRS* in 10 ml of the solvent mixture.

Reference solution (b). Mix equal volumes of the test solution and reference solution (a).

Place the dry plate in a tank containing a shallow layer of the solvent mixture, allow the solvent mixture to ascend to the top, remove the plate from the tank and allow the solvent to evaporate. Use within 2 hours, with the flow of the mobile phase in the direction in which the aforementioned treatment was done.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of warm air, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulphuric acid (20 per cent v/v)*. Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

C. Shake about 10 mg of the residue obtained in test B with 2 ml of *sulphuric acid (50 per cent)*; an orange solution with an intense yellowish fluorescence is produced. Heat the solution gently; the colour becomes deep red and hydrogen sulphide is evolved which turns *lead acetate paper* black. Add the solution to 10 ml of *water*; a greenish yellow solution is produced which shows opalescence or a precipitate.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 1000 ml of *0.1 M hydrochloric acid* containing 0.1 per cent w/v of *sodium dodecyl sulphate*,

Speed and time. 75 rpm and 60 minutes.

Withdraw a suitable volume of the medium, filter through a membrane filter disc with an average pore diameter not greater than 1.0 µm. Measure the absorbance of the filtrate, suitably diluted if necessary, at the maximum at about 242 nm (2.4.7). Calculate the content of $C_{24}H_{32}O_4S$ in the medium taking 445 as the specific absorbance at 242 nm.

Q. Not less than 70 per cent of the stated amount of $C_{24}H_{32}O_4S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing about 62.5 mg of Spironolactone with 25 ml of *chloroform*, sonicate for 15 minutes, centrifuge and filter the supernatant. Repeat the procedure on the residue with a further 25 ml of *chloroform*. Combine the chloroform extracts and evaporate to dryness using a rotary evaporator. Add 2.5 ml of *tetrahydrofuran* and 22.5 ml of the mobile phase to the residue.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 25 mg of *canrenone IPRS* in 1.0 ml of *tetrahydrofuran* and dilute to 10.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 1.0 ml of the test solution and 1.0 ml of reference solution (b) to 100.0 ml with the mobile phase.

Reference solution (e). Dilute 0.5 ml of reference solution (a) to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with endcapped octylsilane bonded to porous silica (5 µm) (Such as Spherisorb C8),
- mobile phase: a mixture of 8 volumes of *acetonitrile*, 18 volumes of *tetrahydrofuran* and 74 volumes of *water*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 254 nm and 283 nm,
- injection volume: 20 µl.

Inject reference solution (d). The test is not valid unless the resolution between the peaks due to canrenone and spironolactone is not less than 1.4 and the signal-to-noise ratio of the principal peak is not less than 6.

Inject reference solution (a), (c), (d), (e) and the test solution. At 254 nm, run the chromatogram twice the retention time of principal peak. In the chromatogram obtained with the test solution, the sum of the areas of all the secondary peaks other than the canrenone, is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (e). At 283 nm, the area of the peak due to canrenone is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (1.0 per cent). Calculate the content of canrenone at 283 nm and the content of the other impurities at 254 nm. The sum of areas of all the peaks is not more than 1.0 per cent.

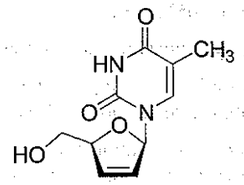
Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 25 mg of Spironolactone, add 100 ml of *methanol* and heat just to boiling, with swirling. Cool, add sufficient *methanol* to produce 250.0 ml, dilute 10.0 ml to 100.0 ml with *methanol* and measure the absorbance of the resulting solution at the maximum at about 238 nm (2.4.7).

Calculate the content of $C_{24}H_{32}O_4S$ taking 470 as the specific absorbance at 238 nm.

Storage. Store protected from light and moisture.

Stavudine



$C_{10}H_{12}N_2O_4$ Mol. Wt. 224.2

Stavudine is 2',3'-didehydro-3'-deoxythymidine.

Stavudine contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{10}H_{12}N_2O_4$, calculated on the dried basis.

Category. Antiretroviral.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *stavudine IPRS* or with the reference spectrum of stavudine.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to stavudine in the chromatogram obtained with reference solution (c).

C. Melts at about 164° (2.4.21).

Tests

Specific optical rotation (2.4.22). –46.0° to –39.0°, determined in a 0.7 per cent w/v solution in *water*.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay.

Inject reference solution (a), (b) and the test solution and record the chromatograms for at least three times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to thymine is not more than the area of the peak in the chromatogram obtained with reference solution (b) (1 per cent); the area of any peak corresponding to β-thymidine is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (1 per cent); the area of any individual impurity peak other than thymine and β-thymidine is not more than 0.5 per cent and the sum of the areas of all the impurity peaks other than thymine and β-thymidine is not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

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Sulphated ash (2.3.18). Not more than 0.3 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying at 105° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50.0 mg of the substance under examination in 100.0 ml of the mobile phase.

Reference solution (a). A solution containing 0.05 per cent w/v of *stavudine IPRS*, 0.01 per cent w/v of *thymine* and 0.0005 per cent w/v of *β-thymidine* in the mobile phase.

Reference solution (b). A 0.0005 per cent w/v solution of *thymine* in the mobile phase.

Reference solution (c). A 0.05 per cent w/v solution of *stavudine IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: a mixture of 20 volumes of *methanol* and 80 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 20 µl.

Inject reference solution (a) and (c). The order of elution in the chromatogram obtained with reference solution (a) is thymine, β-thymidine and stavudine with relative retention times of about 0.5, 0.7 and 1.0 respectively. The test is not valid unless the column efficiency determined from the stavudine peak in the chromatogram obtained with reference solution (a) is not less than 3000 theoretical plates, the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections of reference solution (c) is not more than 2.0 per cent.

Inject reference solution (c) and the test solution and measure the responses of the principal peak.

Calculate the content of $C_{10}H_{12}N_2O_4$.

Storage. Store protected from light and moisture.

Stavudine Capsules

Stavudine Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of stavudine, $C_{10}H_{12}N_2O_4$.

Usual strengths. 30 mg; 40 mg.

Identification

A. Shake a quantity of the mixed contents of the capsules containing about 50 mg of Stavudine with 80 ml of *water* for 10 minutes. Add sufficient *water* to produce 100 ml, mix and filter. Dilute 10 ml of the filtrate to 100 ml with *water*.

When examined in the range 200 nm to 300 nm (2.4.7), the resulting solution shows an absorption maximum at about 265 nm.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to stavudine in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.01 M hydrochloric acid*,

Speed and time. 75 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. The filtrate obtained as given above.

Reference solution. In the case of capsules containing 30 mg of Stavudine, weigh 30 mg of *stavudine IPRS* and transfer to a 100-ml volumetric flask. Dissolve and dilute to volume with *0.01 M hydrochloric acid*. Dilute 5.0 ml of the solution to 50.0 ml with *0.01 M hydrochloric acid*; in the case of capsules containing 40 mg of stavudine, weigh 40 mg of *stavudine IPRS* and transfer to a 100-ml volumetric flask. Dissolve and dilute to volume with *0.01 M hydrochloric acid*. Dilute 5.0 ml of the solution to 50.0 ml with *0.01 M hydrochloric acid*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 85 volumes of *water* and 15 volumes of *methanol*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 266 nm,
- injection volume: 20 µl.

Inject the reference solution. Run the chromatogram twice the retention time of stavudine. The test is not valid unless the column efficiency determined from the stavudine peak is not less than 3000 theoretical plates, the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.5 per cent.

Inject separately the reference solution and the test solution and measure the responses for the major peak.

Calculate the content of $C_{10}H_{12}N_2O_4$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{10}H_{12}N_2O_4$.

Related substances. Determine by liquid chromatography (2.4.14).

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Test solution. Mix well the contents of 20 capsules and transfer a weighed quantity containing about 50 mg of Stavudine to a 100-ml volumetric flask. Add about 60 ml of *water*, mix with the aid of ultrasound for 10 minutes, dilute to volume with *water*, mix and filter.

Reference solution. Weigh 5 mg each of *stavudine IPRS* and *thymine IPRS* and transfer to a 25-ml volumetric flask. Add 5 ml of *methanol*, mix with the aid of ultrasound to dissolve and dilute to volume with *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: A. *0.1 M ammonium acetate*, B.*acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
5	95	5
25	20	80
30	20	80
31	95	5
35	95	5

Inject the reference solution. The test is not valid unless the column efficiency determined from the peaks of stavudine and thymine is not less than 7000 theoretical plates and the tailing factor is not more than 2.0.

Inject water and the test solution. Examine the water chromatogram for any extraneous peaks and ignore the corresponding peaks observed in the chromatogram obtained with the test solution.

Any secondary peak observed in the chromatogram obtained with the test solution corresponding to thymine is not more than 3.0 per cent. Any other secondary peak observed in the chromatogram obtained with the test solution is not more than 0.5 per cent and the sum of the areas of all the secondary peaks is not more than 4.0 per cent when calculated by percentage area normalisation.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the mixed contents of 20 capsules containing about 50 mg of Stavudine and transfer to a 100-ml volumetric flask. Add about 60 ml of *water*, mix with the aid of ultrasound for 10 minutes, dilute to volume with *water*, mix and filter. Further dilute 10.0 ml of the filtrate to 25.0 ml with *water*.

Reference solution. Weigh 50 mg of *stavudine IPRS* and transfer to a 100-ml volumetric flask. Add about 60 ml of *water*, mix with the aid of ultrasound to dissolve and dilute to volume with *water*. Dilute 10.0 ml of the solution to 25.0 ml with *water*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 5 volumes of *acetonitrile* and 95 volumes of *0.1 M ammonium acetate*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency determined from the stavudine peak is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject separately the reference solution and the test solution and measure the responses for the major peak.

Calculate the content of $C_{10}H_{12}N_2O_4$ in the capsules.

Storage. Store protected from moisture.

Stavudine Oral Solution

Stavudine Oral Solution is a mixture consisting of Stavudine with buffering agents and other excipients. It contains a suitable flavouring agent. It is filled in a sealed container.

The oral solution is constituted by dispersing the contents of the sealed container in the specified volume of water just before issue.

Stavudine Oral Solution contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of stavudine $C_{10}H_{12}N_2O_4$.

The constituted solution, when stored at the temperature and for the period stated on the label during which it may be expected to be satisfactory for use, contains not less than 90.0 per cent of the stated amount of stavudine, $C_{10}H_{12}N_2O_4$.

Usual strength. 1 mg per ml.

The contents of the sealed container comply with the requirements stated under Oral Liquids and with the following requirements.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

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Tests

pH (2.4.24). 5.0 to 7.0, determined in the reconstituted solution.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Dilute a volume of the reconstituted solution containing 10 mg of stavudine and dissolve in 20 ml of *water*.

Reference solution (a). A 0.05 per cent w/v solution of *stavudine IPRS* in *water*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *water*.

Reference solution (c). A solution containing 0.0125 per cent w/v each of thymine and thymidine in *water*. Dilute 2.0 ml of the solution to 100.0 ml of *water*.

Use the chromatographic system described under Assay.

Inject reference solution (a). The test is not valid unless the tailing factor is not more than 2.0 and the column efficiency in not less than 2000 theoretical plates.

Inject reference solution (c). The test is not valid unless the resolution between thymine and thymidine peak is not less than 8.4.

Inject reference solution (b) and the test solution. Run the chromatogram for 4 times the retention time (about 9 minutes) of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 1.5 times the area of the peak in the chromatogram obtained with the reference solution (b) (1.5 per cent) and the sum of areas of all the secondary peaks is not more than 3 times the area of the peak in the chromatogram obtained with the reference solution (b) (3.0 per cent).

Other tests. Comply with the tests stated under Oral Liquids.

Water (2.3.43). Not more than 2.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Weigh a quantity of the reconstituted solution containing 10 mg of Stavudine, disperse in sufficient *water* and diluted to 100.0 ml with *water*.

Reference solution (a). A 0.01 per cent w/v solution of *stavudine IPRS* in *water*.

Reference solution (b). A solution containing 0.0125 per cent w/v each of thymine and thymidine in *water*. Dilute 2.0 ml of the solution to 100.0 ml with *water*.

Chromatographic system
- a stainless steel column 3.3 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. 95 volumes of 25 mM *ammonium acetate* and 5 volumes of *methanol*,
  B. 50 volumes of 25 mM *ammonium acetate* and 50 volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 268 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
60	100	0
120	0	100
155	100	0
165	100	0

Inject reference solution (b). The test is not valid unless the resolution between thymine and thymidine is not less than 8.4.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Determine the weight per ml of the oral solution (2.4.29) and calculate the content of $C_{10}H_{12}N_2O_4$, weight in volume. Repeat the procedure using a portion of the constituted solution that has been stored at the temperature and for the period stated on the label.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Stavudine and Lamivudine Tablets

Stavudine and Lamivudine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of stavudine, $C_{10}H_{12}N_2O_4$ and lamivudine, $C_8H_{11}N_3O_3S$.

Usual strengths. Stavudine, 30 mg and Lamivudine, 150 mg; Stavudine, 40 mg and Lamivudine, 150 mg.

Identification

In the Assay, the two principal peaks in the chromatogram obtained with the test solution correspond to the peaks due to stavudine and lamivudine in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.01 M hydrochloric acid*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. The filtrate obtained as given above.

Reference solution. In the case of tablets containing 30 mg of stavudine, weigh 33 mg of *stavudine IPRS* and 167 mg of *lamivudine IPRS* and transfer to a 100 ml volumetric flask. Add about 20 ml of *methanol*, sonicate to dissolve and dilute to volume with a solvent mixture of equal volumes of *methanol* and *water* (diluent). Dilute 5.0 ml of the solution to 50.0 ml with *0.01 M hydrochloric acid*; in the case of tablets containing 40 mg of stavudine, weigh 44 mg of *stavudine IPRS* and 167 mg of *lamivudine IPRS* and transfer to a 100-ml volumetric flask. Add about 20 ml of *methanol*, mix with the aid of ultrasound to dissolve and dilute to volume with the diluent. Dilute 5.0 ml of the solution to 50.0 ml with *0.01 M hydrochloric acid*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of *methanol* and 65 volumes of a buffer prepared by dissolving 0.68 g of *potassium dihydrogen phosphate* and 1.0 g of *sodium octanesulphonate* in 1000.0 ml of *water* to which 1 ml of *triethylamine* is added and the pH of which is adjusted to 2.5 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 266 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency determined from the lamivudine peak is not less than 2000 theoretical plates, the tailing factor for the individual stavudine and lamivudine peaks is not more than 1.5 and the relative standard deviation for replicate injections for each of the peaks corresponding to stavudine and lamivudine is not more than 1.0 per cent.

Inject the reference solution and the test solution and measure the responses for the major peaks due to stavudine and lamivudine.

Calculate the contents of $C_{10}H_{12}N_2O_4$ and $C_8H_{11}N_3O_3S$ in the medium.

Q. Not less than 70 per cent of the stated amounts of $C_{10}H_{12}N_2O_4$ and $C_8H_{11}N_3O_3S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the powdered tablets containing about 100 mg of lamivudine and transfer to a 200-ml volumetric flask. Add about 100 ml of *water*, mix with the aid of ultrasound for 10 minutes with occasional shaking to obtain a uniform dispersion, cool to room temperature, dilute to volume with *water* and mix. Filter through a membrane filter disc with an average pore diameter not greater than 1.0 µm, rejecting the first few ml of the filtrate.

Reference solution (a). Weigh 65 mg of *stavudine IPRS* and 6.5 mg of *thymine IPRS*, and transfer to a 25-ml volumetric flask. Add 5 ml of *methanol*, sonicate to dissolve, dilute to volume with *water* and mix.

Reference solution (b). Weigh 100 mg of *lamivudine IPRS*, transfer to a 200 ml volumetric flask, add about 100 ml of *water* and mix with the aid of ultrasound to dissolve. Add 10 ml of reference solution (a) to this solution and dilute to volume with *water* and mix.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: A. *0.1 M ammonium acetate*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phaseA (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
5	95	5
25	20	80
30	20	80
31	95	5
35	95	5

Inject reference solutions (b) and (c). The test is not valid unless the column efficiency determined from the thymine, stavudine and lamivudine peaks is not less than 3000 theoretical plates and the tailing factor for the same peaks is not more than 2.0.

Inject water and the test solution. Examine the chromatogram obtained with water for any extraneous peaks and ignore the corresponding peaks observed in the chromatogram obtained with the test solution.

Any secondary peak observed in the chromatogram obtained with the test solution corresponding to thymine is not more than 3.0 per cent. Any other secondary peak observed in the chromatogram obtained with the test solution is not more

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than 0.5 per cent and the sum of the areas of all the secondary peaks is not more than 4.0 per cent when calculated by percentage area normalisation.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Transfer a weighed quantity of the powder containing 150 mg of Lamivudine to a 100-ml volumetric flask, add about 20 ml of *methanol* and 50 ml of a mixture of equal volumes of *water* and *methanol*, mix with the aid of ultrasound for 5 minutes with occasional shaking to obtain a uniform dispersion. Cool to room temperature and dilute to volume with the same solvent mixture. Filter the solution through a membrane filter disc with an average pore diameter not greater than 1.0 µm, rejecting the first few ml of the filtrate. Dilute suitably with the same solvent mixture to obtain a solution with a concentration of 0.15 mg per ml of lamivudine.

Reference solution. A similarly prepared solution containing 0.015 per cent w/v of *lamivudine IPRS* and a concentration of *stavudine IPRS* similar to that of the concentration of stavudine in test solution.

Use the chromatographic system described under Dissolution.

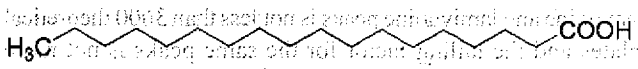
Inject the reference solution. The test is not valid unless the column efficiency determined from the lamivudine peak is not less than 2000 theoretical plates, the tailing factor for the individual peaks due to lamivudine and stavudine is not more than 1.5 and the relative standard deviation for replicate injections for each of the peaks corresponding to stavudine and lamivudine is not more than 2.0 per cent.

Inject separately the reference solution and the test solution and measure the responses for the major peaks.

Calculate the contents of $C_{10}H_{12}N_2O_4$ and $C_8H_{11}N_3O_3S$ in the tablets.

Storage. Store protected from moisture.

Stearic Acid



Stearic Acid consists of a mixture of fatty acids, chiefly stearic acid ($C_{18}H_{36}O_2$) and palmitic acid ($C_{16}H_{32}O_2$). It may contain a suitable antioxidant.

Stearic Acid contains not less than 40.0 per cent of $C_{18}H_{36}O_2$ and the sum of the contents of $C_{18}H_{36}O_2$ and $C_{16}H_{32}O_2$ is not less than 90.0 per cent.

Category. Pharmaceutical aid (tablet lubricant; emulsion adjunct).

Description. A white powder or white, greasy, flaky crystals or hard masses showing signs of crystallisation.

Identification

In the Assay, the chromatogram obtained with the test solution shows two principal peaks which correspond to the two principal peaks in the chromatogram obtained with the reference solution.

Tests

Congealing temperature (2.4.10). Not lower than 54°.

Acid value (2.3.23). 200 to 212, determined on 1.0 g.

Iodine value (2.3.28). Not more than 4.0 *(iodine monochloride method)*.

Mineral acid. Shake 5 g of the melted substance with an equal volume of hot *water* for 2 minutes, cool and filter. To the filtrate add 0.05 ml of *methyl orange solution*; no red colour is produced.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent, determined on 2.0 g.

Assay. Determine by gas chromatography (2.4.13).

Test solution. Dissolve 0.1 g of substance under examination in 5 ml of *boron trifluoride solution* in a conical flask fitted with a reflux condenser. Reflux for 10 minutes, add 4.0 ml of *heptane* through the condenser and boil again for 10 minutes. Allow to cool, add 20 ml of a saturated solution of *sodium chloride*. Shake and allow the layers to separate. Remove about 2 ml of the organic layer and dry it over 0.2 g of *anhydrous sodium sulphate*. Dilute 1.0 ml of the solution to 10.0 ml with *heptane*.

Reference solution. Dissolve 50 mg of *palmitic acid IPRS* and 50 mg of *stearic acid IPRS* in 5 ml of *boron trifluoride solution* in a conical flask fitted with a reflux condenser. Reflux for 10 minutes, add 4.0 ml of *heptane* through the condenser and boil again for 10 minutes. Allow to cool, add 20 ml of a saturated solution of *sodium chloride*. Shake and allow the layers to separate. Remove about 2 ml of the organic layer and dry it over 0.2 g of *anhydrous sodium sulphate*. Dilute 1.0 ml of the solution to 10.0 ml with *heptane*.

Chromatographic system

- a fused-silica column 30 m x 0.32 mm, packed with macrogol 20000 (0.5 µm),
- temperature:
  column 70° from 0 to 2 minutes, 70° to 240° from 2 to 36 minutes and hold at 240° from 36 to 41 minutes,
  inlet port at 220°and detector at 260°,

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– a flame-ionisation detector,
– flow rate: 2.4 ml per minute, using nitrogen as the carrier gas.

The relative retention time with reference to methyl stearate for methyl palmitate is about 0.9.

Inject 1 µl of the reference solution. The test is not valid unless the resolution between the peaks due to methyl palmitate and methyl stearate is not less than 5.0 and the relative standard deviation of methyl stearate and methyl palmitate for replicate injections is not more than 3.0 per cent.

Inject 1 µl of the reference solution and the test solution.

Calculate the content of stearic acid and palmitic acid.

Storage. Store protected from moisture.

Stearyl Alcohol

Stearyl Alcohol is a mixture of solid alcohols consisting chiefly of 1-octadecanol, ($C_{18}H_{38}O$).

Stearyl Alcohol contains not less than 95.0 per cent of $C_{18}H_{38}O$.

Category. Pharmaceutical aid (stiffening agent).

Description. A white, unctuous mass or almost white flakes or granules.

Tests

Appearance of solution. Dissolve 0.5 g in 20 ml of *ethanol (95 per cent)* by heating to boiling and allow to cool. The solution is clear (2.4.1), and not more intensely coloured than reference solution BS6 (2.4.1).

Melting range (2.4.21). 55° to 60°.

Acid value (2.3.23). Not more than 2.0.

Hydroxyl value (2.3.27). 195 to 220.

Iodine value (2.3.28). Not more than 2.0 *(iodine bromide method)*, determined on 2.0 g dissolved in 25 ml of *chloroform*, warming if necessary to effect solution.

Saponification value (2.3.37). Not more than 2.0, determined on 10.0 g.

Assay. Determine by gas chromatography (2.4.13).

Test solution. Dissolve 0.1 g of the substance under examination in 10.0 ml of *ethanol (95 per cent)*.

Reference solution (a). Dissolve 50 mg of *cetyl alcohol* in 10.0 ml of *ethanol (95 per cent)*.

Reference solution (b). Dissolve 50 mg of *stearyl alcohol IPRS* in 5.0 ml of *ethanol (95 per cent)*.

Reference solution (c). To 1.0 ml of reference solution (a), add 1.0 ml of reference solution (b) and dilute to 10.0 ml with *ethanol (95 per cent)*.

Chromatographic system
– a capillary column 30 m x 0.32 mm, packed with poly(dimethyl)siloxane (1 µm),
– temperature:

Column	time (min)	temperature (°)
	0 – 20	150 – 250
	20 – 40	250

– injector port and detector at 250°,
– flame ionization detector,
– flow rate: 1 ml per minute using nitrogen as the carrier gas.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to cetyl alcohol and stearyl alcohol is not less than 5.0.

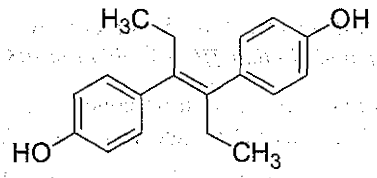
Inject 1 µl of the test solution, reference solution (b) and (c).

Calculate the content of $C_{18}H_{38}O$.

Storage. Store protected from moisture.

Stilboestrol

Diethylstilboestrol



$C_{18}H_{20}O_2$ Mol. Wt. 268.4

Stilboestrol is (*E*)-α,β-diethylstilbene-4,4'-diol.

Stilboestrol contains not less than 97.0 per cent and not more than 101.0 per cent of $C_{18}H_{20}O_2$, calculated on the dried basis.

Category. Oestrogen.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *stilboestrol IPRS* or with the reference spectrum of stilboestrol.

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B. When examined in the range 230 nm to 450 nm (2.4.7), the irradiated solution prepared as directed in the Assay shows absorption maxima at about 292 nm and 418 nm.

C. In the test for Mono- and di-methyl ethers, the principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a).

D. Dissolve 0.5 mg in 0.2 ml of *glacial acetic acid*, add 1 ml of *phosphoric acid* and heat in a water-bath for 3 minutes; a deep yellow colour is produced which almost disappears on dilution with 3 ml of *glacial acetic acid* (distinction from dienoestrol).

Tests

4,4'-Dihydroxystilben and related ethers. Absorbance of a 1.0 per cent w/v solution in *ethanol* at about 325 nm, not more than 0.50 (2.4.7).

Mono- and di-methyl ethers. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 90 volumes of *toluene* and 10 volumes of *diethylamine*.

Test solution. Dissolve 0.5 g of the substance under examination in 100 ml of *ethanol (95 per cent)*.

Reference solution (a). A 0.5 per cent w/v solution of *stilboestrol IPRS in ethanol (95 per cent)*.

Reference solution (b). A 0.05 per cent w/v solution of *stilboestrol monomethyl ether IPRS* in *ethanol (95 per cent)*.

Reference solution (c). A 0.05 per cent w/v solution of *stilboestrol dimethyl ether IPRS* in *ethanol (95 per cent)*.

Reference solution (d). A solution containing 0.25 per cent w/v each of *dienoestrol IPRS* and *stilboestrol IPRS*

Apply to the plate 1 µl of each solution. After development, dry the plate in air, spray with *ethanolic sulphuric acid (20 per cent)* and heat at 120° for 10 minutes. Any secondary spots in the chromatogram obtained with the test solution corresponding to the mono-and di-methyl ethers of stilboestrol are not more intense than the spots in the chromatograms obtained with reference solutions (b) and (c) respectively. Stilboestrol sometimes produces two spots. The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots of approximately the same intensity.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 20 mg in sufficient *ethanol* to produce 100.0 ml and dilute 10.0 ml of the solution to 100.0 ml with *ethanol*. To 25.0 ml of the resulting solution add 25.0 ml of a solution prepared by dissolving 1 g of *dipotassium hydrogen phosphate* in 55 ml of *water*, transfer a portion of the mixture to a 1-cm closed quartz cell, place the cell 10 cm from a 15-watt short-wave, ultraviolet light of mercury lamp and irradiate for 10 minutes. Measure the absorbance of the irradiated solution at the maximum at about 418 nm (2.4.7).

Calculate the content of $C_{18}H_{20}O_2$ from the absorbance obtained by repeating the operation using *stilboestrol IPRS* in place of the substance under examination.

Storage. Store protected from light and moisture.

Stilboestrol Tablets

Diethylstilboestrol Tablets

Stilboestrol Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of stilboestrol, $C_{18}H_{20}O_2$.

Usual strengths. 500 µg; 1 mg; 5 mg; 25 mg.

Identification

A. When examined in the range 230 nm to 450 nm (2.4.7), the irradiated solution prepared as directed in the Assay shows absorption maxima at about 292 nm and 418 nm.

B. Extract a quantity of the powdered tablets containing 3 mg of Stilboestrol with *ether*, filter and evaporate the filtrate to dryness. Dissolve 0.5 mg of the residue in 0.2 ml of *glacial acetic acid*, add 1 ml of *phosphoric acid* and heat in a water-bath for 3 minutes; a deep yellow colour is produced which almost disappears on dilution with 3 ml of *glacial acetic acid* (distinction from dienoestrol).

Tests

Uniformity of content. Complies with the test stated under Tablets.

Finely crush one tablet, add 10 ml of *ethanol*, shake for 30 minutes, add sufficient *ethanol* to produce 25.0 ml and centrifuge. Pipette an aliquot of the supernatant liquid containing 0.5 mg of Stilboestrol add 25.0 ml of a solution prepared by dissolving 1 g of *dipotassium hydrogen phosphate* in 55 ml of *water*, transfer a portion of the mixture to a 1-cm closed quartz cell, place the cell 10 cm from a 15-watt short-wave, ultraviolet light of mercury lamp and irradiate for 10 minutes. Measure the absorbance of the irradiated solution at the maximum at about 418 nm (2.4.7).

Calculate the content of $C_{18}H_{20}O_2$ in the tablet from the absorbance obtained by repeating the operation using *stilboestrol IPRS* in place of the substance under examination.

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Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 5 mg of Stilboestrol, add 5 ml of *ethanol*, shake for 15 minutes, add sufficient *ethanol* to produce 100.0 ml and centrifuge. Dilute 20.0 ml of the clear, supernatant liquid to 50.0 ml with *ethanol* and mix. To 25.0 ml of the resulting solution, add 25.0 ml of a solution prepared by dissolving 1 g of *dipotassium hydrogen phosphate* in 55 ml of *water*, transfer a portion of the mixture to a 1-cm closed quartz cell, place the cell 10 cm from a 15-watt short-wave, ultraviolet light of mercury lamp and irradiate for 10 minutes. Measure the absorbance of the irradiated solution at 418 nm (2.4.7).

Calculate the content of $C_{18}H_{20}O_2$ in the tablet from the absorbance obtained by repeating the operation using *stilboestrol IPRS* in place of the substance under examination.

Storage. Store protected from light and moisture.

Streptokinase

Streptokinase is a preparation of a protein obtained from culture filtrates of certain strains of *Streptococcus haemolyticus* group C. It has the property of combining with human plasminogen to form plasminogen activator and is purified to contain not less than 600 Units of Streptokinase activity per µg of nitrogen before addition of any stabiliser or carrier. It usually contains a buffer and may be stabilised by the addition of suitable substances such as Human Albumin.

Category. Fibrinolytic enzyme.

Description. A white powder or a white, friable solid; hygroscopic.

Identification

A. Place 0.5 ml of citrated human plasma in a haemolysis tube maintained in a water-bath at 37°. Add 0.1 ml of a solution of the substance under examination containing 10,000 Units per ml in *citro-phosphate buffer pH 7.2* and 0.1 ml of a solution of *thrombin* containing 20 Units per ml in *citro-phosphate buffer pH 7.2* and shake immediately; a clot forms and lyses within 30 minutes. Repeat the procedure using citrated bovine plasma; lysis does not occur within 1 hour.

B. Dissolve 0.6 g of *agar* in 50.0 ml of *mixed barbitone buffer pH 8.6*, heating until a clear solution is obtained. Place glass plates (50 mm x 50 mm) that are free from traces of grease on a level surface. Apply to each plate 4 ml of the agar solution and allow to cool until set. Bore a hole 6 mm in diameter in the centre of the agar and an appropriate number of holes (not exceeding six) at distances of 11 mm from the central hole removing the residual agar by means of a cannula connected to a vacuum pump. Place a quantity of 80 µl of goat or rabbit antistreptokinase serum containing 10,000 Units of antistreptokinase activity per ml in the central hole and 80 µl of a solution of the substance under examination containing 125,000 Units of streptokinase activity per ml of each of the surrounding holes. Place the plates in a humidified tank for 24 hours.

Only one precipitation arc is produced which is well-defined and localised between the application point of the serum and each hole containing the solution of the substance under examination.

Tests

pH (2.4.24). 6.8 to 7.5, determined on a solution prepared in carbon dioxide free water containing 5000 Units per ml.

Streptodornase. Introduce 0.5 ml of a 0.1 per cent w/v solution of *sodium deoxyribonucleate* in *imidazole buffer pH 6.5* into each of eight centrifuge tubes. To each of the first two tubes add 0.25 ml of *imidazole buffer pH 6.5* and 0.25 ml of solution of the substance under examination in *imidazole buffer pH 6.5* containing 150,000 Units per ml (solution A) followed immediately by 3.0 ml of *0.25 M perchloric acid*. Mix the contents of each tube, centrifuge for 5 minutes at 3000 rpm and measure the absorbance of each of the supernatant liquids at about 260 nm (2.4.7), using as the blank a mixture of 1.0 ml of *imidazole buffer pH 6.5* and 3.0 ml of *0.25 M perchloric acid*. Calculate the sum of the two absorbances (A_1). To each of the remaining six tubes add, respectively, 0.25, 0.25, 0.125, 0.125, 0 and 0 ml of *imidazole buffer pH 6.5* followed by 0.25 ml of solution A and finally 0, 0, 0.125, 0.125, 0.25 and 0.25 ml respectively of a solution of the Standard Preparation containing 20 Units of streptodornase activity per ml in *imidazole buffer pH 6.5*. [The standard preparation is the Second WHO International standard preparation for streptodornase, established in 2010, consisting of streptodornase supplied in ampoules containing 3200 IU of streptodornase reconstituted with 1 ml of *water*. *NOTE — Current international standard preparation must be used for comparison of the preparation under examination*]. Mix the contents of each tube, incubate at 37° for 15 minutes and add to each tube 3.0 ml of *0.25 M perchloric acid*. Mix the contents of each tube, centrifuge and measure the absorbance of each of the supernatant liquids at about 260 nm (2.4.7), using as the blank the mixture specified above. If the sum of the absorbances of the liquids in the third and fourth tubes is A_2, that of the liquids in the fifth and six tubes is A_3, and that of the liquids in the seventh and eighth tubes is (A_4), ($A_2 - A_1$) is less than $0.5(A_3+A_4)-A_2$.

Streptolysin. Dissolve a quantity of the substance under examination containing 500,000 Units in 0.5 ml of a mixture of 90 volumes of *saline solution* and 10 volumes of *citro-*

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phosphate buffer pH 7.2 in a haemolysis tube. Add 0.4 ml of a 2.3 per cent w/v solution of *sodium thioglycollate* and incubate in a water-bath at 37° for 10 minutes. Add 0.1 ml of a solution of a reference preparation of human antistreptolysin O containing 5 Units per ml and incubate at 37° for 5 minutes. Add 1 ml of *rabbit erythrocyte suspension,* continue the incubation for 30 minutes and centrifuge at about 1000 rpm. The absorbance of the supernatant liquid at about 550 nm (2.4.7), is not more than 1.5 times the absorbance obtained by repeating the above procedure using 0.5 ml of the mixture of saline solution and citro-phosphate buffer pH 7.2 in place of the solution containing the substance under examination.

Loss on drying (2.4.19). Not more than 4.0 per cent, determined on 1.0 g by drying over *phosphorus pentoxide* at a pressure not exceeding 2.7 kPa for 24 hours.

Assay

Potency. The potency of streptokinase is determined by comparing its capacity to activate plasminogen to form plasmin with the same capacity of a reference preparation of streptokinase calibrated in International Units; the formation of plasmin is determined using a suitable chromogenic substrate.

The International Unit is the activity of a stated amount of the International Standard for streptokinase. The equivalence in International Units of the International Standard is stated by the competent authority.

Reference and test solutions. Prepare 2 independent series of 4 dilutions of each of the substance under examination and of the reference preparation of Streptokinase in *tris (hydroxymethyl) aminomethane sodium chloride buffer pH 7.4* in the range of 0.5 to 4.0 IU per ml. Prepare and maintain all solutions at 37°.

Substrate solution. Mix 1.0 ml of *tris (hydroxymethyl) aminomethane buffer pH 7.4* with 1.0 ml of chromophore substrate. Add 5 µl of a 10 per cent w/v solution of *polysorbate 20*. Keep at 37° in a water-bath. Immediately before commencing the activation assay, add 45 µl of a 1 mg per ml solution of human plasminogen.

Analyse each streptokinase dilution, maintained at 37°, in duplicate. Initiate the activation reaction by adding 60 µl of each dilution to 40 µl of substrate solution. For blank wells, use 60 µl of *tris (hydroxymethyl) aminomethane sodium chloride buffer solution pH 7.4* instead of the reference and test solutions. Allow the reaction to proceed at 37° for 20 minutes and read the absorbance at 405 nm. If a suitable thermostated plate reader is available, this may be used to monitor the reaction. Alternatively, it may be necessary to stop the reaction after 20 minutes using 50 µl of a 50 per cent v/v solution of *glacial acetic acid*. Best results are obtained when the absorbance for the highest streptokinase concentration is between 0.1 and 0.2 (after blank subtraction). If necessary, adjust the time of incubation in order to reach this range of absorbances.

Calculate the regression of the absorbance on log concentrations of the solutions of the substance under examination and of the reference preparation of streptokinase and calculate the potency of the substance under examination using the usual statistical methods for parallel-line assays.

The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits (P = 0.95) of the estimated potency are not less than 80 per cent and not more than 125 per cent of the stated potency.

Streptokinase intended for use in the manufacture of parenteral preparations complies with the following additional requirements.

Abnormal toxicity (2.2.1). Determine by Method A, using a solution containing 50,000 Units in 0.5 ml of *water for injections* administered in 15 to 20 seconds.

Bacterial endotoxins (2.2.3). Dissolve the contents of the sealed container in *water BET* to give a solution containing 10,000 Units of Streptokinase per ml. Carry out the test on the resulting solution; the maximum allowable endotoxin concentration of the solution is 23.33 Units of endotoxin per ml. Carry out the test using the maximum valid dilution of the prepared solution calculated from the declared sensitivity of the lysate used in the test.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store in sealed containers, protected from light. The containers should be sterile, tamper-evident and sealed so as to exclude micro-organisms. Under these conditions the contents may be expected to retain their potency for 2 years.

Labelling. The label states (1) the number of Units of streptokinase activity in the container; (2) the number of Units of streptokinase activity per mg, calculated with reference to the dried preparation; (3) the name and quantity of any added substances; (4) the storage conditions; (5) whether or not it is intended for use in the manufacture of parenteral preparations.

Streptokinase Injection

Streptokinase Injection is a sterile material consisting of Streptokinase with or without auxiliary agents. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

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The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Usual strengths. 50,000 Units; 100,000 Units; 750,000 Units; 1,500,000 Units.

Identification

A. Place 0.5 ml of citrated human plasma in a haemolysis tube maintained in a water-bath at 37°. Add 0.1 ml of a solution of the contents of the container containing 10,000 Units per ml in *citro-phosphate buffer pH 7.2* and 0.1 ml of a solution of *thrombin* containing 20 Units per ml in *citro-phosphate buffer pH 7.2* and shake immediately; a clot forms and lyses within 30 minutes. Repeat the procedure using citrated bovine plasma; lysis does not occur within 1 hour.

B. Dissolve 0.6 g of *agar* in 50.0 ml of *mixed barbitone buffer pH 8.6*, heating until a clear solution is obtained. Place glass plates (50 mm x 50 mm) that are free from traces of grease on a level surface. Apply to each plate 4 ml of the agar solution and allow to cool until set. Bore a hole 6 mm in diameter in the centre of the agar and an appropriate number of holes (not exceeding six) at distances of 11 mm from the central hole removing the residual agar by means of a cannula connected to a vacuum pump. Place a quantity of 80 µl of goat or rabbit antistreptokinase serum containing 10,000 Units of antistreptokinase activity per ml in the central hole and 80 µl of a solution of the contents of the container containing 125,000 Units of streptokinase activity per ml of each of the surrounding holes. Place the plates in a humidified tank for 24 hours.

Only one precipitation arc is produced which is well-defined and localised between the application point of the serum and each hole containing the solution of the substance under examination.

Tests

NOTE — Tests for Streptolysin and Streptodornase may be omitted if the Streptokinase injection is prepared from Streptokinase complied as per monograph in current edition of IP.

pH (2.4.24). 6.8 to 7.5, determined on a freshly constituted injection containing 5000 Units per ml, using carbon dioxide free water.

Streptodornase. Introduce 0.5 ml of a 0.1 per cent w/v solution of *sodium deoxyribonucleate* in *imidazole buffer pH 6.5* into each of eight centrifuge tubes. To each of the first two tubes add 0.25 ml of *imidazole buffer pH 6.5* and 0.25 ml of solution of the contents of the container with the substance under examination in *imidazole buffer pH 6.5* containing 150,000 Units per ml (solution A) followed immediately by 3.0 ml of *0.25 M perchloric acid*. Mix the contents of each tube, centrifuge for 5 minutes at 3000 rpm and measure the absorbance of each of the supernatant liquids at about 260 nm (2.4.7), using as the blank a mixture of 1.0 ml of *imidazole buffer pH 6.5* and 3.0 ml of *0.25 M perchloric acid*. Calculate the sum of the two absorbances (A_1). To each of the remaining six tubes add, respectively, 0.25, 0.25, 0.125, 0.125, 0 and 0 ml of *imidazole buffer pH 6.5* followed by 0.25 ml of solution A and finally 0, 0, 0.125, 0.125, 0.25 and 0.25 ml respectively of a solution of the Standard Preparation containing 20 Units of streptodornase activity per ml in *imidazole buffer pH 6.5*. [The standard preparation is the 2nd WHO International standard preparation for streptodornase, established in 2010, consisting of streptodornase supplied in ampoules containing 3200 IU of streptodornase reconstituted with 1 ml of *water*. *NOTE — Current international standard preparation must be used for comparison of the preparation under examination*]. Mix the contents of each tube, incubate at 37° for 15 minutes and add to each tube 3.0 ml of *0.25 M perchloric acid*. Mix the contents of each tube, centrifuge and measure the absorbance of each of the supernatant liquids at about 260 nm (2.4.7), using as the blank the mixture specified above. If the sum of the absorbances of the liquids in the third and fourth tubes is A_2, that of the liquids in the fifth and six tubes is A_3, and that of the liquids in the seventh and eighth tubes is (A_4), ($A_2 - A_1$) is less than $0.5(A_3+A_4)-A_2$.

Streptolysin. Dissolve a quantity of the contents of the container containing 500,000 Units in 0.5 ml of a mixture of 90 volumes of *saline solution* and 10 volumes of *citro-phosphate buffer pH 7.2* in a haemolysis tube. Add 0.4 ml of a 2.3 per cent w/v solution of *sodium thioglycollate* and incubate in a water-bath at 37° for 10 minutes. Add 0.1 ml of a solution of a reference preparation of human antistreptolysin O containing 5 Units per ml and incubate at 37° for 5 minutes. Add 1 ml of *rabbit erythrocyte suspension*, continue the incubation for 30 minutes and centrifuge at about 1000 rpm. The absorbance of the supernatant liquid at about 550 nm (2.4.7), is not more than 1.5 times the absorbance obtained by repeating the above procedure using 0.5 ml of the mixture of saline solution and citro-phosphate buffer pH 7.2 in place of the solution containing the substance under examination.

Bacterial endotoxins (2.2.3). Dissolve the contents of the sealed container in *water BET* to give a solution containing 10,000 Units of Streptokinase per ml. Carry out the test on the

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resulting solution; the maximum allowable endotoxin concentration of the solution is 23.33 Units of endotoxin per ml. Carry out the test using the maximum valid dilution of the prepared solution calculated from the declared sensitively of the lysate used in the test.

Abnormal toxicity (2.2.1). Determine by using a solution containing 50,000 Units in 0.5 ml of water for injection administered in 15 to 20 minutes.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay

Potency. The potency of streptokinase is determined by comparing its capacity to activate plasminogen to form plasmin with the same capacity of a reference preparation of streptokinase calibrated in International Units; the formation of plasmin is determined using a suitable chromogenic substrate.

The International Unit is the activity of a stated amount of the International Standard for streptokinase. The equivalence in International Units of the International Standard is stated by the competent authority.

Reference and test solutions. Prepare 2 independent series of 4 dilutions of each of the substance under examination and of the reference preparation of Streptokinase in *tris (hydroxymethyl) aminomethane sodium chloride buffer pH 7.4* in the range of 0.5 to 4.0 IU per ml. Prepare and maintain all solutions at 37°.

Substrate solution. Mix 1.0 ml of *tris (hydroxymethyl) aminomethane buffer pH 7.4* with 1.0 ml of chromophore substrate. Add 5 µl of a 10 per cent w/v solution of *polysorbate 20*. Keep at 37° in a water-bath. Immediately before commencing the activation assay, add 45 µl of a 1 mg per ml solution of human plasminogen.

Analyse each streptokinase dilution, maintained at 37°, in duplicate. Initiate the activation reaction by adding 60 µl of each dilution to 40 µl of substrate solution. For blank wells, use 60 µl of *tris (hydroxymethyl) aminomethane sodium chloride buffer solution pH 7.4* instead of the reference and test solutions. Allow the reaction to proceed at 37° for 20 minutes and read the absorbance at 405 nm. If a suitable thermostated plate reader is available, this may be used to monitor the reaction. Alternatively, it may be necessary to stop the reaction after 20 minutes using 50 µl of a 50 per cent v/v solution of *glacial acetic acid*. Best results are obtained when the absorbance for the highest streptokinase concentration is between 0.1 and 0.2 (after blank subtraction). If necessary, adjust the time of incubation in order to reach this range of absorbances.

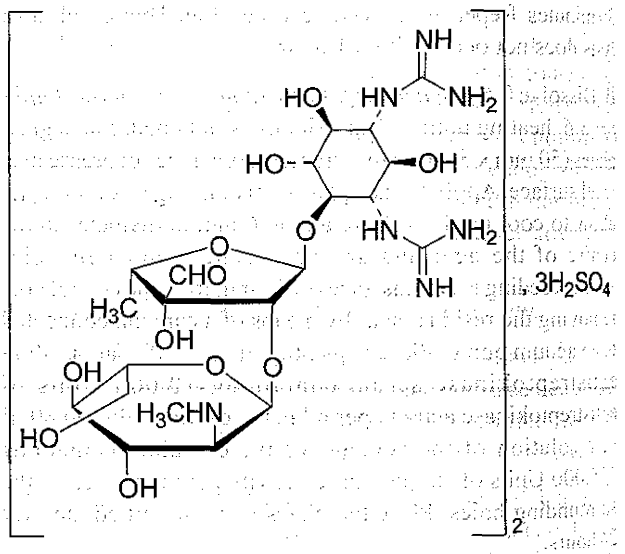
Calculate the regression of the absorbance on log concentrations of the solutions of the substance under examination and of the reference preparation of streptokinase and calculate the potency of the substance under examination using the usual statistical methods for parallel-line assays.

The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits (P = 0.95) of the estimated potency are not less than 80 per cent and not more than 125 per cent of the stated potency.

Storage. Store in sealed containers, protected from light in a refrigerator (2° to 8°). The containers should be sterile and sealed so as to exclude micro-organisms. Under these conditions the contents may be expected to retain their potency for 2 years.

Labelling. The label states the total number of Units of streptokinase activity contained in it.

Streptomycin Sulphate



$(C_{21}H_{39}N_7O_{12})_2,3H_2SO_4$ Mol. Wt. 1457.4

Streptomycin Sulphate is the sulphate of *O*-2-deoxy-2-methylamino-α-L-glucopyranosyl-(1→ 2)-*O*-5-deoxy-3-*C*-formyl-α-L-lyxofuranosyl-(1→ 4)-*N*^{1},*N*^{3}-diamidino-D-streptamine, a substance produced by the growth of certain strains of *Streptomyces griseus* or obtained by any other means.

Streptomycin Sulphate has a potency equivalent to not less than 720 µg of streptomycin per mg, calculated on the dried basis.

Category. Antitubercular.

Description. A white or almost white powder; hygroscopic.

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Identification

A. Determine by thin-layer chromatography (2.4.17). Prepare the plate by mixing 0.3 g of *carbomer* with 240 ml of *water*, allowing to stand with moderate stirring for 1 hour, adjusted to pH 7.0 by the gradual addition with constant shaking of *2 M sodium hydroxide* and adding 30 g of *silica gel H*. Spread a uniform layer of the resulting suspension 0.75 mm thick. Heat the plate at 110° for 1 hour, allow to cool and use immediately.

Mobile phase. A 7 per cent w/v solution of *potassium dihydrogen phosphate*.

Test solution. Dissolve 0.1 g of the substance under examination in 100 ml of *water*.

Reference solution (a). A 0.1 per cent w/v of *streptomycin sulphate IPRS* in *water*.

Reference solution (b). A solution containing 0.1 per cent w/v of *streptomycin sulphate IPRS*, 0.1 per cent w/v of *neomycin sulphate IPRS* and 0.1 per cent w/v of *kanamycin monosulphate IPRS* in *water*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in warm air, spray with a mixture of equal volumes of a 0.2 per cent w/v solution of *naphthalene-1,3-diol* in *ethanol (95 per cent)* and *sulphuric acid (45 per cent)* and heat at 150° for 5 to 10 minutes. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

B. Dissolve 5 to 10 mg in 4 ml of *water* and add 1 ml of *1 M sodium hydroxide*. Heat in a water-bath for 4 minutes. Add a slight excess of *2 M hydrochloric acid* and 0.1 ml of a 10 per cent w/v solution of *ferric chloride*; a violet colour is produced.

C. Dissolve 0.1 g in 2 ml of *water* and add 1 ml of *dilute 1-naphthol solution* and 2 ml of a mixture of equal volumes of *dilute sodium hypochlorite solution* and *water*; a red colour is produced.

D. Dissolve 10 mg in 5 ml of water and add 1 ml of *1 M hydrochloric acid*. Heat in a water-bath for 2 minutes. Add 2 ml of a 0.5 per cent w/v solution of *1-naphthol* in *1 M sodium hydroxide* and heat in a water-bath for 1 minute; a faint yellow colour is produced.

E. It gives the reactions of sulphates (2.3.1).

Tests

Appearance of solution. A 25.0 per cent w/v solution in *carbon dioxide-free water* is not more intensely coloured than degree 3 of the appropriate range of reference solutions (2.4.1). The solution, after standing protected from light at a temperature of about 20° for 24 hours, is not more opalescent than opalescence standard OS2 (2.4.1).

pH (2.4.24). 4.5 to 7.0, determined in a 25.0 per cent w/v solution.

Sulphates. 18.0 to 21.5 per cent, calculated on the dried basis, when determined by the following method. Dissolve 0.25 g in 100 ml of *water*, adjusted to pH 11 with *strong ammonia solution* and add 10.0 ml of *0.1 M barium chloride* and 0.5 mg of *metalphthalein*. Titrate the excess of barium chloride with *0.1 M disodium edetate*, adding 50 ml of *ethanol (95 per cent)* when the colour of the solution begins to change and continuing the titration until the violet-blue colour disappears.

1 ml of *0.1 M barium chloride* is equivalent to 0.009606 g of sulphate, SO_4.

Colorimetric test. Dissolve 0.1 g in sufficient *water* to produce 100 ml. To 5 ml, add 5 ml of *0.2 M sodium hydroxide* and heat in a water-bath for exactly 10 minutes. Cool in ice for exactly 5 minutes, add 3 ml of a 1.5 per cent w/v solution of *ferric ammonium sulphate* in *0.25 M sulphuric acid* and sufficient *water* to produce 25 ml and mix. Exactly 20 minutes after the addition of the *ferric ammonium sulphate solution*, measure the absorbance of a 2-cm layer of the solution at the maximum at about 525 nm (2.4.7), using as the blank a solution prepared in the same manner but omitting the substance under examination. The absorbance is not less than 90.0 per cent of that obtained by carrying out the procedure at the same time and in the same manner using *streptomycin sulphate IPRS* in place of the substance under examination, each absorbance being calculated on the dried basis.

Streptomycin B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 50 volumes of *toluene*, 25 volumes of *glacial acetic acid* and 25 volumes of *methanol*.

Test solution. Dissolve 0.2 g of the substance under examination in 5 ml of a freshly prepared mixture of 97 volumes of *methanol* and 3 volumes of *sulphuric acid*, heat under a reflux condenser for 1 hour, cool, wash down the condenser with *methanol* and add sufficient *methanol* to produce 20 ml.

Reference solution. Dissolve 36 mg of *D-mannose* in 5 ml of a freshly prepared mixture of 97 volumes of *methanol* and 3 volumes of *sulphuric acid*, heat under a reflux condenser for 1 hour, cool, wash down the condenser with *methanol* and add sufficient *methanol* to produce 50 ml. Dilute 5 ml of the resulting solution to 50 ml with *methanol*; this solution contains the equivalent of 0.03 per cent w/v of streptomycin B (1 mg of D-mannose is equivalent to 4.13 mg of streptomycin B).

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 13 to 15 cm. Dry the plate in air, and spray with a

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freshly prepared mixture of equal volumes of a 0.2 per cent w/v solution of *naphthalene-1,3-diol* in *ethanol (95 per cent)* and a 20 per cent v/v solution of *sulphuric acid* and heat at 110° for 5 minutes. Any spot in the chromatogram obtained with the test solution is not more intense than the corresponding spot in the chromatogram with the reference solution.

Methanol. Determine by gas chromatography (2.4.13).

Test solution. A 4 per cent w/v solution of the substance under examination in *water*.

Reference solution. A 0.012 per cent w/v solution of *methanol* in *water*.

Chromatographic system

- a glass column 1.5 to 2.0 m × 2 to 4 mm, packed with ethylvinylbenzene-divinylbenzene copolymer (150 to 180 µm) porous polymer beads (Such as Porapak Q),
- temperature: column 120° to 140°,
  inlet port and detector at least 50° higher than that of the column,
- flow rate: 30 to 40 ml per minute, using nitrogen as the carrier gas.

The area of any peak corresponding to methanol in the chromatogram obtained with the test solution is not more than that of the peak in the chromatogram obtained with reference solution (0.3 per cent).

Sulphated ash (2.3.18). Not more than 1.0 per cent.

Loss on drying (2.4.19). Not more than 7.0 per cent, determined on 1.0 g by drying over *phosphorus pentoxide* at 60° at a pressure not exceeding 0.1 kPa for 24 hours.

Assay. Determine by the microbiological assay of antibiotics, Method A or B (2.2.10), and express the results in µg of streptomycin per mg.

Streptomycin Sulphate intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.25 Endotoxin Unit per mg of streptomycin.

Streptomycin Sulphate intended for use in the manufacture of parenteral preparations without a further appropriate sterilisation procedure complies with the following additional requirement.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store protected from light and moisture. If it is intended for use in the manufacture of parenteral preparations the container should be sterile and sealed so as to exclude micro-organisms.

Labelling. The label states (1) the equivalent weight of streptomycin contained in it; (2) whether or not the contents are intended for use in the manufacture of parenteral preparations; (3) the name and quantity of any added stabiliser.

Streptomycin Injection

Streptomycin Sulphate Injection

Streptomycin Injection is a sterile material consisting of Streptomycin Sulphate with or without auxiliary agents. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Streptomycin Injection contains not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of streptomycin, $C_{21}H_{39}N_7O_{12}$.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Usual strengths. The equivalent of 750 mg and 1 g of streptomycin.

Description. A white or almost white powder which yields a clear, colourless or faintly yellow coloured solution when dissolved in water.

Identification

A. Determine by thin-layer chromatography (2.4.17). Prepare the plate by mixing 0.3 g of *carbomer* with 240 ml of *water*, allowing to stand with moderate stirring for 1 hour, adjusted to pH 7.0 by the gradual addition with constant shaking of 2 *M sodium hydroxide* and adding 30 g of *silica gel H*. Spread a uniform layer of the resulting suspension 0.75 mm thick. Heat the plate at 110° for 1 hour, allow to cool and use immediately.

Mobile phase. A 7 per cent w/v solution of *potassium dihydrogen phosphate*.

Test solution. Dissolve 0.1 g of the substance under examination in 100 ml of *water*.

Reference solution (a). A 0.1 per cent w/v of *streptomycin sulphate IPRS* in *water*.

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Reference solution (b). A solution containing 0.1 per cent w/v of *streptomycin sulphate IPRS*, 0.1 per cent w/v of *neomycin sulphate IPRS* and 0.1 per cent w/v of *kanamycin monosulphate IPRS* in *water*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in warm air, spray with a mixture of equal volumes of a 0.2 per cent w/v solution of *naphthalene-1,3-diol* in *ethanol (95 per cent)* and *sulphuric acid (45 per cent)* and heat at 150° for 5 to 10 minutes. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

B. Dissolve 5 to 10 mg in 4 ml of *water* and add 1 ml of *1 M sodium hydroxide*. Heat in a water-bath for 4 minutes. Add a slight excess of *2 M hydrochloric acid* and 0.1 ml of a 10 per cent w/v solution of *ferric chloride*; a violet colour is produced.

C. Dissolve 0.1 g in 2 ml of *water* and add 1 ml of *dilute 1-naphthol solution* and 2 ml of a mixture of equal volumes of *dilute sodium hypochlorite solution* and *water*; a red colour is produced.

D. Dissolve 10 mg in 5 ml of water and add 1 ml of *1 M hydrochloric acid*. Heat in a water-bath for 2 minutes. Add 2 ml of a 0.5 per cent w/v solution of *1-naphthol* in *1 M sodium hydroxide* and heat in a water-bath for 1 minute; a faint yellow colour is produced.

Tests

pH (2.4.24). 4.5 to 7.0, determined in a 25.0 per cent w/v solution.

Loss on drying (2.4.19). Not more than 7.0 per cent, determined on 1.0 g by drying in an oven at 60° over *phosphorus pentoxide* at a pressure not exceeding 0.1 kPa for 24 hours.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine on the mixed contents of ten containers by the microbiological assay of antibiotics, Method A or B (2.2.10).

Bacterial endotoxins (2.2.3): Not more than 0.25 Endotoxin Unit per mg of streptomycin.

Storage. Store protected from moisture.

Labelling. The label states the strength in terms of the equivalent amount of streptomycin.

Streptomycin Tablets

Streptomycin Sulphate Tablets

Streptomycin Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of streptomycin, $C_{21}H_{39}N_7O_{12}$.

Usual strength. The equivalent of 500 mg of streptomycin.

Identification

A. Triturate a quantity of the powdered tablets containing 0.2 g of streptomycin in a mixture of 2 ml of *methanol* and 0.1 ml of *sulphuric acid*, filter, if necessary, and allow to stand at about 25°; crystals of streptidine sulphate separate in the course of 2 to 3 days. Dissolve the crystals in a solution of 0.1 g of *picric acid* in 10 ml of hot *water* and cool; the precipitate, after recrystallisation from hot *water*, melts at about 283° (2.4.21).

B. Boil a small quantity of the powdered tablets containing 0.1 g of streptomycin with 5 ml of *1 M sodium hydroxide* for a few minutes, add a slight excess of *2 M hydrochloric acid* and 0.15 ml of a 10 per cent w/v solution of *ferric chloride*; a brilliant violet colour is produced.

Tests

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 0.3 g of streptomycin and, triturate with 20 ml of *buffer solution pH 8.0*. Dilute to 100.0 ml with *water*.

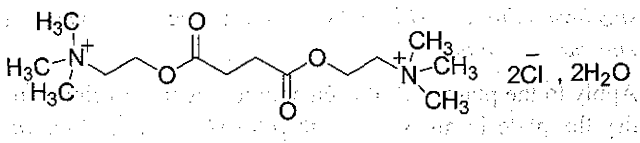
Determine by the microbiological assay of antibiotics, Method A or B (2.2.10).

Storage. Store protected from moisture.

Labelling. The label states the strength in terms of the equivalent amount of streptomycin.

Succinylcholine Chloride

Suxamethonium Chloride



$C_{14}H_{30}Cl_2N_2O_4,2H_2O$ Mol. Wt. 397.3

Succinylcholine Chloride is 2,2'-succinyldioxybis(ethyltrimethylammonium) dichloride dihydrate.

Succinylcholine Chloride contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{14}H_{30}Cl_2N_2O_4$, calculated on the anhydrous basis.

Category. Depolarising skeletal muscle relaxant.

Description. A white or almost white, crystalline powder; hygroscopic.

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Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *succinylcholine chloride IPRS* or with the reference spectrum of succinylcholine chloride.

B. Dissolve about 25 mg in 1 ml of *water*, add 0.1 ml of a 1 per cent w/v solution of *cobalt chloride* and 0.1 ml of *potassium ferrocyanide solution*; a green colour is produced.

C. Dissolve 1 g in sufficient *carbon dioxide-free water* to produce 20 ml. To 1 ml of the solution add 9 ml of *water*, 10 ml of *1 M sulphuric acid* and 30 ml of *ammonium reineckate solution*; a pink precipitate is produced. Allow to stand for 30 minutes, filter and wash with *water*, then with *ethanol (95 per cent)* and finally with *ether*. The residue, after drying at 80° melts at 180° to 185° (2.4.21).

D. It gives the reactions of chlorides (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution in *carbon dioxide-free water* (solution A) is clear (2.4.1). 4 ml of solution A diluted to 10 ml with *water* is colourless (2.4.1).

pH (2,4,24). 4.0 to 5.0, determined in a 0.5 per cent w/v solution.

Choline chloride. Determine by thin-layer chromatography (2.4.17), coating the plate with *microcrystalline cellulose*.

Mobile phase. A mixture of 50 volumes of *1-butanol*, 40 volumes of *water* and 10 volumes of *anhydrous formic acid*, shake for 10 minutes, allow to separate. Use the upper layer as the mobile phase.

Test solution. Dissolve 0.4 g of the substance under examination in 10 ml of *methanol*.

Reference solution. A solution containing 4 per cent w/v of *succinylcholine chloride IPRS* and 0.02 per cent w/v of *choline chloride* in *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air, spray with *potassium iodobismuthate solution*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot due to choline chloride in the chromatogram obtained with the reference solution. The test is not valid unless the chromatogram obtained with the reference solution shows two clearly separated spots.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). 8.0 to 10.0 per cent, determined on 0.3 g.

Assay. Dissolve 0.3 g in 30 ml of *anhydrous glacial acetic acid*, add 30 ml of *acetic anhydride* and 10 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator, until a bluish green colour is produced. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01807 g of $C_{14}H_{30}Cl_2N_2O_4$.

Storage. Store protected from light and moisture.

Succinylcholine Injection

Suxamethonium Chloride Injection

Succinylcholine Injection is a sterile solution of Succinylcholine Chloride in Water for Injections.

Succinylcholine Injection contains not less than 90.0 per cent and not more than 107.5 per cent of the stated amount of succinylcholine chloride, $C_{14}H_{30}Cl_2N_2O_4,2H_2O$.

Usual strength. 50 mg per ml.

Identification

Dilute a volume containing 20 mg of Succinylcholine Chloride to 50 ml with *water*. To 0.5 ml add 2 ml of *chloroform*, 2 ml of a solution containing 0.16 per cent w/v of *citric acid* and 6.6 per cent w/v of *disodium hydrogen phosphate* and 0.1 ml of a solution containing 0.15 per cent w/v of each of *bromothymol blue* and *anhydrous sodium carbonate*. Shake for 2 minutes and allow to separate. The chloroform layer is yellow.

Tests

pH (2.4.28). 3.0 to 5.0.

Hydrolysis products. The volume of *0.1 M sodium hydroxide* required for the preliminary neutralisation in the Assay is not more than one tenth of the total volume of *0.1 M sodium hydroxide* required for the preliminary neutralisation and the hydrolysis.

Bacterial endotoxins (2.2.3). Not more than 2.0 Endotoxin Units per mg of succinylcholine chloride.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. To a measured volume containing about 0.25 g of Succinylcholine Chloride add 30 ml of *carbon dioxide-free water* and shake with five quantities, each of 25 ml, of *ether*. Wash the combined ether solutions with two quantities, each of 10 ml, of *water* and discard the ether. Shake the combined washings with two quantities, each of 10 ml, of *ether*, add the washings to the original aqueous solution and neutralise with *0.1 M sodium hydroxide* using *bromothymol blue solution* as indicator. Add 25.0 ml of *0.1 M sodium hydroxide*, heat

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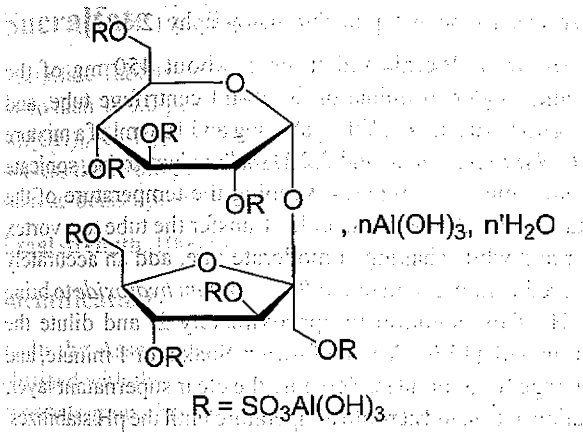
under a reflux condenser for 40 minutes, allow to cool and titrate the excess of alkali with *0.1 M hydrochloric acid* using *bromothymol blue solution* as indicator. Repeat the operation using 40 ml of *carbon dioxide-free water* beginning at the words "Add 25.0 ml of *0.1 M sodium hydroxide*.....". The difference between the titrations represents the amount of sodium hydroxide required.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01987 g of $C_{14}H_{30}Cl_2N_2O_4,2H_2O$.

Storage. Store protected from light, at a temperature between 2° to 8°. The injection should not be allowed to freeze.

Sucralfate



Sucralfate is $Al_8(OH)_{16}(C_{12}H_{14}O_{35}S_8)[Al(OH)_3]_n[H_2O]_{n'}$ in which n = 8 to 10, and n' = 22 to 31. α-D-Glucopyranoside, β-D-fructofuranosyl-, octakis (hydrogensulphate), aluminium complex.

Sucralfate is the hydrous basic aluminium salt of sucrose octasulfate. It contains the equivalent of not less than 30.0 per cent and not more than 38.0 per cent of sucrose octasulfate, $C_{12}H_{14}O_{35}S_8$.

Category. Antiulcer.

Description. A white or almost white, amorphous powder.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Dissolve 500 mg of the substance under examination in 100.0 ml of *0.1 M hydrochloric acid*, and boil the mixture gently, with stirring, for 20 minutes until the sample is completely dissolved. Neutralize with *0.1 M sodium hydroxide*, and allow to cool. Add 4 ml of *alkaline cupric tartrate solution*. Boil a small amount of the solution: a red copious precipitate of cuprous oxide is produced.

C. A solution in *3 M hydrochloric acid* gives the reactions of aluminium salts (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution in *1 M sulphuric acid* is clear and colourless (2.4.1).

Neutralising capacity. Weigh and transfer 0.25 g of the substance under examination in 250 ml screw-capped bottle, add 100.0 ml of *0.1 M hydrochloric acid* previously heated to 37°, cap the bottle and place in water-bath, maintaining the temperature at 37° in water-bath, and stir continuously for 1 hour. Cool to room temperature and transfer 20.0 ml of this resulting solution to a 100-ml beaker add 30 ml of *water* and titrate with *0.1 M sodium hydroxide* to a pH of 3.5. Carry out a blank titration using a mixture of 30 ml of *water* and 20.0 ml of *0.1 M hydrochloric acid*.

Calculate the mEq of acid consumed per g of sucralfate taken by the formula:

$$\frac{5M(V_B - V_T)}{W}$$

Where M is the exact molarity of the *0.1 M sodium hydroxide*, V_B and V_T are the volumes, in ml of *0.1 M sodium hydroxide* consumed by the blank and the test solution, respectively; and W is the weight, in g, of sample taken.

Not less than 12.0 mEq of *acid* is consumed.

Chloride (2.3.12). Transfer 0.5 g to a 100.0 ml volumetric flask, add 30 ml of *2 M nitric acid*, dissolve and dilute to volume with *water*. 10.0 ml of the solution complies with the limit test for chlorides (0.5 per cent).

Arsenic (2.3.10). Dissolve 2.5 g in 15 ml of *brominated hydrochloric acid*, add 45 ml of *water* and remove excess of *bromine* with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit for arsenic (4 ppm).

Pyridine and 2-methylpyridine. Not more than 0.05 per cent each of pyridine and 2-methylpyridine, determine by gas chromatography (2.4.13).

Internal standard solution. Dilute 1.0 ml of *3-methylpyridine* into a 50.0 ml volumetric flask with *chloroform* and mix. Further dilute 1.0 ml of the solution to 50.0 ml with *chloroform*.

Test solution. Disperse 1.0 g of the substance under examination in 10.0 ml of *1 M sodium hydroxide* with the aid of ultrasound until a uniformly cloudy mixture is obtained, extract

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with three 5 ml quantities of *chloroform* and collect the chloroform layer in 20.0 ml volumetric flask, add 1.0 ml of internal standard solution , dilute to volume with *chloroform* and mix.

Reference solution (a). Weigh and dissolve 0.5 g of each of, *2-methylpyridine* and *pyridine* in sufficient *chloroform* to produce 50.0 ml. Dilute 5.0 ml of the solution to 50.0 ml with *chloroform*. Further dilute 5.0 ml of this resulting solution to 50.0 ml with *chloroform* and mix.

Reference solution (b). Dilute 5.0 ml of reference solution (a), add 1.0 ml of internal standard solution to 20.0 ml with *chloroform*.

Chromatographic system
- a capillary column 10 m x 0.53 mm, coated with 5 per cent phenyl and 95 per cent methylpolysiloxane (film thickness 2.65 µm),
- column temperature: 50°,
- injection port at 150° and detector at 200°,
- flame ionization detector,
- pressure: 0.7 psi using helium or nitrogen as carrier gas
- injection volume: 1 µl.

Inject reference solution (b). The relative retention times for pyridine, 2-methylpyridine and 3-methylpyridine are about 0.42, 0.72 and 1.0 respectively. The test is not valid unless the resolution between the peaks due to pyridine and 2-methylpyridine is not less than 3.5 and for the peaks due to 2-methylpyridine and 3-methylpyridine is not less than 2.5. The relative standard deviation for the ratio of the area of analyte peak to internal standard for replicate injections is not more than 2.0 per cent.

Inject reference solution (b) and the test solution. Calculate the content of pyridine and 2-methylpyridine from the peak response ratios of pyridine to the internal standard and 2-methylpyridine to internal standard obtained with reference solution (b) and the test solution respectively.

Calculate the content of pyridine and 2-methylpyridine.

Sucrose Heptasulphate: Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Mobile phase. A buffer solution prepared by dissolving 99.1 g of *ammonium sulphate* in 900 ml of *water*, dilute to 1000 ml with *water* and adjusted to pH 3.5 with *orthophosphoric acid.*

Inject the test solution. The relative retention time for sucrose heptasulphate and sucrose octasulfate is 0.6 and 1.0 respectively. The ratio of the peak response of the sucrose heptasulphate peak to that of the sucrose octasulfate peak is not more than 0.1.

Aluminum: 15.5 to 18.5 per cent of aluminium calculated on as is basis and determined by the following method.

Dissolve about 1.0 g of the substance under examination accurately weighed in a 250.0 ml volumetric flask, add 10 ml of *6.0 M hydrochloric acid*, mix, and heat with continuous stirring in a water-bath at 70° for 5 minutes. Cool to room temperature, dilute to volume with *water* and mix. Filter the solution, discarding the first portion of the filtrate. Transfer 25.0 ml of the filtrate to a 250-ml beaker, add 25.0 ml of *0.05 M disodium edetate disodium*, add 20 ml of *acetic acid-ammonium acetate buffer* and mix. Heat in a water-bath at 70° for 5 minutes. Cool to room temperature add 50 ml of *ethanol (95 per cent)* and 2 ml of *dithizone solution* and mix. Titrate with *0.05 M zinc sulphate* to a bright rose-pink colour. Carry out a blank titration.

1 ml of 0.05 M *disodium edetate* is equivalent to 0.001349 g of aluminium.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and transfer about 450 mg of the substance under examination in 50-ml centrifuge tube, and shake on a vortex mixer. While shaking add 10.0 ml of a mixture of *2.0 M sulphuric acid* and *2.2 M sodium hydroxide*, sonicate with swirling for 5 minutes, keeping the temperature of the mixture below 30°. Without delay transfer the tube to a vortex mixer and while shaking at moderate rate, add an accurately measured volume, V, in ml, of *0.1 M sodium hydroxide* to bring the pH of the solution to approximately 2, and dilute the solution with (15.0 – V) ml of *water*. Shake for 1 minute, and centrifuge for 5 minutes. Separate the clear supernatant layer, and allow it to stand at room temperature until the pH stabilizes. If the pH is not between 2.3 and 3.5, repeat the test using a different volume of *0.1 M sodium hydroxide*.

Reference solution. A 1.0 per cent w/v solution of *anhydrous potassium sucrose octasulphate IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm, packed with aminopropylsilane bonded to porous silica (5 µm),
- mobile phase: a buffer solution prepared by dissolving 132 g of *ammonium sulphate* in 900 ml of *water*, dilute to 1000 ml with *water* and adjusted to pH 3.5 with *orthophosphoric acid,*
- flow rate: 1 ml per minute,
- refractive index detector maintained at 30°,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the column efficiency due to sucrose octasulfate peak is not less than 400 theoretical plates, the tailing factor is not more than 4.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{12}H_{14}O_{35}S_8$ as

$$\frac{974.75}{1287.53} \times 25C \times \frac{r_u}{r_s}$$

where 974.75 and 1287.53 are the molecular weight of sucrose octasulfate and anhydrous potassium sucrose octasulfate, respectively, C is the concentration in mg per ml of anhydrous potassium sucrose octasulfate in reference solution and r_u and r_s are the peak responses of sucrose octasulfate obtained from the test solution and the reference solution respectively.

1 mg of *anhydrous potassium sucrose octasulfate* is equivalent to 0.757 mg of sucrose octasulfate.

Storage. Store protected from moisture.

Sucralfate Tablets

Sucralfate Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of sucralfate, $Al_8(OH)_{16}(C_{12}H_{14}O_{35}S_8)[Al(OH)_3]_n[H_2O]_n$) corresponding to not less than 30.6 per cent and not more than 37.4 per cent of sucrose octasulphate, $C_{12}H_{14}O_{35}S_8$.

Usual strength. 1000 mg.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Dissolve 1.0 g of sucralfate in *3 M hydrochloric acid*. The resulting solution gives the reactions of aluminium salts (2.3.1).

Tests

Neutralising Capacity. Weigh and powder 20 tablets. Transfer 0.25 g of the powder in a 250 ml screw-capped bottle, add 100.0 ml of *0.1 M hydrochloric acid* previously heated to 37°, cap the bottle, and place in water-bath, maintaining the temperature at 37°, and stir continuously for 1 hour. Cool to room temperature and transfer 20.0 ml of this resulting solution to a 100-ml beaker add 30 ml of *water* and titrate with *0.1 M sodium hydroxide* to pH 3.5. Carry out a blank titration using a mixture of 30 volumes of *water* and 20 volumes of *0.1 M hydrochloric acid*.

Calculate the mEq of acid consumed per g of Sucralfate calculated by the formula:

$$\frac{5M(V_B - V_T)}{W}$$

Where M is the exact molarity of the *sodium hydroxide* VS, V_B and V_T are the volumes, in ml of *sodium hydroxide* consumed by the blank and the test solution, respectively; and W is the weight, in g , of equivalent amount of sample taken.

Not less than 12.0 mEq of *acid* is consumed.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 450 mg of Sucralfate in 35-ml centrifuge tube, and shake on a vortex mixer. While shaking add 10.0 ml of a mixture of *2.0 M sulphuric acid* and *2.2 M sodium hydroxide* Sonicate with swirling for 5 minutes, keeping the temperature of mixture below 30°. Without delay transfer the tube to a vortex mixer and while shaking at moderate rate, add an accurately measured volume, V, in ml, of *0.1M sodium hydroxide* to bring the pH of the solution to approximately 2, and dilute the solution with (15.0 – V) ml of *water*. Shake for 1 minute, and centrifuge for 5 minutes. Separate the clear supernatant layer, and allow to stand at room temperature until the pH stabilizes. If the pH is not between 2.3 and 3.5, repeat the test using a different volume of *0.1 M sodium hydroxide.*

Reference solution. A 1.0 per cent w/v solution of *anhydrous potassium sucrose octasulfate IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm, packed with aminopropylsilane bounded to porous silica (5 µm),
- mobile phase: a buffer solution prepared by dissolving 132 g of *ammonium sulphate* in 900 ml of *water*, dilute to 1000 ml with *water* and adjusted to pH 3.5 with *orthophosphoric acid.*
- flow rate: 1 ml per minute,
- refractive index detector maintained at 30°,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the column efficiency due to sucrose octasulphate peak is not less than 400 theoretical plates, the tailing factor is not more than 4.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{12}H_{14}O_{35}S_8$ in the of tablets as,

$$\frac{974.75}{1287.53} \times 25C \times \frac{r_u}{r_s}$$

where 974.75 and 1287.53 are the molecular weight of sucrose octasulphate and anhydrous potassium sucrose octasulphate, respectively, C is the concentration in mg per ml of anhydrous potassium sucrose octasulphate in reference solution and r_u and r_s are the peak responses of sucrose octasulphate obtained from the test solution and the reference solution respectively.

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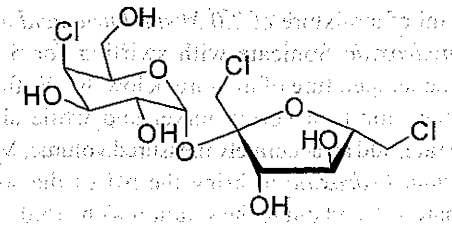
1 mg of *anhydrous potassium sucrose octasulfate* is equivalent to 0.757 mg of sucrose octasulfate.

Storage. Store protected from moisture.

Sucralose



$C_{12}H_{19}Cl_3O_8$ Mol. Wt. 397.6

Sucralose is 1,6-dichloro-1,6-dideoxy-β-D-fructofuranosyl-4-chloro-4-deoxy-α-D-galactopyranoside.

Sucralose contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{12}H_{19}Cl_3O_8$, calculated on the anhydrous basis.

Category. Pharmaceutical aid (sweetening vehicle).

Description. A white to off-white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sucralose IPRS* or with the reference spectrum of sucralose.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). +84.0° to +87.5°, determined at 20° in a 1.0 per cent w/v solution in *water*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with octadecylsilanized silica gel.

Mobile phase. A mixture of 70 volumes of 5 per cent w/v solution of *sodium chloride* and 30 volumes of *acetonitrile*.

Test solution. Dissolve about 1.0 g of the substance under examination in 10 ml of *methanol*.

Reference solution (a). A 1.0 per cent w/v solution of *sucralose IPRS* in *methanol*.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100.0 ml with *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and spray the plate with 15 per cent w/v solution of *sulphuric acid* in *methanol*. Heat the plate at 125° for 10 minutes. The R_f value of the principal spot obtained with the test solution corresponds to the spot in the chromatogram obtained with reference solution (a). Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Hydrolysis products. Determine by thin-layer chromatography (2.4.17), coating the plate with silica gel.

NOTE—This test does not require a developing solvent.

Test solution. Dissolve about 2.5 g of the substance under examination in 10 ml of *methanol*.

Reference solution (a). A 10.0 per cent w/v solution of *mannitol* in *water*.

Reference solution (b). A solution containing 0.04 per cent w/v of *fructose* and 10 per cent w/v of *mannitol* in *water*.

Apply to the plate 5 µl of each solution. Spray with a solution containing 1.23 g of *p-anisidine* and 1.66 g of *phthalic acid* in 100 ml of *methanol*, heat the plate at 105° for 15 minutes. If the spot from reference solution (a) has darkened, repeat the test, heating for a shorter period of time. Immediately after heating, view the plate against a dark background: the color of the spot obtained from the test solution is not more intense than that obtained from reference solution (b) (0.1 per cent).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.7 per cent.

Water (2.3.43). Not more than 2.0 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 250 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution. A 1.0 per cent w/v solution of *sucralose IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 85 volumes of *water* and 15 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- refractive index detector
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 per cent.

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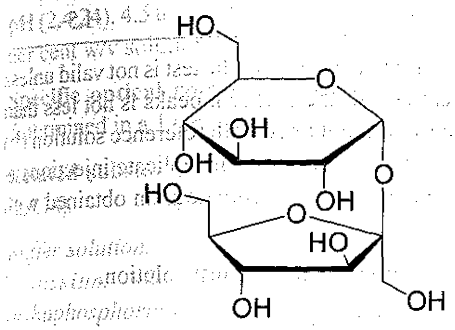
Inject the reference solution and the test solution.

Calculate the content of $C_{12}H_{19}Cl_3O_8$.

Storage. Store protected from moisture, at a temperature not exceeding 21°.

Sucrose

Refined Sugar



$C_{12}H_{22}O_{11}$ Mol. Wt. 342.3

Sucrose is β-D-fructofuranosyl α-D-glucopyranoside.

Category. Pharmaceutical aid (sweetening agent; tablet excipient).

Description. An almost white or colourless crystals, dry crystalline powder.

Identification

Dissolve 150.0 g in sufficient *carbon dioxide-free water* prepared from *distilled water* to produce 300 ml (solution A). Dilute 1 ml of solution A to 100 ml with *water*. To 5 ml of the solution add 2 ml of freshly prepared *2 M sodium hydroxide* and 0.15 ml of a freshly prepared *copper sulphate solution*; the solution is clear and blue and remains so on boiling. To the hot solution add 4 ml of *2 M hydrochloric acid*, heat to boiling and add 4 ml of *2 M sodium hydroxide*; an orange precipitate is produced immediately.

Tests

Acidity or alkalinity. To 10 ml of solution A add 0.3 ml of *phenolphthalein solution*. The solution is colourless and not more than 0.6 ml of *0.01 M sodium hydroxide* is required to change the colour of the solution to pink.

Specific optical rotation (2.4.22). +65.9° to +67.0°, determined in a 10 per cent w/v solution.

Barium. To 10 ml of solution A add 1 ml of *1 M sulphuric acid*. When examined immediately and after 1 hour any opalescence is not more intense than that of a mixture of 1 ml of *distilled water* and 10 ml of solution A.

Calcium. To 1 ml of solution A add 9 ml of *water* and 1 ml of *ammonium oxalate solution*; the solution remains clear for at least 1 minute.

Heavy metals (2.3.13). Add 0.1 ml of *dilute hydrochloric acid* to 4 ml of solution A and dilute with sufficient *water* to produce 25 ml. The solution complies with the limit test for heavy metals, Method A (10 ppm).

Sulphites. To 4 ml of solution A add sufficient *water* to produce 20 ml, add 0.05 ml of *0.1 M iodine* and 0.05 ml of *starch solution*; a blue colour develops.

Dextrins. To 2 ml of solution A add 8 ml of *water*, 0.05 ml of *2 M hydrochloric acid* and 0.05 ml of *0.05 M iodine*; the solution remains yellow or becomes faint bluish green.

Glucose and invert sugar. Dissolve 50 g in *water* and dilute to 100.0 ml with the same solvent. To 5.0 ml of the solution in a test-tube about 150 mm long and 16 mm in diameter add 5 ml of *water*, 1.0 ml of *1 M sodium hydroxide* and 1.0 ml of a 0.1 per cent solution of *methylene blue*. Mix and place in a water-bath. After exactly 2 minutes, take the tube out of the bath and examine the solution immediately. The blue colour does not disappear completely. Ignore any blue colour at the air/solution interface.

Colouring matter. A. To 100 ml of solution A in a ground-glass-stoppered tube add 1 ml of *dilute hypophosphorous acid* and allow to stand for 1 hour; no unpleasant odour is detectable.

B. Examine solution A under ultraviolet light at 365 nm. Any fluorescence is not more intense than that of a solution containing 0.4 µg per ml of *quinine sulphate* in *0.005 M sulphuric acid*.

Sulphated ash (2.3.18). Not more than 0.1 per cent determined by dissolving 5.0 g in 5 ml of *water*, adding 2 ml of *sulphuric acid*, evaporating to dryness and igniting to constant weight.

Sucrose intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.25 Endotoxin unit per mg of Sucrose.

Storage. Store protected from light and moisture.

Sugar Spheres

Sugar Spheres contain not less than 62.5 per cent and not more than 91.5 per cent of sucrose, $C_{12}H_{22}O_{11}$, calculated on the dried basis, the remainder consisting mainly of starch. They consist of approximately spherical particles of a labeled

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nominal size range. They may contain colour additives permitted for use in drugs.

Category. Pharmaceutical aid.

Description. Hard, brittle, free flowing, spherical masses ranging generally in size from 10 to 60 mesh. Usually white, but may be coloured.

Identification

A. A 10 per cent w/v solution produce a violet to deep blue colour on addition of *iodine*.

B. Specific optical rotation (see Test).

Tests

Particle size distribution estimation (2.5.7). Not less than 90 per cent of it passes the coarser sieve size stated in the labeling; all of it passes the next coarser sieve size listed in Table 1 of the chapter; and not more than 10 per cent passes the finer sieve size stated on the label.

Specific optical rotation (2.4.22). + 41.0° to + 61.0°, determined in a 10 per cent w/v solution prepared by dissolving 20 g in 160 ml of *water* and dilute to 200.0 ml with *water*. Pass the solubilised sucrose solution by vacuum filtration through fine filter paper.

Sulphated ash (2.3.18). Not more than 0.25 per cent, determined on 2.0 g at a temperature of about 700°.

Loss on drying (2.4.19). Not more than 4.0 per cent, determined on 1.0 g by drying in an oven at 105° for 4 hours.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 100 CFU per g. 1 g is free from *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. 10 g is free from *Salmonella*.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 1.0 g of Sugar Spheres in 20 ml of *water* and dilute to 50.0 ml with *water*.

Reference solution (a). A 2.0 per cent w/v solution of *sucrose IPRS* in *water*.

Reference solution (b). A solution containing 2.0 per cent w/v of *sucrose IPRS*, 0.1 per cent w/v of *dextrose IPRS*, 0.06 per cent w/v of *fructose IPRS*, 0.06 per cent w/v of *maltose monohydrate IPRS*, and 0.08 per cent w/v of *anhydrous lactose IPRS* in *water*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with amino propylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: a mixture of 80 volumes of *acetonitrile* and 20 volumes of *water*,
- flow rate: 2 ml per minute,
- refractive index detector at 40°,
- injection volume: 15 µl.

Name	Relative retention time
Fructose	0.5
Dextrose (glucose)	0.6
Sucrose	1.0
Maltose	1.3
Lactose	1.5

Inject reference solution (a) and (b). The test is not valid unless the resolution between all the adjacent peaks is not less than 1.3 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 2.0 per cent the chromatogram obtained with reference solution (a).

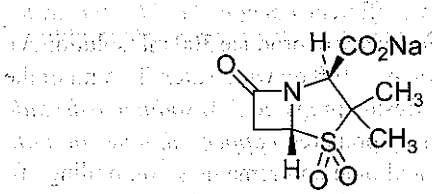
Inject reference solution (a) and the test solution.

Calculate the content of $C_{12}H_{22}O_{11}$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the nominal particle size range. Label it to indicate the name and amount of any added color additives.

Sulbactam Sodium



$C_8H_{10}NNaO_5S$ Mol. Wt. 255.2

Sulbactam Sodium is (2*S*,5*R*)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate 4,4-dioxide.

Sulbactam Sodium contains not less than 97.0 per cent and not more than 102.0 per cent of $C_8H_{10}NNaO_5S$, calculated on the anhydrous basis.

Category. β-lactamase inhibitor.

Description. A white or almost white, hygroscopic, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sulbactam*

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sodium IPRS or with the reference spectrum of sulbactam sodium.

B. It gives reaction (A) of sodium (2.3.1).

Tests

Appearance of solution. A 10 per cent w/v solution is clear (2.4.1).

Light absorption (2.4.7). Absorbance of 1.0 per cent w/v solution at 430 nm is not more than 0.1.

pH (2.4.24). 4.5 to 7.2, if the substance is sterile: 5.2 to 7.2, of 5 per cent w/v solution in *carbon dioxide-free water*.

Specific optical rotation (2.4.22). + 219.0° to + 233.0°, determined in a 1 per cent w/v solution in *water*.

Related substances. Determine by liquid chromatography (2.4.14).

Buffer solution. A 0.27 per cent w/v solution of *monobasic potassium phosphate*, adjusted to pH 4.0 with *orthophosphoric acid*.

Solvent mixture. 98 volumes of the buffer solution and 2 volumes of *acetonitrile*.

NOTE — Protect the solution from light.

Test solution. Dissolve 77 mg of the substance under examination in 2 ml of *acetonitrile* and dilute to 100.0 ml with the buffer solution.

Reference solution (a). Dissolve 70 mg of *sulbactam IPRS* in 2 ml of *acetonitrile* and dilute to 100.0 ml with the buffer solution.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution (c). A solution containing 0.007 per cent w/v each of *sulbactam related substance A IPRS, sulbactam related substance D IPRS, sulbactam related substance E IPRS* and *sulbactam related substance F IPRS* in *acetonitrile*. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 40°,
- mobile phase: A. a 0.54 per cent w/v solution of *monobasic potassium phosphate*, adjusted to pH 4.0 with *orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	98	2
7.5	50	50
8.5	50	50
9.0	98	2
13	98	2

Name	Relative retention time	Correction factor
Sulbactam related compound A[1]	0.4	0.59
Amoxicillin related compound A[2]	0.6	0.50
Sulbactam	1.0	—
6-Bromopenicillanic acid sulfone[3]	1.6	—
Sulbactam related compound D[4]	2.0	0.5
Sulbactam related compound E[5]	2.1	—
Sulbactam related compound F[6]	2.5	0.59

[1]3-Sulfino-D-valine; (2*S*)-2-Amino-3-methyl-3-sulfinobutanoic acid,

[2]6-Aminopenicillanic acid; (2*S*,5*R*,6*R*)-6-Amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,

[3](2*S*,5*R*,6*R*)-6-Bromo-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide,

[4]6-Bromopenicillanic acid; (2*S*,5*R*,6*R*)-6-Bromo-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,

[5]6,6-Dibromopenicillanic acid sulfone; (2*S*,5*R*)-6,6-Dibromo-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide,

[6]6,6-Dibromopenicillanic acid; (2*S*,5*R*)-6,6-Dibromo-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid.

Inject reference solution (b) and (c). The test is not valid unless the resolution between sulbactam related compound D and sulbactam related compound E peaks is not less than 1.5 in the chromatogram obtained with reference solution (c) and the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to sulbactam related compound A is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any peak corresponding to 6-bromopenicillanic acid sulfone and sulbactam related compound E, each of is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any peak corresponding to amoxicillin related compound A, sulbactam related compound D and sulbactam related compound F, each of is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per

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cent) and the sum of areas of all peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

2-Ethylhexanoic acid (2.3.51). Not more than 0.5 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 1 g.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modifications.

Inject reference solution (a) and the test solution.

1 mg of sulbactam is equivalent to 1.094 mg of $C_8H_{10}NNaO_5S$.

Calculate the content of $C_8H_{10}NNaO_5S$.

Sulbactam Sodium intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

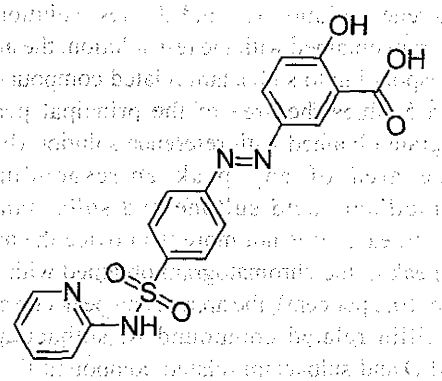
Bacterial endotoxins (2.2.3). Not more than 0.17 Endotoxin Units per mg of sulbactam sodium.

Sulbactam Sodium intended for use in the manufacture of parenteral preparations without a further appropriate sterilisation procedure complies with the following additional requirement.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store protected from moisture. If the substance is sterile, store in a sterile, moisture free container.

Sulfasalazine



$C_{18}H_{14}N_4O_5S$ Mol. Wt. 398.4

Sulfasalazine is 2-hydroxy-5-[2-[4-(pyridin-2-ylsulfamoyl) phenyl]diazenyl]benzoic acid.

Sulfasalazine contains not less than 97.0 per cent and not more than 101.5 per cent of $C_{18}H_{14}N_4O_5S$, calculated on the dried basis.

Category. Disease-modifying antirheumatic drug (DMARD)

Description. A bright yellow or brownish-yellow, fine powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sulfasalazine IPRS* or with the reference spectrum of sulfasalazine.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in *dilute ammonia* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *dilute ammonia*.

Reference solution (b). Dissolve 1 mg of *sulfasalazine derivative for resolution IPRS* in 10.0 ml of reference solution (a). Dilute 1.0 ml of the solution to 10.0 ml with reference solution (a).

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. dissolve 1.13 g of *sodium dihydrogen phosphate* and 2.5 g of *sodium acetate* in 900 ml of *water* ; adjusted to pH 4.8 with *glacial acetic acid* and dilute to 1000 ml with *water*,
  B. a mixture of 80 volumes of *methanol* and 20 volumes of mobile phase A,
- a gradient programme using the condition given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 320 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	60	40
15	45	55
25	45	55
60	0	100
65	0	100
70	60	40

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Name	Relative retention time
Sulfasalazine impurity H[8]	0.16
Sulfasalazine impurity I[9]	0.28
Sulfasalazine impurity C[3]	0.80
Sulfasalazine impurity F[6]	0.85
Sulfasalazine	1.0
Sulfasalazine impurity G[7]	1.39
Sulfasalazine impurity E[5]	1.63
Sulfasalazine impurity B[2]	1.85
Sulfasalazine impurity D[4]	1.9
Sulfasalazine impurity A[1]	2.0

[1]4,4'-[(4-hydroxy-1,3-phenylene)bis(diazenediyl)]bis[*N*-(pyridin-2-yl)benzenesulfonamide],

[2]2-hydroxy-3,5-bis[2-[4-(pyridin-2-ylsulfamoyl)phenyl]diazenyl]benzoic acid,

[3]2-hydroxy-5-[2-[4-(2-iminopyridin-1(2*H*)-yl)phenyl]diazenyl]benzoic acid,

[4]4-[2-(2-hydroxyphenyl)diazenyl]-*N*-(pyridin-2-yl) benzenesulfonamide,

[5]2-hydroxy-4'-(pyridin-2-ylsulfamoyl)-5-[2-[4-(pyridin-2-ylsulfamoyl)phenyl]diazenyl]biphenyl-3-carboxylic acid,

[6]2-hydroxy-3-[2-[4-(pyridin-2-ylsulfamoyl)phenyl]diazenyl]benzoic acid,

[7]5-[2-[4',5-bis(pyridin-2-ylsulfamoyl)biphenyl-2-yl]diazenyl]-2-hydroxybenzoic acid,

[8]2-hydroxybenzenecarboxylic acid (salicylic acid),

[9]2-hydroxy-5-[2-(4-sulfophenyl)diazenyl]benzoic acid.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to sulfasalazine and its derivatives is not less than 3.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of each peak due to impurities A, B, C, D, E, F, G and I is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent) and the sum of areas of all the secondary peaks is not more than four times the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent), and also ignore any peak with a retention time less than 6 minutes (due to impurities H and J).

Impurities H and J. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in *dilute ammonia* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *salicylic acid* (impurity H) and 5 mg of *sulfapyridine IPRS* (H_2N-R: 4-amino-*N*-(pyridin-2-yl)benzenesulfonamide: impurity J) in *dilute ammonia* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 100.0 ml with *dilute ammonia*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 30 volumes of mobile phase B and 70 volumes of mobile phase A (as described under Related substances),
- flow rate: 1 ml per minute,
- spectrophotometer set at 300 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to sulfasalazine impurity H and sulfasalazine impurity J is not less than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of each peak due to impurities H and J is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Chlorides (2.3.12). Shake 1.8 g with 30 ml of *water*, heat at about 70° for 5 minutes, cool and filter, add 1 ml of *nitric acid*, allow to stand for 5 minutes and filter, if necessary. The solution complies with the limit test for chlorides (140 ppm).

Sulphates (2.3.17). Shake 0.375 g with 30 ml of *water*, heat at about 70° for 5 minutes, cool and filter, add 1 ml of *dilute hydrochloric acid* and allow to stand for 5 minutes and filter, if necessary. The solution complies with the limit test for sulphates (400 ppm).

Sulphated ash (2.3.18). Not more than 0.5 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1 g by drying in an oven at 105° for 2 hours.

Assay. Dissolve 150 mg in sufficient quantity of *0.1 M sodium hydroxide* and dilute to 100.0 ml with *0.1 M sodium hydroxide*. Dilute 5.0 ml of the solution with 750 ml of *water* add 20 ml of *0.1 M acetic acid* and dilute to 1000.0 ml with *water*. Measure the absorbance of the resulting solution at the maximum at about 359 nm (2.4.7).

Calculate the content of $C_{18}H_{14}N_4O_5S$ from the absorbance obtained by repeating the operation using *sulfasalazine IPRS* in the place of the substance under examination.

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Sulfasalazine Gastro-resistant Tablets

Sulfasalazine Gastro-resistant Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sulfasalazine, $C_{18}H_{14}N_4O_5S$. They are made gastro-resistant by enteric-coating or by any other means.

Usual strength. 500 mg.

Identification

A. To a quantity of the powdered tablets containing 0.2 g of Sulfasalazine, add 100 ml of *methanol*, shake thoroughly and filter. Evaporate the filtrate to dryness and dry at about 35° the resulting precipitate at a pressure of 2kPa for 1 hour. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sulfasalazine IPRS* or with the reference spectrum of sulfasalazine.

B. In the test of Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (c).

Tests

Dissolution (2.5.2).

A. Apparatus No. 1 (Basket),
Medium. 900 ml of *0.1 M hydrochloric acid,*
Speed and time. 100 rpm and 120 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.0056 per cent w/v solution of *sulfasalazine IPRS* in *0.1M sodium hydroxide.*

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porus silica (5 µm),
- mobile phase: a mixture of 11 volumes of *isopropanol*, 7 volumes of *acetronitrile*, 22 volumes of *water* and 0.4 volume of *glacial acetic acid*,
- flow rate: 1 ml per minute
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{18}H_{14}N_4O_5S$:

Complies with the acceptance criteria given under acid stage.

B. Apparatus No. 1 (Basket),
Medium. 900 ml of *phosphate buffer pH 7.5,*
Speed and time. 100 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.0056 per cent w/v solution of *sulfasalazine IPRS* in *0.1M sodium hydroxide.*

Use chromatographic system as described under test A.

Inject the reference solution. The test is not valid unless the relative standard deviation of replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Q. Not less than 85 per cent of the stated amount of $C_{18}H_{14}N_4O_5S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets containing 0.1 g of sulfasalazine with 100.0 ml of *0.1 M ammonia* for 30 minutes, centrifuge and use the supernatant liquid.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *0.1M ammonia.*

Reference solution (b). A solution containing 0.001per cent w/v of *sulfasalazine derivative for resolution IPRS* in reference solution (a).

Reference solution (c). A solution containing 0.001per cent w/v of *sulfasalazine IPRS* in *0.1M ammonia.*

Reference solution (d). Dilute 1 ml of reference solution (a) to 20 ml with *0.1M ammonia.*

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Nucleosil C18),
- mobile phase: A. a solution containing 0.013per cent w/v of *sodium dihydrogen orthophosphate* and 0.25per cent w/v of *sodium acetate* adjusted to pH 4.8 with *glacial acetic acid*,
  B. a mixture of 80 volumes of *methanol* and 20 volumes of mobile phase A,
- a gradient programme using the condition given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 320 nm,
- injection volume: 20 µl.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	60	40
15	45	55
25	45	55
60	0	100
65	0	100
67	60	40
77	60	40

Name	Relative retention time
Sulfasalazine impurity H[8]	0.16
Sulfasalazine impurity I[9]	0.28
Sulfasalazine impurity C[3]	0.80
Sulfasalazine impurity F[6]	0.85
Sulfasalazine (Retention time is about 24 minutes)	1.0
Sulfasalazine impurity G[7]	1.39
Sulfasalazine impurity E[5]	1.63
Sulfasalazine impurity B[2]	1.85
Sulfasalazine impurity D[4]	1.9
Sulfasalazine impurity A[1]	2.0

[1]4,4'-[(4-hydroxy-1,3-phenylene)bis(diazenediyl)]bis[*N*-(pyridin-2-yl) benzenesulfonamide],

[2]2-hydroxy-3,5-bis[2-[4-(pyridin-2-ylsulfamoyl)phenyl]diazenyl] benzoic acid,

[3]2-hydroxy-5-[2-[4-(2-iminopyridin-1(2*H*)-yl)phenyl]diazenyl]benzoic acid,

[4]4-[2-(2-hydroxyphenyl)diazenyl]-*N*-(pyridin-2-yl) benzenesulfonamide,

[5]2-hydroxy-4,2'-(pyridin-2-ylsulfamoyl)-5'-[2-[4-(pyridin-2-yl sulfamoyl)phenyl]diazenyl]biphenyl-3-carboxylic acid,

[6]2-hydroxy-3-[2-[4-(pyridin-2-ylsulfamoyl)phenyl]diazenyl]benzoic acid,

[7]5-[2-[4,2'-bis(pyridin-2-ylsulfamoyl)biphenyl-2-yl]diazenyl]-2-hydroxybenzoic acid,

[8]2-hydroxybenzenecarboxylic acid (salicylic acid),

[9]2-hydroxy-5-[2-(4-sulfophenyl)diazenyl]benzoic acid.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to sulfasalazine and its derivatives is not less than 3.0.

Inject reference solution (a), (d) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent) and the sum of areas of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent), and also ignore any peak with a retention time less than 6 minutes (due to impurities H and J).

Salicylic acid and Sulfapyridine. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets containing 0.1 g of sulfasalazine with 100.0 ml of *0.1 M ammonia* for 30 minutes, centrifuge and use the supernatant liquid.

Reference solution. A solution containing 0.05 per cent w/v of *salicylic acid* and 0.05 per cent w/v of *sulfapyridine IPRS* (H_2N-R: 4-amino-*N*-(pyridin-2-yl)benzenesulfonamide) in *0.1 M ammonia*. Dilute 1.0 ml of the solution to 50.0 ml with *0.1M ammonia*.

Use chromatographic system as described under Related substances with the following modifications

– spectrophotometer set at 300 nm,

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
12	70	30
15	30	70
25	30	70
27	70	30
35	70	30

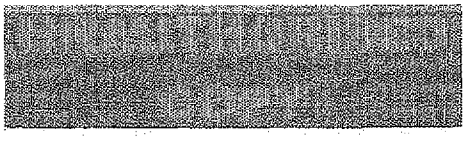
Inject the reference solution. The test is not valid unless the resolution between the peaks corresponding to salicylic acid and sulfapyridine is not less than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak due to impurities salicylic acid and sulfapyridine is not more than 0.5 times the areas of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent of each).

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 150 mg in 100.0 ml of *0.1 M sodium hydroxide*. Dilute 5.0 ml of the solution with 750 ml of *water* add 20 ml of *0.1 M acetic acid* and dilute to 1000.0 ml with *water*. Measure the absorbance of the resulting solution at the maximum at about 359 nm (2.4.7).

Calculate the content of $C_{18}H_{14}N_4O_5S$ from the absorbance obtained by repeating the operation using *sulfasalazine IPRS* in the place of the substance under examination.



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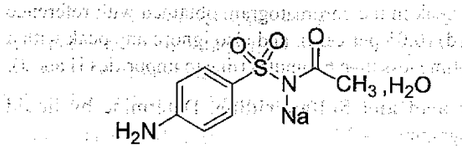
Sulphacetamide Sodium



$C_8H_9N_2NaO_3S,H_2O$ Mol. Wt. 254.2

Sulphacetamide Sodium is the monohydrate of the sodium salt of *N'*-acetylsulphanilamide.

Sulphacetamide Sodium contains not less than 99.0 per cent and not more than 101.0 per cent of $C_8H_9N_2NaO_3S$, calculated on the anhydrous basis.

Category. Antibacterial.

Description. A white or yellowish white, crystalline powder.

Identification

Test A may be omitted if tests B, C, D, E and F are carried out. Tests B, C, D and E may be omitted if tests A and F are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sulphacetamide sodium IPRS* or with the reference spectrum of sulphacetamide sodium.

B. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

C. Dissolve 1 g in 10 ml of *water*, add 6 ml of *2 M acetic acid* and filter. Wash the precipitate with a small volume of *water* and dry at 105° for 4 hours. The melting range of the precipitate is 181° to 185° (2.4.21).

D. Dissolve 0.1 g of the precipitate obtained in test C in 5 ml of *ethanol (95 per cent)*, add 0.2 ml of *sulphuric acid* and heat; ethyl acetate, recognizable by its odour, is produced.

E. Dissolve 1 mg of the precipitate obtained in test C in 5 ml of *water* with the aid of heat. The solution gives the reaction of primary aromatic amines (2.3.1), producing an orange-red precipitate.

F. A 5 per cent w/v solution gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution GYS4 (2.4.1).

pH (2.4.28). 8.0 to 9.5, determined in a 5.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Prepare the solutions immediately before use and protected from light.

Test solution. Dissolve 0.2 g of the substance under examination in 10.0 ml of the mobile phase.

Reference solution (a). Dissolve 5 mg each of *sulphacetamide sodium IPRS* and *sulphanilamide* (sulphacetamide sodium impurity A) in 1.0 ml of the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 12.5 cm x 4 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm)
- mobile phase: a mixture of 1 volumes of *glacial acetic acid*, 10 volumes of *methanol* and 89 volumes of *water*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

The relative retention time with reference to sulphacetamide for sulphacetamide impurity A is about 0.5.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to sulphacetamide impurity A and sulphacetamide is not less than 5.0.

Inject reference solution (b) and the test solution. Run the chromatogram 7 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of the peak due to sulphacetamide impurity A, multiplied by correction factor of 0.5 is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphates (2.3.17). Dissolve 1.5 g in sufficient *distilled water* to produce 25 ml, add 25 ml of *2 M acetic acid*, shake for 30 minutes and filter. 25 ml of the filtrate complies with the limit test for sulphates (200 ppm).

Water (2.3.43). 6.0 to 8.0 per cent, determined on 0.2 g.

Assay. Dissolve 0.25 g in a mixture of 50 ml of *water* and 20 ml of *2 M hydrochloric acid*, add 3 g of *potassium bromide*, cool in ice and carry out the nitrite titration (2.3.31).

1 ml of *0.1 M sodium nitrite* is equivalent to 0.02362 g of $C_8H_9N_2NaO_3S$.

Storage. Store protected from light and moisture.

Sulphacetamide Eye Drops

Sulphacetamide Sodium Eye Drops

Sulphacetamide Eye Drops are a sterile solution of Sulphacetamide Sodium in Purified Water. It may contain a suitable antimicrobial agent.

Sulphacetamide Eye Drops contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sulphacetamide sodium, $C_8H_9N_2NaO_3S,H_2O$.

Usual strengths. 10 per cent w/v; 20 per cent w/v; 30 per cent w/v.

Identification

To a volume containing 0.5 g of Sulphacetamide Sodium add 6 ml of *5 M acetic acid*, stirring constantly. Filter the precipitate, wash with *water* and dry at 105° for 4 hours. The residue complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sulphacetamide sodium IPRS* treated in the same manner or with the reference spectrum of sulphacetamide.

B. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

C. Dissolve 10 mg in 2 ml of *2 M hydrochloric acid*. The solution gives the reaction of primary aromatic amines (2.3.1).

Tests

Appearance of solution. Dilute the eye drops, if necessary, to contain 10.0 per cent w/v of Sulphacetamide Sodium. The solution is not more intensely coloured than reference solution BYS4 (2.4.1).

pH (2.4.24). 6.6 to 8.6.

Related substances. Determine by liquid chromatography (2.4.14).

Note—Prepare the solutions immediately before use and protected from light.

Test solution. Dissolve a volume of solution containing about 0.2 g of Sulphacetamide Sodium in 10.0 ml of the mobile phase.

Reference solution (a). Dissolve 5 mg each of *sulphacetamide sodium IPRS* and *sulphanilamide* (sulphacetamide sodium impurity A) in 1.0 ml of the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 12.5 cm x 4 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm)
- mobile phase: a mixture of 1 volumes of *glacial acetic acid*, 10 volumes of *methanol* and 89 volumes of *water*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to sulphacetamide impurity A and sulphacetamide is not less than 5.0. The relative retention time with reference to sulphacetamide for sulphacetamide impurity A is about 0.5.

Inject reference solution (b) and the test solution. Run the chromatogram 7 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of the peak due to sulphacetamide impurity A, multiplied by correction factor of 0.5 is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Other tests. Comply with the tests stated under Eye Drops.

Assay. To a measured volume containing about 0.5 g of Sulphacetamide Sodium add 75 ml of *water* and 10 ml of *hydrochloric acid*. Add 3 g of *potassium bromide*, cool in ice and carry out the nitrite titration (2.3.31).

1 ml of *0.1 M sodium nitrite* is equivalent to 0.02542 g of $C_8H_9N_2NaO_3S,H_2O$.

Storage. Store protected from light and moisture. The Eye Drops should not be allowed to freeze.

Labelling. The label states (1) the name and concentration of any antimicrobial agent used; (2) that it is not meant for injection; (3) that the solution should be used within one month of opening the container; (4) that the solution should not be used if it is dark brown in colour; (5) that it should not be allowed to freeze.

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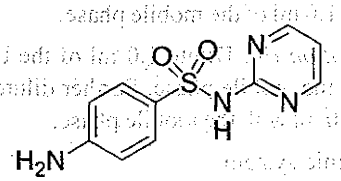
Sulphadiazine



$C_{10}H_{10}N_4O_2S$ Mol. Wt. 250.3

Sulphadiazine is *N*¹-(pyrimidin-2-yl)sulphanilamide.

Sulphadiazine contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{10}H_{10}N_4O_2S$, calculated on the dried basis.

Category. Antibacterial.

Description. White, yellowish white or pinkish white crystals or crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests C and D may be omitted if tests A and B are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sulphadiazine IPRS* or with the reference spectrum of sulphadiazine.

B. In the test for Related substances, the principal spot in the chromatogram obtained with reference solution (a) corresponds to that in the chromatogram obtained with the reference solution (c).

C. Dissolve about 5 mg in 10 ml of *1 M hydrochloric acid* and dilute 1 ml of the solution to 10 ml with *water*. The solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

D. Heat 3 g in a test-tube inclined at an angle of 45° with the lower part immersed in a silicone oil-bath at about 270°. It decomposes and a white or yellowish white sublimate is produced. The sublimate, after recrystallisation from *toluene* and drying at 100° melts at 123° to 127° (2.4.21).

Tests

Appearance of solution. Dissolve 0.8 g in 10 ml of *1 M sodium hydroxide*. The solution is not more intensely coloured than reference solution YS5, BYS5 or GYS5 (2.4.1).

Acidity. Heat 1.25 g of the finely powdered substance at about 70° with 25 ml of *carbon dioxide-free water* for 5 minutes. Cool for about 15 minutes in ice and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution*. Not more than 0.2 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution.

Related substances (2.3.7). Complies with test C for Related substances in Sulphonamides (2.3.7), Method C.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh 0.2 g, dissolve in a mixture of 20 ml of *2 M hydrochloric acid* and 50 ml of *water*. Add 3 g of *potassium bromide*, cool in ice and carry out the nitrite titration (2.3.3).

1 ml of *0.1 M sodium nitrite* is equivalent to 0.02503 g of $C_{10}H_{10}N_4O_2S$.

Storage. Store protected from light and moisture.

Sulphadiazine Tablets

Sulphadiazine Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sulphadiazine, $C_{10}H_{10}N_4O_2S$.

Usual strength. 500 mg.

Identification

A. Triturate a quantity of the powdered tablets containing 0.5 g Sulphadiazine with two successive quantities, each of 5 ml, of *chloroform* and reject the chloroform. Triturate the residue with 10 ml of *dilute ammonia solution* for 5 minutes, add 10 ml of *water* and filter. Warm the filtrate until most of the ammonia has been expelled, cool and acidify with *acetic acid*. Collect the precipitate, wash with *water* and dry at about 100°; the residue melts at about 256°, with decomposition (2.4.21).

B. On the residue obtained in test A determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sulphadiazine IPRS* or with the reference spectrum of sulphadiazine.

C. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 100 rpm and 60 minutes.

Withdraw a suitable volume of the sample and filter promptly through a membrane filter disc with an average pore diameter not greater than 1.0 mm. Reject the first few ml of the filtrate and dilute a suitable volume of the filtrate with the same

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solvent. Dilute suitably with *0.01 M sodium hydroxide*. Measure the absorbances of the resulting solution and of a standard solution of *sulphadiazine IPRS* of similar concentration in the same medium at the maximum at about 254 nm (2.4.7).

Calculate the content of $C_{10}H_{10}N_4O_2S$ in the medium.

Q. Not less than 70.0 per cent of the stated amount of $C_{10}H_{10}N_4O_2S$.

Related substances (2.3.7). Complies with test C for Related substances in Sulphonamides (2.3.7), Method C, but using the following solutions.

Test solution (a). Extract a quantity of the powdered tablets containing 0.5 g of Sulphadiazine with 25 ml of a mixture of 90 volumes of *methanol* and 10 volumes of *strong ammonia solution* by shaking for 10 minutes, filter and use the filtrate.

Test solution (b). Dilute 1 volume of test solution (a) to 5 volumes with a mixture of 24 volumes of *methanol* and 1 volume of *strong ammonia solution*.

Test solution (c). Dilute 1 volume of test solution (a) to 200 volumes with the same solvent mixture.

Reference solution. A 0.4 per cent w/v solution of *sulphadiazine IPRS* in the same solvent mixture.

Any secondary spot in the chromatogram obtained with the test solution (a) is not more intense that the spot obtained with test solution (c).

Other tests. Comply with the tests stated under Tablets.

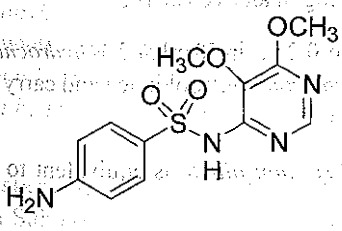
Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 0.5 g of Sulphadiazine and dissolve as completely as possible in a mixture of 50 ml of *water* and 10 ml of *hydrochloric acid*, add 3 g of *potassium bromide*, cool in ice. Carry out the nitrite titration (2.3.31).

1 ml of *0.1 M sodium nitrite* is equivalent to 0.02503 g of $C_{10}H_{10}N_4O_2S$.

Storage. Store protected from light and moisture.

Sulphadoxine

Sulphormethoxine; Sulphoethomidine



$C_{12}H_{14}N_4O_4S$ Mol. Wt. 310.3

Sulphadoxine is N^1-(5,6-dimethoxypyrimidin-4-yl) sulphanilamide.

Sulphadoxine contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{12}H_{14}N_4O_4S$, calculated on the dried basis.

Category. Antibacterial; antimalarial in combination with pyrimethamine.

Description. A white or yellowish white crystals or crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and D may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sulphadoxine IPRS* or with the reference spectrum of sulphadoxine.

B. In the test for Related substances, the principal spot in the chromatogram obtained with reference solution (a) corresponds to that in the chromatogram obtained with the reference solution (c).

C. Dissolve 0.5 g in 1 ml of *sulphuric acid (40 per cent)*, heating gently to effect solution, and continue heating until a crystalline precipitate is produced. Allow to cool, add 10 ml of *2 M sodium hydroxide*, cool again, add 25 ml of *ether* and shake for 5 minutes. Dry the upper layer over *anhydrous sodium sulphate*, filter and evaporate the solvent by heating on a water-bath. The residue melts either at 80° to 82° or at 90° to 92° (2.4.21).

D. Dissolve about 5 mg in 10 ml of *1 M hydrochloric acid* and dilute 1 ml to 10 ml with *water*. The resulting solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

Tests

Appearance of solution. Dissolve 0.8 g in 10 ml of *1 M sodium hydroxide*. The solution is not more intensely coloured than reference solution YS5, BYS5 or GYS5 (2.4.1).

Acidity. Heat 1.25 g of the finely powdered substance at about 70° with 25 ml of *carbon dioxide-free water* for 5 minutes. Cool for about 15 minutes in ice and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution*. Not more than 0.2 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution.

Related substances. Complies with the test for Related substances in Sulphonamides (2.3.7), Method C, but using the following solution.

Test solution. A 2 per cent w/v solution of the substance under examination in a mixture of 24 volumes of *methanol* and 1 volume of *strong ammonia solution*.

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Heavy metals (2.3.13).1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

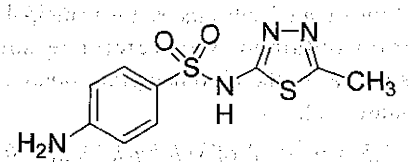
Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.25 g in 50 ml of *2 M hydrochloric acid*, add 3 g of *potassium bromide*, cool in ice and carry out the nitrite titration (2.3.31).

1 ml of *0.1 M sodium nitrite* is equivalent to 0.03103 g of $C_{12}H_{14}N_4O_4S$.

Storage. Store protected from light and moisture.

Sulphamethizole



$C_9H_{10}N_4O_2S_2$ Mol. Wt. 270.3

Sulphamethizole is *N*^{1}-(5-methyl-1,3,4-thiadiazol-2-yl) sulphanilamide.

Sulphamethizole contains not less than 99.0 per cent and not more than 101.0 per cent of $C_9H_{10}N_4O_2S_2$, calculated on the dried basis.

Category. Antibacterial.

Description. White or yellowish white crystals or crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests C and D may be omitted if tests A and B are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sulphamethizole IPRS* or with the reference spectrum of sulphamethizole.

B. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (b).

C. Dissolve 50 mg in 4 ml of *methanol* and add 0.2 ml of a 4.0 per cent w/v solution of *cupric acetate*; a flocculent, yellowish green precipitate is produced which becomes dark green.

D. Dissolve about 5 mg in 10 ml of *1 M hydrochloric acid* and dilute 1 ml of the solution to 10 ml with *water*. The solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

Tests

Appearance of solution. Dissolve 0.8 g in 10 ml of *1 M sodium hydroxide*. The solution is not more intensely coloured than reference solution YS5, BYS5 or GYS5 (2.4.1).

Acidity. Heat 1.25 g of the finely powdered substance at about 70° with 25 ml of *carbon dioxide-free water* for 5 minutes. Cool for about 15 minutes in ice and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution*. Not more than 0.2 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 80 volumes of *chloroform* and 15 volumes of *methanol*.

Test solution (a). Dissolve 0.3 g of the substance under examination in 10 ml of *acetone*.

Test solution (b). Dissolve 0.3 g of the substance under examination in 100 ml of *acetone*.

Reference solution (a). Dissolve 15 mg of the substance under examination in 100 ml of *acetone*.

Reference solution (b). A 0.3 per cent w/v solution of *sulphamethizole IPRS* in *acetone*.

Apply to the plate 2 µl of each solution. After development, dry the plate at 105° and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.25 g in 50 ml of *2 M hydrochloric acid*, add 3 g of *potassium bromide*, cool in ice and carry out the nitrite titration (2.3.31).

1 ml of *0.1 M sodium nitrite* is equivalent to 0.02703 g of $C_9H_{10}N_4O_2S_2$.

Storage. Store protected from light and moisture.

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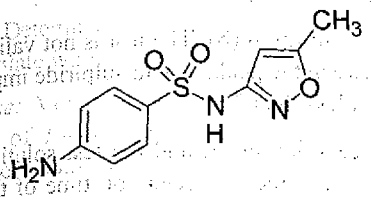
Sulphamethoxazole



$C_{10}H_{11}N_3O_3S$ Mol. Wt. 253.3

Sulphamethoxazole is *N'*-(5-methylisoxazol-3-yl) sulphanilamide.

Sulphamethoxazole contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{10}H_{11}N_3O_3S$, calculated on the dried basis.

Category. Antibacterial.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sulphamethoxazole IPRS* or with the reference spectrum of sulphamethoxazole.

B. In the test for Related substances, the principal spot in the chromatogram obtained with reference solution (a) corresponds to that in the chromatogram obtained with the reference solution (c).

C. Dissolve about 5 mg in 10 ml of *1 M hydrochloric acid* and dilute 1 ml to 10 ml with *water*. The resulting solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

D. Melting range (2.4.21). 169° to 172°.

Tests

Appearance of solution. Dissolve 0.8 g in 10 ml of *1 M sodium hydroxide*. The solution is not more intensely coloured than reference solution YS5, BYS5 or GYS5 (2.4.1).

Acidity. Heat 1.25 g of the finely powdered substance with 25 ml of *carbon dioxide-free water* at 70° for 5 minutes. Cool for about 15 minutes in ice and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution*. Not more than 0.3 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution.

Related substances. Complies with the test for Related substances in Sulphonamides (2.3.7), Method C, but using the following solution.

Test solution. Dissolve 0.1 g of the substance under examination in sufficient of a mixture of 1 volume of *strong ammonia solution* and 24 volumes of *methanol* to produce 5 ml.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

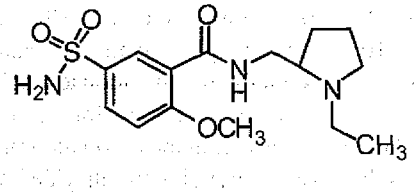
Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.2 g in 50 ml of *2 M hydrochloric acid*, add 3 g of *potassium bromide*, cool in ice and carry out the nitrite titration (2.3.31).

1 ml of *0.1 M sodium nitrite* is equivalent to 0.02533 g of $C_{10}H_{11}N_3O_3S$.

Storage. Store protected from light and moisture.

Sulpiride



$C_{15}H_{23}N_3O_4S$ Mol. Wt. 341.4

Sulpiride is (*RS*)-5-(Aminosulfonyl)-*N*-[(1-ethyl-2-pyrrolidinyl) methyl]-2-methoxybenzamide.

Sulpiride contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{15}H_{23}N_3O_4S$, calculated on the dried basis.

Category. Neuroleptic.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sulpiride IPRS* or with the reference spectrum of sulpiride.

B. In the test for impurity A, the principal spot in the chromatogram obtained with the test solution corresponds to the principal spot in the chromatogram obtained with reference solution (a).

C. To 1 mg in a porcelain dish, add 0.5 ml of *sulphuric acid* and 0.05 ml of *formaldehyde solution*. When examined under

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ultraviolet light at 365 nm (2.4.7); the solution shows blue fluorescence.

Tests

Appearance of solution. A 10.0 per cent w/v solution in *dilute acetic acid* is clear (2.4.1) and not more intensely coloured than YS6 (2.4.1).

Impurity A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 2 volumes of *concentrated ammonia*, 10 volumes of *dioxan*, 14 volumes of *methanol* and 90 volumes of *dichloromethane*.

Test solution. Dissolve 0.2 g of the substance under examination in *methanol* and dilute to 10 ml with *methanol*. Dilute 1.0 ml of the solution to 10.0 ml with *methanol*.

Reference solution (a). A 0.2 per cent w/v solution of *sulpiride IPRS* in *methanol*.

Reference solution (b). A 0.0002 per cent w/v solution of *sulpiride impurity A IPRS ([(2RS)-1-ethylpyrrolidin-2-yl]methanamine IPRS)* in *methanol*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 10 cm. Dry the plate in air and examine under ultraviolet light at 254 nm for identification test B. Then spray with *ninhydrin solution*, heat at 105° for 15 minutes and examine in daylight. Any spot in the chromatogram obtained with the test solution corresponding to sulpiride impurity A is not more intense than the correponding spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 3.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.01 per cent w/v each of *sulpiride IPRS* and *sulpiride impurity B IPRS (methyl 2-methoxy-5-sulfamoylbenzoate IPRS)* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 10 volumes of *acetonitrile*, 10 volumes of *methanol* and 80 volumes of a solution containing 0.68 per cent w/v of *potassium dihydrogen phosphate* and 0.1 per cent w/v of *sodium octanesulphonate*, adjusted to pH 3.3 using *orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to sulpiride impurity B and sulpiride is not less than 2.5.

Inject reference solution (a) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the sum of areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent).

Chlorides (2.3.12). Shake 1.0 g with 20 ml of *water*, filter. To 10 ml of the filtrate, add 5 ml of *water*. The solution complies with the limit test for chlorides (100 ppm), using 2 ml of *chloride standard solution (25 ppm Cl)*.

Iron (2.3.14). Ignite 1.0 g in a silica crucible. To the residue add 1 ml of *1 M hydrochloric acid*, 3 ml of *water* and 0.1 ml of *nitric acid*. Heat on a water-bath for a few minutes. Place the solution in a test-tube. Rinse the crucible with 4 ml of *water*. Collect the rinsings in the test-tube and dilute to 10 ml with *water*. The solution complies with the limit test for iron (10 ppm) using 0.5 ml of *iron standard solution (20 ppm Fe)*.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.25 g in 80 ml of *anhydrous acetic acid*. Titrate with *0.1 M perchloric acid*, determining the endpoint potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03414 g of $C_{15}H_{23}N_3O_4S$.

Sulpiride Tablets

Sulpiride Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sulpiride, $C_{15}H_{23}N_3O_4S$.

Usual strengths. 100 mg; 200 mg; 400 mg.

Identification

To a quantity of the powdered tablets containing 0.2 g of Sulpiride add 20 ml of *methanol*, shake for 5 minutes, filter and evaporate the filtrate to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the

spectrum with that obtained with *sulpiride IPRS* or with the reference spectrum of sulpiride.

Impurity A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 2 volumes of *13.5 M ammonia*, 10 volumes of *1,4-dioxan*, 14 volumes of *methanol* and 90 volumes of *dichloromethane*.

Test solution. Disperse a quantity of the powdered tablets containing 0.2 g of Sulpiride with 20 ml of *methanol*, shake for 5 minutes, filter, evaporate the filtrate to dryness and dissolve the residue in 10 ml of *methanol*.

Reference solution (a). A 0.02 per cent w/v solution of *sulpiride impurity A IPRS ([(2RS)-1-ethylpyrrolidin-2-yl]methanamine IPRS)* in *methanol*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with *methanol*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 10 cm. Dry the plate in air, spray with *ninhydrin solution*, heat at 105° for 15 minutes. Any spot in the chromatogram obtained with the test solution corresponding to the principal spot in the chromatogram obtained with reference solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

Related substances. Determine by liquid chromatography.

Test solution. Disperse a quantity of the powdered tablets containing 0.2 g of Sulpiride with 20 ml of *methanol*, shake for 5 minutes, filter, evaporate the filtrate to dryness and dissolve the residue in the mobile phase and dilute to 200.0 ml with the mobile phase.

Reference solution (a). Dilute 3.0 ml of the test solution to 100.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.01 per cent w/v each of *sulpiride IPRS* and *sulpiride impurity B IPRS (methyl 2-methoxy-5-sulphamoylbenzoate IPRS)* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 10 volumes of *acetonitrile*, 10 volumes of *methanol* and 80 volumes of a solution containing 6.8 per cent w/v of *potassium dihydrogen orthophosphate* and 0.1 per cent w/v of *sodium octanesulphonate*, adjusted to pH 3.3 with *orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 20 µl.

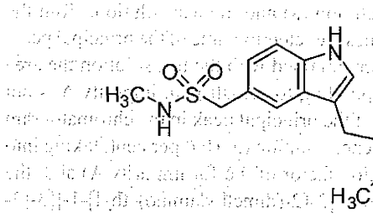
Inject reference solution (b). The test is not valid unless the resolution between the peaks corresponding to sulpiride impurity B and sulpiride is not less than 2.5.

Inject reference solution (a) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the sum of the areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.1 g of Sulpiride with 50 ml of *0.1 M sodium hydroxide* with the aid of ultrasound for 5 minutes, and dilute to 100.0 ml with *0.1 M sodium hydroxide*, filter, discarding the first 10 ml of filtrate. Dilute 5.0 ml of the filtrate to 100.0 ml with *0.1 M sodium hydroxide* and measure the absorbance of the solution at the maximum at 291 nm (2.4.7). Calculate the content of $C_{15}H_{23}N_3O_4S$ in the tablets from the absorbance of a 0.005 per cent w/v solution of *sulpiride IPRS* in *0.1 M sodium hydroxide*.

Sumatriptan



$C_{14}H_{21}N_3O_2S$ Mol. Wt. 295.4

Sumatriptan is 3-(2-dimethylaminoethyl)indol-5-yl-*N*-methylmethanesulphonamide.

Sumatriptan contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{14}H_{21}N_3O_2S$, calculated on the anhydrous basis.

Category. Antimigraine.

Description. A white to pale yellow powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sumatriptan IPRS* or with the reference spectrum of sumatriptan.

Tests

Impurities A and H. Determine by liquid chromatography (2.4.14).

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Solvent mixture. 1 volume of *acetonitrile* and 3 volumes of *0.025 M sodium dihydrogen orthophosphate*, adjusted to pH 6.5.

Test solution. A 0.2 per cent w/v solution of the substance under examination in the solvent mixture.

Reference solution (a). Dilute 1 ml of test solution (a) to 100 ml with the solvent mixture.

Reference solution (b). Dissolve the contents of a vial of *sumatriptan for system suitability IPRS* to 1 ml with *1 M hydrochloric acid.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with silica gel (5 µm) (Such as Spherisorb silica S5W),
- mobile phase: a mixture of 10 volumes of *10 M ammonium acetate* and 90 volumes of *methanol*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 282 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the resolution between [3-[2-(dimethylamino)ethyl]-2-[[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]-1*H*-indol-5-yl]-*N*- methylmethanesulphonamide (sumatriptan impurity A) and sumatriptan is not less than 1.5.

Inject reference solution (a) and the test solution. Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any peak corresponding to sumatriptan impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent, taking into account the correction factor of 0.6 for impurity A) and the area of any peak due to [3-[2-(dimethylamino)ethyl]-1-[[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]-1*H*-indol-5-yl]-*N*- methylmethanesulphona-mide (sumatriptan impurity H) is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 25 volumes of *acetonitrile* and 75 volumes of *0.025 M sodium dihydrogen orthophosphate*, adjusted to pH 6.5.

Test solution. A 0.2 per cent w/v solution of the substance under examination in the solvent mixture.

Reference solution (a). Dilute 1 ml of test solution (a) to 100 ml with the solvent mixture and further dilute 1 ml of the resulting solution to 10 ml with the solvent mixture.

Reference solution (b). Dilute the contents of a vial of *sumatriptan impurity mixture IPRS* to 1 ml with *0.1 M hydrochloric acid.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Spherisorb ODS 1),
- mobile phase: a mixture of 25 volumes of *acetonitrile* and 75 volumes of buffer solution prepared by dissolving 0.97 g of *dibutylamine*, 0.735 g of *orthophosphoric acid* and 2.93 g of *sodium dihydrogen phosphate* in 750 ml of *water*, adjusted to pH 6.5 with *sodium hydroxide solution* and diluted to 1000 ml with *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 282 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to [3-[2-(dimethylamino) ethyl]-1-(hydroxymethyl)-1*H*-indol-5-yl]-*N*- methylmethane-sulphonamide (sumatriptan impurity C) and sumatriptan is not less than 1.5.

Inject the reference solution (a) and the test solution. In the chromatogram obtained with the test solution the areas of any peaks corresponding to *N*-methyl[3-[2-(methylamino) ethyl]-1*H*-indol-5-yl]methanesulphonamide (sumatriptan impurity B), [3-[2-(dimethylamino)ethyl]-1-(hydroxymethyl)-1*H*-indol-5-yl]-*N*- methylmethanesulphonamide (sumatriptan impurity C) and *N*,*N*-dimethyl-2-[5-[(methylsulphamoyl) methyl]-1*H*-indol-3-yl]ethanamine *N*-oxide (sumatriptan impurity D) is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the area of any peak corresponding to [3-(2-aminoethyl)-1*H*-indol-5-yl]-*N*-methylmethanesulphonamide (sumatriptan impurity E) is not more than the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); the area of any other secondary peak is not more than the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent). The total impurity content in the test for Impurities A and H and in the test for Related substances is not more than 1.5 per cent. Ignore any peak with an area less than 0.5 times the area of the peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.3.43). Not more than 1.0 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14) as described under Related substances, using the following solutions.

Test solution. Dissolve 10 mg of the substance under examination in 100.0 ml of the solvent mixture.

Reference solution (a). A 0.014 per cent w/v solution of *sumatriptan succinate IPRS* in the solvent mixture.

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Reference solution (b). Dilute the contents of a vial of *sumatriptan impurity mixture IPRS* to 1 ml with *0.1 M hydrochloric acid.*

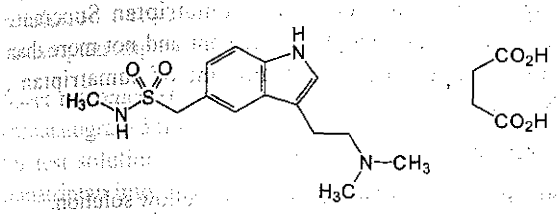
Inject reference solution (b). The test is not valid unless the resolution between the peaks due to sumatriptan and sumatriptan impurity C is not less than 1.5.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{14}H_{21}N_3O_2S$.

Storage. Store protected from light.

Sumatriptan Succinate



$C_{14}H_{21}N_3O_2S,C_4H_6O_4$ Mol. Wt. 413.5

Sumatriptan Succinate is [3-[2-(Dimethylamino)ethyl]-1*H*-indol-5-yl]-*N*-methylmethanesulphonamide hydrogen butanedioate.

Sumatriptan Succinate contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{18}H_{27}N_3O_6S$, calculated on the anhydrous basis.

Category. Antimigraine.

Description. A white or almost white powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sumatriptan succinate IPRS* or with the reference spectrum of sumatriptan succinate.

Tests

Solution A. Dissolve 1.0 g in *carbon dioxide-free water* and dilute to 25.0 ml with the same solvent.

pH (2.4.24). 4.5 to 5.3, Dilute 2.5 ml of solution A to 10 ml with *carbon dioxide-free water.*

Light absorption (2.4.7). Not more than 0.1, measured at 440 nm on solution A.

Impurities A and H. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 30 mg of the substance under examination in the mobile phase and dilute to 10.0 ml with the same solvent.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the same solvent.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with silica gel (5 µm),
- mobile phase: a mixture of 10 volumes of 77.1 per cent w/v solution of *ammonium acetate* and 90 volumes of *methanol,*
- flow rate: 2 ml per minute,
- spectrophotometer set at 282 nm,
- injection volume: 20 µl.

The relative retention time with reference to sumatriptan for sumatriptan impurity A ([3-[2-(dimethylamino)ethyl]-2-[[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]- 1*H*-indol-5-yl]-*N*-methylmethanesulphonamide) is about 2.2 and for sumatriptan impurity H ([3-[2-(dimethylamino)ethyl]-1-[[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]- 1*H*-indol-5-yl]-*N*-methylmethanesulphonamide) is about 3.0.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to sumatriptan impurity A is not more than 6 times the area of the principal peak in the chromatogram obtained with the reference solution (0.6 per cent) and the area of the peak due to sumatriptan impurity H is not more than 3 times the area of the principal peak in the chromatogram obtained with the reference solution (0.3 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Solution A. Dissolve 2.925 g of *sodium dihydrogen phosphate* in 600 ml of *water*, adjusted to pH 6.5 with *sodium hydroxide solution*, dilute to 750 ml with *water*, add 250 ml of *acetonitrile.*

Test solution (a). Dissolve 30 mg of the substance under examination in the mobile phase and dilute to 10.0 ml with the mobile phase.

Test solution (b). Dissolve 15 mg of the substance under examination in solution A and dilute to 100.0 ml with solution A.

Reference solution (a). Dilute 1.0 ml of test solution (a) to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

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Reference solution (b). Dissolve 15 mg of *sumatriptan succinate IPRS* in solution A and dilute to 100.0 ml with solution A.

Chromatographic system
- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 25 volumes of *acetonitrile* and 75 volumes of buffer solution prepared by dissolving 0.97 g of *dibutylamine*, 0.735 g of *phosphoric acid* and 2.93 g of *sodium dihydrogen phosphate* in 750 ml of *water*, adjusted to pH 6.5 with *sodium hydroxide solution* and dilute to 1000 ml with *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 282 nm,
- injection volume: 10 µl.

The relative retention time with reference to sumatriptan for sumatriptan impurity E ([3-(2-aminoethyl)-1*H*-indol-5-yl]-*N*-methylmethanesulphonamide) is about 0.5, for sumatriptan impurity B (*N*-methyl[3-[2-(methylamino)ethyl]-1*H*-indol-5- yl]methanesulphonamide) is about 0.6, for sumatriptan impurity D (*N,N*-dimethyl-2-[5-[(methylsulphamoyl)methyl]-1*H*-indol-3-yl]ethanamine *N*- oxide) is about 0.7 and for sumatriptan impurity C ([3-[2-(dimethylamino)ethyl]-1-(hydroxymethyl)-1*H*-indol-5-yl]-*N*-methylmethanesulphonamide) is about 0.8.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (a) and test solution (a). Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with test solution (a), the area of any peak due to sumatriptan impurity B, C and D is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). The area of any peak due to sumatriptan impurity E is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.3.43). Not more than 1.0 per cent, determined on 0.5 g.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Determine by liquid chromatography (2.4.14), as described under test for Related substances with the following modification.

Inject reference solution (b) and test solution (b).

Calculate the content of $C_{18}H_{27}N_3O_6S$.

Storage. Store protected from light.

Sumatriptan Injection

Sumatriptan Succinate Injection

Sumatriptan Injection is a sterile isotonic solution of Sumatriptan Succinate in Water for Injections.

Sumatriptan Injection contains Sumatriptan Succinate equivalent to not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of sumatriptan, $C_{14}H_{21}N_3O_2S$.

Usual strength. 6 mg per 0.5 ml.

Description. A clear, colourless to pale yellow solution.

Identification

To a volume of the injection containing equivalent to 30 mg of sumatriptan, add 1 ml of *saturated sodium chloride solution* and 1ml of *saturated sodium carbonate solution*. Shake vigorously for 30 seconds, add two quantities of 2 ml of *propan-2-ol*, shake, allow to separate (this may take up to 24 hours) and discard the aqueous layer. Evaporate under a stream of nitrogen and dry at 100°. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum obtained with *sumatriptan succinate IPRS* treated in the same manner or with the reference spectrum of sumatriptan.

Tests

pH (2.4.24). 4.2 to 5.3.

Impurities A and H. Determine by liquid chromatography (2.4.14).

Solvent mixture. 75 volumes of *0.025 M sodium dihydrogen orthophosphate*, adjusted to pH 6.5 and 25 volumes of *acetonitrile*.

Test solution. Dilute a volume of injection in the solvent mixture to obtain a solution containing 0.214 per cent w/v of sumatriptan.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture.

Reference solution (b). Dilute the contents of a vial of *sumatriptan for system suitability IPRS* (containing

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sumatriptan impurity A ([3-[2-(dimethylamino)ethyl]-2-[[3-[2-(dimethyl-amino)ethyl]-1*H*-indol-5-yl]methyl]-1*H*-indol-5-yl]-*N*-methylmethane sulphonamide) and sumatriptan impurity H ([3-[2-(dimethylamino)ethyl]-1-[[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]-1*H*-indol-5-yl]-*N*-methylmethane-sulphonamide) to 1.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with silica (5 µm) (Such as Spherisorb silica S5W),
- mobile phase: a mixture of 10 volumes of *10 M ammonium acetate* and 90 volumes of *methanol*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 282 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to sumatriptan and sumatriptan impurity A is not less than 1.5.

Inject reference solution (a) and the test solution. Run the chromatogram 5 times the retention time of the principal peak for test solution. The area of any peak corresponding to sumatriptan impurity A, multiplied with correction factor of 0.6, is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent) and the area of any peak corresponding to sumatriptan impurity H is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 75 volumes of *0.025 M sodium dihydrogen orthophosphate*, adjusted to pH 6.5 and 25 volumes of *acetonitrile*.

Test solution. Dilute a volume of injection in the solvent mixture to obtain a solution containing 0.21 per cent w/v of sumatriptan.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Spherisorb ODS 1),
- mobile phase: a mixture of 75 volumes of a solution containing 0.97 g of *dibutylamine*, 0.735 g of *orthophosphoric acid* and 2.93 g of *sodium dihydrogen orthophosphate* in 750 ml of *water*, adjusted to pH 7.5 with *10 M sodium hydroxide*, diluted to 1000 ml with *water* and 25 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 282 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Sumatriptan impurity D[1]	0.2	—
Sumatriptan impurity 2[2]	0.3	0.3
Sumatriptan impurity 1[3]	0.4	0.2
Sumatriptan impurity E[4]	0.5	—
Sumatriptan impurity B[5]	0.6	—
Sumatriptan impurity F[6]	0.7	—
Sumatriptan impurity C[7]	0.8	—
Sumatriptan (Retention time is about 17 minutes)	1.0	—

[1] *N,N*-dimethyl-2-[5-[(methylsulfamoyl)methyl]-1*H*-indol-3-yl]ethanamine N-oxide,

[2] 1-{3-[2-(dimethylamino)ethyl]-3-hydroxy-2-oxo-2,3-dihydro-1*H*-indol-5-yl}-*N*-methylmethanesulfonamide,

[3] 3a-hydroxy-1,1-dimethyl-5-{[(methylamino)sulfonyl]methyl}-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indol-1-ium trifluoroacetate,

[4] [3-(2-aminoethyl)-1*H*-indol-5-yl]-*N*-methylmethanesulfonamide,

[5] R1 = R2 = H: N-methyl[3-[2-(methylamino)ethyl]-1*H*-indol-5-yl] methanesulfonamide,

[6] R = H: N-methyl(2,3,4,9-tetrahydro-1*H*-pyrido[3,4-b]indol-6-yl) methanesulfonamide,

[7] R1=CH2-OH, R2 = CH3: [3-[2-(dimethylamino)ethyl]-1-(hydroxymethyl)-1*H*-indol-5-yl]-*N*-methylmethanesulfonamide.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. Run the chromatogram 4 times the retention time of the principal peak for test solution. The area of any peak corresponding to sumatriptan impurity 1, multiplied with correction factor 0.2, is not more than 1.5 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent), the area of any peak corresponding to sumatriptan impurity 2, multiplied with correction factor 0.3, is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent) and the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent). The total impurity content in test for impurities A and H and the test for Related substances is not more than 4.0 per cent.

Bacterial endotoxins (2.2.3). Not more than 29.2 Endotoxin Units per mg of sumatriptan.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

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Solvent mixture. 75 volumes of *0.025 M sodium dihydrogen orthophosphate*, adjusted to pH 6.5 and 25 volumes of *acetonitrile*.

Test solution. Dilute a volume of injection in the solvent mixture to obtain a solution containing 0.021 per cent w/v of sumatriptan.

Reference solution. A 0.03 per cent w/v solution of *sumatriptan succinate IPRS* in the solvent mixture.

Use chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{14}H_{21}N_3O_2S$ in the injection.

1 mg of $C_{14}H_{21}N_3O_2S$ is equivalent to 1.4 mg of $C_{14}H_{21}N_3O_2S$, $C_4H_6O_4$.

Storage. Store protected from light.

Surgical Spirit

Surgical Spirit contains 0.45 per cent to 0.55 per cent v/v of methyl salicylate, $C_8H_8O_3$ and 1.8 cent to 2.2 per cent v/v of diethyl phthalate, $C_{12}H_{14}O_4$.

Surgical Spirit contains	
Methyl Salicylate	5 ml
Diethyl Phthalate	20 ml
Castor Oil	25 ml
Industrial Methylated Spirit	Sufficient to produce 1000 ml.

Category. Skin disinfectant.

Identification

A. To 1 ml, add 0.5 ml of 0.4 M *iron (III) chloride hexahydrate*, a violet colour is produced.

B. To 1 ml, add 50 mg of *resorcinol* and 1 ml of *sulphuric acid*, heat for 1 minute, cool, pour into *water* and make alkaline with *5 M sodium hydroxide*; a distinct green fluorescence is produced which disappears when the solution is made acidic and reappears when it is made alkaline.

Tests

Weight per ml (2.4.29). 0.817 to 0.827 g.

Assay. *For methyl salicylate* —

Dilute 5 ml to 100 ml with *ethanol*, further dilute 10 ml of the solution to 100 ml with *ethanol* and measure the absorbance at the maximum at 306 nm (2.4.7). Calculate the content of $C_8H_8O_3$ taking 335 as the specific absorbance at 306 nm.

For diethyl phthalate—

Further dilute 10 ml of the solution prepared in the Assay for methyl salicylate to 50 ml with *ethanol* and measure the absorbance of the resulting solution at 227 nm (2.4.7). Subtract from the observed absorbance the absorbance due to the methyl salicylate present, as determined above, taking 432 as the specific absorbance of methyl salicylate. For calculation, take 419 as the specific absorbance of diethyl phthalate at 227 nm.

Storage. Store protected from moisture.

Labelling. The label states that the preparation is flammable and should be kept away from a naked flame.

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T

Tacrolimus 3701
Tacrolimus Capsules 3703
Tadalafil 3706
Tadalafil Tablets 3708
Talc 3709
Tamoxifen Citrate 3710
Tamoxifen Tablets 3711
Tamsulosin Hydrochloride 3713
Tamsulosin Hydrochloride Prolonged-release Capsules 3714
Tamsulosin Hydrochloride Prolonged-release and Dutasteride Capsules 3715
Tapentadol Hydrochloride 3718
Tartaric Acid 3719
Tauroursodeoxycholic Acid 3719
Tazobactam 3720
Tazobactam Sodium 3721
Teicoplanin 3722
Teicoplanin Injection 3724
Telmisartan 3726
Telmisartan Tablets 3727
Telmisartan and Amlodipine Tablets 3728
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Temozolomide 3732
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Teneligliptin Hydrobromide Hydrate 3735
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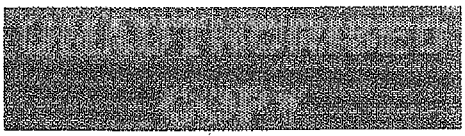
Tenofovir Alafenamide Fumarate 3740
Tenofovir Alafenamide Tablets 3743
Tenofovir Disoproxil Fumarate 3745
Tenofovir Disoproxil Fumarate Tablets 3746



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Tenofovir and Emtricitabine Tablets 3748
Tenofovir Disoproxil Fumarate, Lamivudine and Efavirenz Tablets 3749
Tenoxicam 3751
Tenoxicam Tablets 3752
Terazosin Hydrochloride 3753
Terazosin Tablets 3755
Terbinafine Hydrochloride 3756
Terbinafine Cream 3757
Terbinafine Tablets 3758
Terbutaline Sulphate 3760
Terbutaline Inhalation 3761
Terbutaline Injection 3762
Terbutaline Tablets 3762
Testosterone Propionate 3764
Testosterone Propionate Injection 3764
Tetracycline 3765
Tetracycline Hydrochloride 3766
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Theophylline Prolonged-release Tablets 3771
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Thiabendazole Tablets 3773
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Thiocolchicoside Capsules 3776
Thioguanine 3777
Thioguanine Tablets 3779
Thiomersal 3780
Thiopentone Sodium 3781
Thiopentone Injection 3782
Thiotepa 3784

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Thiotepa Injection 3785
Thymol 3786
Thyroxine Sodium 3787
Thyroxine Tablets 3788
Tibolone 3790
Tibolone Tablets 3791
Ticagrelor 3792
Ticagrelor Tablets 3794
Ticarcillin Monosodium 3796
Ticarcillin and Clavulanic Acid Injection 3797
Tigecycline 3798
Tigecycline Injection 3800
Timolol Maleate 3802
Timolol Eye Drops 3804
Timolol Tablets 3804
Tinidazole 3806
Tinidazole Tablets 3807
Tiotropium Bromide Monohydrate 3807
Tiotropium Powder for Inhalation 3809
Titanium Dioxide 3809
Tizanidine Hydrochloride 3810
Tizanidine Tablets 3811
Tobramycin 3812
Tobramycin Injection 3814
Tobramycin Inhalation Solution 3815
Tobramycin and Fluorometholone Acetate Ophthalmic Suspension 3817
Tofacitinib Citrate 3818
Tofacitinib Tablets 3820
Tofluprost 3822
Tolazamide 3823
Tolazamide Tablets 3823
Tolbutamide 3824
Tolbutamide Tablets 3825

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Tolnaftate 3826
Tolnaftate Cream 3827
Tolnaftate Gel 3828
Tolnaftate Topical Powder 3828
Tolnaftate Topical Solution 3829
Tolterodine Tartrate 3829
Tolterodine Tartrate Tablets 3830
Tolvaptan 3832
Topiramate 3833
Topiramate Tablets 3833
Topotecan Hydrochloride 3834
Topotecan Injection 3836
Torsemide 3836
Torsemide Tablets 3837
Tramadol Hyrochloride 3839
Tramadol Capsules 3840
Tramadol Prolonged-release Tablets 3841
Trandolapril 3842
Trandolapril Tablets 3843
Tranexamic Acid 3845
Tranexamic Acid Injection 3846
Tranexamic Acid Tablets 3847
Tranilast 3848
Travoprost 3849
Travoprost Eye Drops 3850
Trazodone Hydrochloride 3851
Trazodone Tablets 3853
Triamcinolone 3854
Triamcinolone Tablets 3855
Triamcinolone Acetonide 3856
Triamcinolone Acetonide Injection 3858
Triamterene 3859
Triamterene Capsules 3860
Triamterene and Hydrochlorothiazide Tablets 3860

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Tributyl Citrate 3862
Trichloromonofluoromethane 3862
Triclofos Sodium 3863
Triclofos Oral Solution 3864
Triethyl Citrate 3865
Trifluoperazine Hydrochloride 3866
Trifluoperazine Injection 3866
Trifluoperazine Tablets 3867
Triflupromazine Hydrochloride 3868
Triflupromazine Injection 3868
Triflupromazine Tablets 3869
Trifluridine 3870
Trifluridine Eye Drops 3870
Trimebutine Maleate 3871
Trimetazidine Hydrochloride 3872
Trimetazidine Tablets 3874
Trimethobenzamide Hydrochloride 3874
Trimethoprim 3875
Trimethoprim Tablets 3877
Trimethoprim and Sulphamethoxazole Oral Suspension 3878
Trimethoprim and Sulphamethoxazole Tablets 3878
Triprolidine Hydrochloride 3880
Triprolidine Tablets 3881
Trisodium Edetate Concentrate for Injection 3881
Tropicamide 3882
Tropicamide Eye Drops 3883
Trospium Chloride 3884
Trospium Chloride Tablets 3885
Troxidone 3886
Troxidone Capsules 3887
Tubocurarine Chloride 3888
Tubocurarine Injection 3889
Tyrothricin 3890

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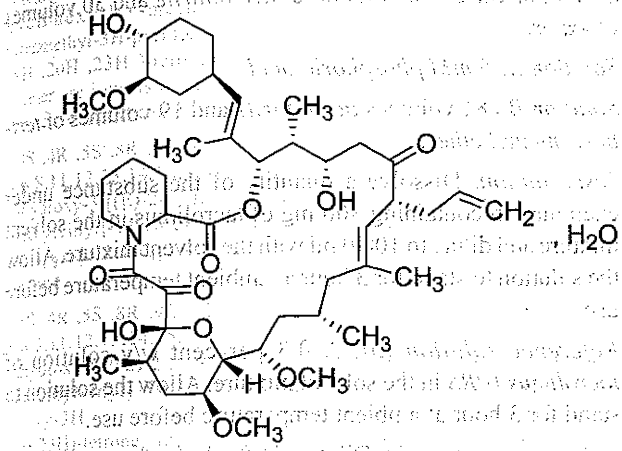
Tacrolimus



$C_{44}H_{69}NO_{12},H_2O$ Mol. Wt. 822.03

Tacrolimus is *E*-(7*R*,8*R*,10*S*,11*R*,12*S*,14*S*,18*R*,21*S*,22*R*,23*S*)-18-Allyl-1,2,3,4,6,7,8,9,10,11,12,13,14,15,18,19,20,21,22,23,25,25a-docosahydro-7,21-dihydroxy-23-{(*E*)-2-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl}-10,12-dimethoxy-8,14,16,22-tetramethyl-7,11-epoxy-5*H*-24-oxa-4a-azabenzocyclotricosene-5,6,19,25-tetrone.

Tacrolimus contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{44}H_{69}NO_{12}$, calculated on the anhydrous and solvent-free basis.

Category. Immunosuppressive; immunomodulator.

Description. White Crystals or white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tacrolimus IPRS* or with the reference spectrum of tacrolimus.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Specific optical rotation (2.4.22). – 115.0° to – 110.0°, calculated on as is basis and determined in a 1.0 per cent w/v solution in *dimethylformamide*.

Related substances. Determine by liquid chromatography (2.4.14).

A. Determine by liquid chromatography (2.4.14).

NOTE — Used when the impurity profile includes tacrolimus methylacrylaldehyde and tacrolimus diene. Condition the new column with 500 ml of ethanol before use.

Test solution. Dissolve 50 mg of substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution. A 0.01 per cent w/v solution each of *tacrolimus IPRS* and *tacrolimus impurity A IPRS* in the mobile phase.

Chromatographic system

- two stainless steel columns 25 cm x 4.6 mm, packed with dihydroxy propane chemically bonded to porous silica (3 to 10 µm),
- mobile phase: a mixture of 70 volumes of *hexane*, 20 volumes of *n-butyl chloride* and 10 volumes of *acetonitrile* (*NOTE — Add n-butyl chloride to hexane, mix well before adding acetonitrile. After adding acetonitrile, mix the mobile phase for 2 hours to get a clear solution*),
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Tacrolimus methylacryl aldehyde[1]	0.55	0.06
Tacrolimus diene[2]	0.79	0.45
Tacrolimus impurity A[3]	0.96	1.0
Tacrolimus impurity B[4]	0.96	—
Tacrolimus	1.0	1.0
Tacrolimus 19-epimer[5]	1.1	—
Tacrolimus open ring[6]	1.3	—

[1](E)-3-[[(1R ,3R ,4R)-4-Hydroxy-3-methoxycyclohexyl]-2-methylacrylaldehyde,

[2](14E ,18E)-17-Allyl-1-hydroxy-12-[(E)-2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.04,9] octacosa-14,18-diene-2,3,10,16-tetrone,

[3]Specified unidentified impurity,

[4](E)-8-Ethyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-Hexadecahydro-5,19-dihydroxy-3-[(E)-2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-3H -pyrido[2,1-c][1,4]oxaazacyclotricosine-1,7,20,21-(4H ,23H)-tetrone For informational purposes only; not to be reported,

[5](3S, 4R, 5S, 8R, 9E, 12S, 14S, 15R, 16S, 18R, 19S, 26aS)-8-Allyl 5,6,8, 11, 12, 13, 14, 15, 16, 17, 18, 19, 24, 25, 26, 26a, hexadecahydro-5,19-dihydroxy-3-{(E)-2-[1R,3R,4R)-4-hydroxy-3-methoxy-cyclohexyl]-1-methylvinyl}-14,16,dimethoxy-4,10,12,18-tetramethyl-15,19-expoxy-3H-pyrido[2,1-c][1,4]oxaazacyclotricosine 1,7,20,21(4H, 23H)-tetrone. For informational purposes only; not to be reported,

[6](3S, 4R, 5S, 8R, 9E, 12S, 14S, 15R, 16S, 18R, 19S, 26aS, E)-)-8-Allyl 5,6,8, 11, 12, 13, 14, 15, 16, 17, 18, 19, 24, 25, 26, 26a-tetradecahydro-5,15,20,20-tetrahydroxy-3-{(E)-2-[1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl}-14,16-dimethoxy-4,10,12,18-tetramethyl-3H-pyrido[2,1-c][c][1,4], oxaazacyclotricosine-1,7,19,21 (4H, 8H, 20H, 23H)-tetrone. For informational purposes only; not to be reported.

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Inject the reference solution. The test is not valid unless the resolution between tacrolimus and tacrolimus impurity A is not less than 1.1 and the tailing factor is not more than 1.5.

Inject the test solution. The area of any peak due to tacrolimus methylacryl aldehyde, tacrolimus diene and tacrolimus impurity A is not more than 0.2 per cent, the area of any other secondary peak is not more than 0.2 per cent and the sum of the areas of all the secondary peaks is not more than 0.3 per cent, calculated by area normalization method.

NOTE — If the related substance test other than A is used the label states the article complies with related substance test B.

B. Determine by liquid chromatography (2.4.14). Use solvent mixture, solution A, solution B, reference solution (a), reference solution (b), test solution and Chromatographic system as described under Assay with the following modifications.

NOTE — Used when the impurity profile includes ascomycin, desmethyl tacrolimus, tacrolimus 8-epimer, and tacrolimus 8-propyl analog.

Inject reference solution (b). The test is not valid unless the relative standard deviation for replicate injections for sum of the responses of tacrolimus and tacrolimus 19-epimer is not more than 10.0 per cent.

Inject reference solution (b) and the test solution. In the chromatogram obtained with test solution the area of any peak due to ascomycin 19-epimer and desmethyl tacrolimus is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent), the area of any peak due to ascomycin is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any peak due to tacrolimus 8-epimer is not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent), the area of any peak due to tacrolimus 8-propyl analog is not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent), the area of any other secondary peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of the areas of all the secondary peaks excluding tacromilus open ring and tacrolinus epimer is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore any peak with area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 4.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Protect the solutions from light by using low-actinic glassware.

Solvent mixture. 70 volumes of *acetonitrile* and 30 volumes of *water*.

Solution A. 6 mM phosphoric acid.

Solution B. 81 volumes *acetonitrile* and 19 volumes of *tert-butyl methyl ether*.

Test solution. Dissolve a quantity of the substance under examination containing 300 mg of tacrolimus in the solvent mixture and dilute to 100.0 ml with the solvent mixture. Allow the solution to stand for 3 hour at ambient temperature before use.

Reference solution (a). A 0.3 per cent w/v solution of *tacrolimus IPRS* in the solvent mixture. Allow the solution to stand for 3 hour at ambient temperature before use.

Reference solution (b). Dilute of 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (3 µm),
- column temperature: 60°,
- sample temperature: 4°,
- mobile phase: A. 80 volumes of solution A and 20 volumes of solution B,
  B. 80 volumes of solution B and 20 volumes of solution A,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	72	28
30	72	28
53	15	85
54	72	28
60	72	28

Name	Relative retention time
Tacrolimus open ring[1]	0.52
Ascomycin 19-epimer[2]	0.54
Tacrolimus 19-epimer[3]	0.63
Ascomycine	0.87
Desmethyl Tacrolimus[4]	0.94
Tacrolimus	1.0
Tacrolimus 8-epimer[5]	1.28
Tacrolimus 8-propyl analog[6]	1.33

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(3S,4R,5S,8R,12S,14S,15R,16S,18R,26aS,E)-8-Allyl-5,6,11,12,13,14,15,16,17,18,24,25,26,26a-tetradecahydro-5,15,20,20-tetrahydroxy-3-{(E)-2-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl}-14,16-dimethoxy-4,10,12,18-tetramethyl-3H-pyrido[2,1-c][1,4]oxaaza-cyclotricosine-1,7,19,21(4H,8H,20H,23H)-tetrone. Tacrolimus open ring and tacrolimus 19-epimer are isomers of tacrolimus, which are present in equilibrium with the active ingredient. They are not to be reported as degradation products

(3S,4R,5S,8R,9E,12S,14S,15R,16S,18R,19S,26aS)-8-Ethyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-[(E)-2-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclotricosine-1,7,20,21-(4H,23H)-tetrone.

(3S,4R,5S,8R,9E,12S,14S,15R,16S,18R,19S,26aS)-8-Allyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-{(E)-2-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl}-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclotricosine-1,7,20,21(4H,23H)-tetrone. Tacrolimus open ring and tacrolimus 19-epimer are isomers of tacrolimus, which are present in equilibrium with the active ingredient. They are not to be reported as degradation products.

(3S,4R,5S,8R,9E,12S,14S,15R,16S,18R,19R,26aS)-8-Ethyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-[(E)-2-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclotricosine-1,7,20,21-(4H,23H)-tetrone.

(3S,4R,5S,8R,9E,12S,14S,15R,16S,18R,19R,26aS)-8-Allyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-[(E)-2-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl]-14,16-dimethoxy-4,12,18-trimethyl-15,19-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclotricosine-1,7,20,21-(4H,23H)-tetrone.

(3S,4R,5S,8S,9E,12S,14S,15R,16S,18R,19R,26aS)-8-Allyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-{(E)-2-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl}-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclotricosine 1,7,20,21(4H,23H)-tetrone.

Inject reference solution (a). The test is not valid unless the relative standard deviation of replicate injections is not more than 2.0 per cent for the sum of responses of tacrolimus, tacrolimus open ring, and tacrolimus 19-epimer.

Inject reference solution (a) and the test solution.

Calculate the content of tacrolimus $C_{44}H_{69}NO_{12}$ in the portion of tacrolimus taken by the formula

$$\frac{rU}{rS} \times \frac{CS}{CU} \times 100$$

in which *rU* is the sum of the peak responses of tacrolimus and tacrolimus 19-epimer from the test solution, *rS* is the sum of the peak responses of tacrolimus and tacrolimus 19-epimer from the reference solution and *CS* and *CU* are the concentration of reference solution and test solution respectively.

Storage. Store protected from moisture and at a controlled temperature.

Tacrolimus Capsules

Tacrolimus Capsules contain not less than 93.0 per cent and not more than 105.0 per cent of the stated amount of tacrolimus, $C_{44}H_{69}NO_{12}$.

Usual strengths. 0.5 mg; 1 mg; 2 mg; 5 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

NOTE — Allow reference solution (b) to stand for 3 hour at ambient temperature before use. Protect the solution from light by using low-actinic glassware.

Apparatus No. 2 (Paddle) (Use sinkers, if required);

Medium. 900 ml of buffer solution prepared by dissolving 6 g of *sodium dodecylsulphate* and 8.28 g *monobasic sodium phosphate* in 6000 ml of *water,* adjusted to pH 7.0 with *2 M sodium hydroxide,*

Speed and time. 50 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate as given above, suitably diluted if necessary

Reference solution (a). Dissolve 20 mg of *tacrolimus IPRS* in 30 ml of *ethanol (95 per cent)* and dilute to 100.0 ml with the dissolution medium.

Reference solution (b). Dilute reference solution (a) to obtain a 0.0005 per cent w/v solution of *tacrolimus IPRS* in the dissolution medium. Dilute further with dissolution medium, if necessary.

Chromatographic system

- a stainless steel column 5.5 cm x 4.0 mm, packed with endcapped octadecylsilane bonded to porous silica (3 µm),
- column temperature: 60°,
- mobile phase: a mixture of 600 volumes of *6mM orthophosphoric acid,* 50 volumes of *tert-butyl ether* and 335 volumes of *acetonitrile,*
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 100 µl.

The relative retention time for tacrolimus 19 epimer and tacrolimus are about 0.67 and 1.0 respectively.

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Inject reference solution (b). The test is not valid unless the tailing factor is not more than 2 and the relative standard deviation for replicate injections is not more than 5.0 per cent for the sum of the areas of tacrolimus and tacrolimus 19-epimer.

Inject reference solution (b) and the test solution.

Calculate the content of $C_{44}H_{69}NO_{12}$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{44}H_{69}NO_{12}$.

Related substances. A. Determine by liquid chromatography (2.4.14).

NOTE — Used when the impurity profile includes tacrolimus diene and tacrolimus regioisomer. Conditioned the new column with 500 ml of ethanol before use to meet the resolution criteria. Prepare the solution immediately before use.

Test solution. Transfer the mixed contents of the capsules containing about 10 mg of Tacrolimus (5 mg, in case of capsules having label claim of 0.5 mg) to a centrifuge tube. Add 1.5 ml of a mixture of 2 volumes of *n-butyl chloride* and 1 volume of *acetonitrile*, mix with the aid of ultrasound add 3.5 ml of *n-hexane*, mix. Centrifuge the solution, and collect the supernatant or pass the solution through a 0.45-µm membrane filter.

Reference solution. A 0.01 per cent w/v solution each of *tacrolimus IPRS* and *tacrolimus impurity A* in mobile phase.

Chromatographic system

- two stainless steel column 25 cm x 4.6 mm, packed with dihydoxypropane groups bonded to porous silica (5 µm),
- mobile phase: a mixture of 70 volumes of *hexane*, 20 volumes of *n-butyl chloride* and 10 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Tacrolimus diene[1]	0.79	0.45
Tacrolimus regioisomer[2]	0.88	—
Tacrolimus impurity A[3]	0.96	—
Tacrolimus impurity B[4]	0.96	—
Tacrolimus	1.0	—
Tacrolimus 19- epimer[5]	1.1	—
Tacrolimus open ring[6]	1.3	—

[1](14E,18E)-17-Allyl-1-hydroxy-12-[(E)-2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.04,9]octacosa-14,18-diene-2,3,10,16-tetrone.

[2](4E,11E)-10-Allyl-7,8,10,13,14,15,16,17,18,19,20,21,26,22,28,28a-hexadecahydro-7,21-dihydroxy-3-(4-hydroxy-3-methoxycyclohexyl)-16,18-dimethoxy-4,6,12,14,20-pentamethyl-17,21-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclopentacosine-1,9,22,23(6H,25H)-tetrone.

[3]Tacrolimus impurity A is a specified, unidentified impurity.

[4]For information only, not to be reported.

[5](3S,4R,5S,8R,9E,12S,14S,15R,16S,18R,19S,26aS)-8-Allyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-{(E)-2-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl}-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-3H-pyrido[2,1c][1,4]oxaazacyclotricosine-1,7,20,21(4H,23H)-tetrone. For information only, not to be reported.

[6](3S,4R,5S,8R,12S,14S,15R,16S,18R,26aS,E)-8-Allyl5,6,11,12,13,14,15,16,17,18,24,25,26,26a-tetradecahydro-5,15,20,20-tetrahydroxy-3-{(E)-2-[(1R,3R,4R)-4-hydroxy-3methoxycyclohexyl]-1-methylvinyl}-14,16-dimethoxy-4,10,12,18-tetramethyl-3H pyrido[2,1c][1,4]oxaazacyclotricosine-1,7,19,21(4H,8H,20H,23H)-tetrone. For information only, not to be reported.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to tacrolimus and tacrolimus impurity A is not less than 1.1 and the tailing factor is not more than 2.0.

Inject the test solution. Run the chromatogram 3 times the retention time of tacrolimus. The area of the peak corresponding to tacrolimus diene is not more than 0.3 per cent, the area of any peak due to tacrolimus regioisomer is not more than 0.5 per cent, the area of any peak due to tacrolimus impurity A is not more than 0.3 per cent. The area of any secondary peak is not more than 0.2 per cent and the sum of areas of all the secondary peaks excluding tacrolimus open ring and tacrolimus 19 epimer is not more than 1.0 per cent, calculated by area normalization.

NOTE — If the related substance test other than A is used the label states the article complies with related substance test B.

B. Determine by liquid chromatography (2.4.14).

NOTE — Used when the impurity profile includes tacrolimus 21-carboxylic acid and tacrolimus 8-epimer. It is suggested to equilibrate the column overnight with a mixture of 85 volumes of mobile phase A and 15 volumes of mobile phase B before performing this method. Allow reference solution (a), reference solution (b) and test solution to stand for 3 hour at ambient temperature before use. Protect the solution from light using low-actinic glassware.

Solution A. 81 volumes of *acetonitrile* and 19 volumes of *tert-butyl methyl ether.*

Solution B. Prepared by dissolving 50 g of *polyoxyethylene (23) lauryl ether* in 1000 ml *6 mM orthophosphoric acid.*

Solvent mixture. 70 volumes of *acetonitrile* and 30 volumes of solution B.

Test solution. Disperse a quantity of the mixed content of capsules containing 15 mg of Tacrolimus in the solvent mixture

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with the aid of ultrasound, and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). A 0.00075 per cent w/v solution of *tacrolimus IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 5.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecysilane bonded to porous silica (3 µm),
- column temperature: 60°,
- mobile phase: A. 80 volumes of *6 mM orthophosphoric acid* and 20 volumes of solution A,
  B. 20 volumes of *6 mM orthophosphoric acid* and 80 volumes of solution A,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 40 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	74	26
45	74	26
60	15	85
75	15	85
76	74	26
85	74	26

Name	Relative retention time
Tacrolimus hydroxyl acid[1]	0.18
Tacrolimus open ring[2]	0.49
Ascomycin 19-epimer[3]	0.52
Tacrolimus 19-epimer[4]	0.62
Ascomycin[5]	0.84
Desmethyl Tacrolimus[6]	0.91
Tacrolimus	1.0
Tacrolimus 8-epimer[7]	1.28
Tacrolimus 8-propyl analog[8]	1.3

[1]2-[(2*R* ,3*R* ,5*S* ,6*R*)-6-{(1*S* ,3*S* ,5*E* ,7*R* ,10*S* ,11*R* ,12*S* ,13*E*)-7-Allyl-10-hydroxy-14-{(1*R* ,3*R* ,4*R*)-4-hydroxy-3-methoxycyclohexyl}-1-methoxy-3,5,11,13-tetramethyl-8-oxo-12-[(*S*)-piperidine-2-carbonyloxy]tetradeca-5,13-dienyl}-2-hydroxy-5-methoxy-3-methyltetrahydro-2*H* -pyran-2-yl]-2-oxoacetic acid,

[2](3*S* ,4*R* ,5*S* ,8*R* ,12*S* ,14*S* ,15*R* ,16*S* ,18*R* ,26a*S* ,*E*)-8-Allyl-5,6,11,12,13,14,15,16,17,18,24,25,26,26a-tetradecahydro-5,15,20,20-tetrahydroxy-3-{(*E*)-2-[(1*R* ,3*R* ,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl}-14,16-dimethoxy-4,10,12,18-tetramethyl-3*H* -pyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,19,21(4*H* ,8*H*,20*H*,23*H*)-tetrone. Tacrolimus open ring and tacrolimus 19-epimer are isomers oftacrolimus, which are present in equilibrium with the active ingredient. They are not to be reported as degradation products,

[3](3*S* ,4*R* ,5*S* ,8*R* ,9*E* ,12*S* ,14*S* ,15*R* ,16*S* ,18*R* ,19*S* ,26a*S*)-8-Ethyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-[(*E*)-2-[(1*R* ,3*R* ,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-3*H* -pyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21-(4*H* ,23*H*)-tetrone,

[4](3*S* ,4*R* ,5*S* ,8*R* ,9*E* ,12*S* ,14*S* ,15*R* ,16*S* ,18*R* ,19*S* ,26a*S*)-8-Allyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-{(*E*)-2-[(1*R* ,3*R* ,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl}-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-3*H*-pyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(4*H* ,23*H*)-tetrone. Tacrolimus open ring and tacrolimus 19-epimer are isomers of tacrolimus, which are present in equilibrium with the active ingredient. They are not to be reported as degradation products.,

[5](3*S* ,4*R* ,5*S* ,8*R* ,9*E* ,12*S* ,14*S* ,15*R* ,16*S* ,18*R* ,19*R* ,26a*S*)-8-Ethyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-[(*E*)-2-{(1*R* ,3*R* ,4*R*)-4-hydroxy-3-methoxycyclohexyl}-1-methylvinyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-3*H* -pyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21-(4*H*, 23*H*)-tetrone. These are process impurities that are controlled in the drug substance. They are not to be reported in the drug product,

[6](3*S* ,4*R* ,5*S* ,8*R* ,9*E* ,12*S* ,14*S* ,15*R* ,16*S* ,18*R* ,19*R* ,26a*S*)-8-Allyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-[(*E*)-2-[(1*R* ,3*R* ,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl]-14,16-dimethoxy-4,12,18-trimethyl-15,19-epoxy-3*H* -pyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21-(4*H* ,23*H*)-tetrone. These are process impurities that are controlled in the drug substance. They are not to be reported in the drug product,

[7](3*S* ,4*R* ,5*S* ,8*S* ,9*E* ,12*S* ,14*S* ,15*R* ,16*S* ,18*R* ,19*R* ,26a*S*)-8-Allyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-{(*E*)-2-[(1*R* ,3*R* ,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl}-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-3*H*-pyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(4*H*, 23*H*)-tetrone,

[8](3*S* ,4*R* ,5*S* ,8*R* ,9*E* ,12*S* ,14*S* ,15*R* ,16*S* ,18*R* ,19*R* ,26a*S*)-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-{(*E*)-2-[(1*R* ,3*R* ,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylvinyl}-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-8-propyl-3*H*-pyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(4*H* ,23*H*)-tetrone. These are process impurities that are controlled in the drug substance. They are not to be reported in the drug product.

Inject reference solution (b). The test is not valid unless, the signal to noise ratio not less than 10.0 in the chromatogram obtained with reference solution (b).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of peak due to tacrolimus hydroxyl acid multiplied by correction factor 0.67 and tacrolimus 8-epimer is not more than the area of the peak in the chromatogram obtained with reference solution (a) (0.5 per cent), the area of any other secondary peak is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 3 times the area of the principal peak in the

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chromatogram obtained with reference solution (a) (1.5 per cent). Ignore any peak with area less than the area of the principal peak in the chromatogram obtained with reference solution (b).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Allow the solution to stand for 3 hour at ambient temperature before use. Protect the solution from light by using low-actinic glassware.

Solution A. Prepared by dissolving 50 g of *polyoxyethylene (23) lauryl ether* in 1000 ml of *water.*

Solution B. A mixture of 30 volumes of solution A and 70 volumes of *acetonitrile.*

Test solution. Weigh and mix content of 20 capsules. Disperse a quantity of the mixed content containing 5 mg of tacrolimus in solution B and dilute it to 100.0 ml with the solution B.

Reference solution. A 0.005 per cent w/v solution of tacrolimus in solution B.

Chromatographic system

- a stainless steel column 5.5 cm x 4 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 60°,
- mobile phase: a mixture of 600 volumes of *6 mM orthophosphoric acid*, 55 volumes of *tertbutylmethyl-ether* and 335 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 5 µl.

The relative retention time for tacrolimus 19-epimer and tacrolimus are 0.67 and 1.0 respectively.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections for the sum of the tacrolimus and tacrolimus 19-epimer peaks is not more than 3.0 per cent.

Inject the reference solution and the test solution.

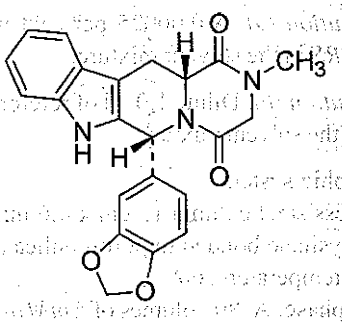
Calculate the content of tacrolimus $C_{44}H_{69}NO_{12}$ in the portion of capsules taken by the formula

$$\frac{rU}{rS} \times \frac{CS}{CU} \times 100$$

in which *rU* is the sum of the peak responses of tacrolimus and tacrolimus 19-epimer from the test solution, *rS* is the sum of the peak responses of tacrolimus and tacrolimus 19-epimer from the reference solution and *CS* and *CU* are the concentration of reference solution and test solution respectively.

Storage. Store protected from moisture.

Tadalafil



$C_{22}H_{19}N_3O_4$ Mol. Wt. 389.4

Tadalafil is (6*R*,12a*R*)-6-(1,3-benzodioxol-5-yl)-2-methyl-2,3,6,7,12,12a-hexahydropyrazino[1',2':1,6]-pyrido[3,4-*b*]indole-1,4-dione.

Tadalafil contains not less than 97.5 per cent and not more than 102.5 per cent of $C_{22}H_{19}N_3O_4$, calculated on the dried basis.

Category. Erectile disfunction.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tadalafil IPRS* or with the reference spectrum of tadalafil.

B. In the test for Enantiomeric and Diastereomeric purity, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the identification solution.

Identification solution. A 0.05 per cent w/v solution of *tadalafil IPRS* in the solvent mixture.

Tests

Enantiomeric and Diastereomeric purity. Determine by liquid chromatography (2.4.14).

Solvent mixture. 40 volumes of *hexane*, 40 volumes of *isopropyl alcohol* and 20 volumes of *acetonitrile.*

Test solution. Dissolve a quantity of the substance under examination in the solvent mixture to obtain a solution containing 0.05 per cent w/v of Tadalafil.

Reference solution (a). A 0.005 per cent w/v solution of *tadalafil IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Reference solution (c). Dissolve 25 mg of the substance under examination in 40 ml of the solvent mixture. Add 1.0 ml of *1.0*

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M tetrabutylammonium hydroxide in *methanol* and allow to stand at room temperature for 20 minutes. Add 1.0 ml of *trifluoroacetic acid* and dilute to 50.0 ml with the solvent mixture to generate 6R,12aS diastereomer of tadalafil.

Reference solution (d). Transfer 1.0 ml of reference solution (c) and 10 ml of reference solution (a) to a 50-ml volumetric flask and dilute to volume with the solvent mixture.

Reference solution (e). Dilute 5 ml of reference solution (b) to 10 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with silica gel AD for chiral separation (10 µm),
- mobile phase: equal volumes of *hexane* and *isopropyl alcohol*,
- flow rate: 0.75 ml per minute,
- spectrophotometer set at 222 nm,
- injection volume: 10 µl.

Name	Relative retention time
6R,12aS diastereomer [1]	0.79
Tadalafil	1.0
6S,12aS enantiomer[2]	1.4
6S,12aR diastereomer[3]	1.7

[1](6*R* ,12a*S*)-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydro-2-methyl-pyrazino[1',2':1,6]pyrido[3,4-*b*]indole-1,4-dione,

[2](6*S* ,12a*S*)-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydro-2-methyl-pyrazino[1',2':1,6]pyrido[3,4-*b*]indole-1,4-dione,

[3](6*S* ,12a*R*)-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydro-2-methyl-pyrazino[1',2':1,6]pyrido[3,4-*b*]indole-1,4-dione.

Inject reference solution (b), (d) and (e). The test is not valid unless the resolution between the peaks due to 6R,12aS diastereomer and tadalafil is not less than 2.0 in the chromatogram obtained with reference solution (d); the tailing factor is not less than 0.8 and not more than 1.5 and the relative standard deviation for replicate injections is not more than 10.0 per cent in the chromatogram obtained with reference solution (b). The signal-to-noise ratio is not less than 20 in the chromatogram obtained with reference solution (e).

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Do not use sonication during the preparation of test solution.

Test solution. Dissolve a quantity of the substance under examination in a mixture of equal volumes of *acetonitrile* and mobile phase A, prepared by first dissolving in *acetonitrile* and then dilute to volume with mobile phase A to obtain a solution containing 0.04 per cent w/v of Tadalafil.

Reference solution (a). A 0.00004 per cent w/v solution of *tadalafil IPRS* in a mixture of equal volumes of *acetonitrile* and mobile phase A, prepared by first dissolving in *acetonitrile* and then dilute to volume with mobile phase A.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with a mixture of equal volumes of *acetonitrile* and mobile phase A.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. add 1.0 ml of *trifluoroacetic acid* to 1000 ml of *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 285 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
3	85	15
30	5	95
33	5	95
35	85	15

The relative retention time with reference to tadalafil for 6R,12aS diastereomer is about 1.03.

Inject reference solution (a) and (b). The test is not valid unless the tailing factor is not more than 1.5 in the chromatogram obtained with reference solution (a) and signal-to-noise ratio is not less than 10 in the chromatogram obtained with reference solution (b).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent). Ignore the peaks due to the 6R ,12aS and 6S ,12aR diastereomers of tadalafil, which co-elute at a retention time of about 1.03 relative to tadalafil.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1 g by drying under vacuum at 105° for 3 hours.

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Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Solution A. Add 1.0 ml of *trifluoroacetic acid* to 1000 ml of *water*.

Test solution. Dissolve a quantity of the substance under examination in a mixture of equal volumes of *acetonitrile* and solution A, prepared by first dissolving in *acetonitrile* and then dilute to volume with solution A to obtain a solution containing 0.01 per cent w/v of tadalafil.

Reference solution. A 0.01 per cent w/v solution of *tadalafil IPRS* in a mixture of equal volumes of *acetonitrile* and solution A, prepared by first dissolving in *acetonitrile* and then dilute to volume with solution A.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 45 volumes of *acetonitrile* and 55 volumes of solution A,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 285 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.0.

Inject the reference solution and the test solution.

Calculate the content of tadalafil, $C_{22}H_{19}N_3O_4$.

Storage. Store protected from moisture.

Tadalafil Tablets

Tadalafil Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of tadalafil, $C_{22}H_{19}N_3O_4$.

Usual strengths. 10 mg; 20 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 1000 ml of 0.5 per cent w/v of *sodium dodecyl sulphate*,

Speed and time. 50 rpm ,10 minutes and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.025 per cent w/v solution of *tadalafil IPRS* in a mixture of equal volumes of *acetonitrile* and *water*. Dilute a volume of the solution with the dissolution medium to obtain 0.0075 mg per ml of *tadalafil IPRS*.

Chromatographic system

- a stainless steel column 5 cm x 4.6 mm, packed with octylsilane bonded to porous silica (3.5 µm),
- column temperature: 40°,
- mobile phase: a mixture of equal volumes of *methanol* and *water*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0.

Q. Not less than 40 per cent of the stated amount of $C_{22}H_{19}N_3O_4$ at 10 minutes and not less than 80 per cent of the stated amount of $C_{22}H_{19}N_3O_4$ at 30 minutes.

Related substances. Determine by liquid chromatography (2.4.14) as described under Assay with the following modifications.

The relative retention time with reference to tadalafil for 6R,12aS diastereomer of tadalafil is about1.2.

Inject reference solution (a) and (b). The test is not valid unless the tailing factor is not more than 1.5 in the chromatogram obtained with reference solution (a) and the resolution between the peaks due to tadalafil and the 6R,12aS diastereomer peak is not less than 3.0 in the chromatogram obtained with reference solution (b).

Inject the test solution. The area of any secondary peak is not more than 0.2 per cent and the sum of the areas of all the secondary peaks is not more than 0.3 per cent. Ignore any peak with an area less the 0.05 per cent, calculated by area normalization method.

Uniformity of content. Complies with the test stated under Tablets.

Solvent mixture. Equal volumes of *acetonitrile* and *water*.

Add 1 tablet to a suitable volumetric flask to obtain a solution containing 0.1 to 0.2 mg per ml of Tadalafil. Add a volume of solvent mixture upto 50 per cent of the volume of the flask and mechanically shake for 15 minutes, dilute with the solvent mixture to volume and filter. Dilute 5.0 ml of the filtrate to

50.0 ml using solvent mixture. Measure the absorbance of the resulting solution at the maximum at about 285 nm (2.4.7). Calculate the content of $C_{22}H_{19}N_3O_4$ from the absorbance obtained by repeating the procedure using a solution of *tadalafil IPRS* of the same concentration as that of the test solution.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *acetonitrile* and *water*.

Test solution. Place not less than 20 tablets into an appropriate size volumetric flask. Fill the flask about 50 per cent with the solvent mixture and disperse with the aid of ultrasound and dilute with the solvent mixture to volume. Allow the solution to stand for 1 hour. Dilute the solution, if necessary to obtain a solution containing 0.25 mg per ml of Tadalafil. Centrifuge or filter the solution. *(NOTE — The initial concentration before a secondary dilution step should not exceed 6 mg per ml)*.

Reference solution (a). A 0.025 per cent w/v solution of *tadalafil IPRS* in the solvent mixture.

Reference solution (b). To partially convert tadalafil to the 6R,12aS diastereomer, transfer 25 ml of the reference solution into a suitable container. Add 0.25 ml of *5 M sodium hydroxide*, mix well, and let stand for 30 minutes. Neutralize the solution to pH 7 by drop-wise addition of *trifluoroacetic acid*. *(NOTE— This solution is stable for 1 month when stored in a refrigerator)*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (3.5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 35 volumes of *acetonitrile*, 65 volumes of *water* and 0.1 volume of *trifluoroacetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 285 nm,
- injection volume: 10 µl.

The relative retention time with reference to tadalafil for 6R,12aS diastereomer is about 1.2.

Inject reference solution (a) and (b). The test is not valid unless resolution between the peaks due to tadalafil and the 6R ,12aS diastereomer peak is not less than 3.0 in the chromatogram obtained with reference solution (b) and the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{22}H_{19}N_3O_4$ in the tablets.

Storage. Store protected from moisture.

Talc

Purified Talc; Talcum

Powdered, selected, natural, hydrated magnesium silicate. Pure talc has the formula $Mg_3Si_4O_{10}(OH)_2$. It may contain variable amounts of associated minerals among which chlorites (hydrated aluminium and magnesium silicates), magnesite (magnesium carbonate), calcite (calcium carbonate) and dolomite (calcium and magnesium carbonate) are predominant.

Category. Pharmaceutical aid (dusting powder).

Description. A white or almost white powder, free from grittiness; readily adheres to the skin; unctuous to the touch.

Production

Talc derived from deposits that are known to contain associated asbestos is not suitable for pharmaceutical use. The manufacturer is responsible for demonstrating by the test for amphiboles and serpentines that the product is free from asbestos. The presence of amphiboles and of serpentines is revealed by infrared spectrophotometry. If detected, the specific morphological criteria of asbestos are investigated by a suitable method of optical microscopy to determine whether tremolite asbestos or chrysotile is present, as described below.

Determine by infrared absorption spectrophotometry (2.4.6).

In the range 740 cm^{-1} to 760 cm^{-1} using scale expansion, any absorption band at 758 cm^{-1} may indicate the presence of tremolite or of chlorite. If the absorption band remains after ignition of the substance under examination at 850 ± 50° for at least 30 minutes, it indicates the presence of the tremolite. In the range 600 cm^{-1} to 650 cm^{-1} using scale expansion, any absorption band or shoulder may indicate the presence of serpentines.

Identification

Test A may be omitted if tests B, C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6), shows absorption bands at 3677 ± 2 cm^{-1}, 1018 ± 2 cm^{-1} and 669 ± 2 cm^{-1}.

B. In a platinum crucible, melt a mixture of 0.2 g of *anhydrous sodium carbonate* and 2.0 g of *potassium carbonate*. To the melted mass add 0.1 g of the substance under examination and heat until the mixture is completely melted. Allow to cool and transfer the melted mass into an evaporating dish with 50 ml of hot *water*. Add *hydrochloric acid* until effervescence

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ceases. Add 10 ml of *hydrochloric acid* and evaporate to dryness on a water-bath. Allow to cool. Add 20 ml of *water*, heat to boiling and filter (the residue is used for identification test C). To 5 ml of the filtrate add 1 ml of *ammonia* and 1 ml of *ammonium chloride solution* and filter. To the filtrate add 1 ml of *disodium hydrogen phosphate solution*. A white, crystalline precipitate is formed.

C. The residue obtained in identification test B gives the reaction of silicates (2.3.1).

Tests

Acidity or alkalinity. Shake 5.0 g with 25 ml of *carbon dioxide-free water* for 1 minute, filter and add to the filtrate 0.5 ml of *bromothymol blue solution*; the solution is not acidic and requires not more than 0.3 ml of *0.1 M hydrochloric acid* to make it acidic.

Iron (2.3.14). Boil 4.0 g with 25 ml of *water* for 30 minutes, replacing the water lost by evaporation, and filter. The filtrate, after the addition of 5 ml of *nitric acid*, complies with the limit test for iron (10 ppm).

Acid-soluble substances. Not more than 2.0 per cent, determined by the following method. Digest 2.0 g with 40 ml of *dilute hydrochloric acid* for 15 minutes, filter, evaporate the filtrate; to the residue add 0.1 ml of *sulphuric acid* and ignite at 800 ± 25° to constant weight.

Water-soluble substances. To 10 g add 50 ml of *carbon dioxide-free water*, heat to boiling and maintain boiling under a reflux condenser for 30 minutes. Allow to cool, filter through a medium-speed filter paper and dilute to 50.0 ml with *carbon dioxide-free water*. Take 25 ml of the filtrate, evaporate to dryness and heat at 105° for 1 hour. The residue weighs a maximum of 10 mg.

Carbonates. To 1 g add 20 ml of *dilute hydrochloric acid*; no effervescence is produced.

Chlorides (2.3.12). Suspend 2.0 g in 10 ml of *water*, add 10 ml of *2 M nitric acid*, shake for 15 minutes and filter. 10 ml of the filtrate complies with the limit test for chlorides (250 ppm).

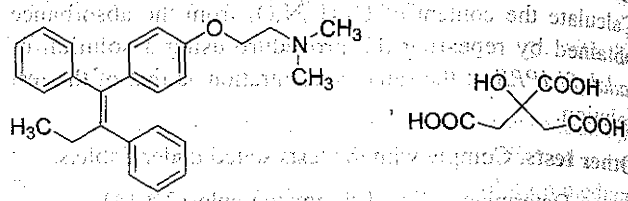
Organic compounds. The residue obtained in the test for Loss on drying is not more than slightly yellow or grey.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 180° for 1 hour.

Microbial contamination (2.2.9). If intended for topical application, the total aerobic viable count is not more than 10^2 CFU per g. If intended for oral administration, the total aerobic viable count is not more than 10^3 CFU per g and total fungal count is not more than 10^2 CFU per g.

Storage. Store protected from moisture.

Tamoxifen Citrate



$C_{26}H_{29}NO,C_6H_8O_7$ Mol. Wt. 563.6

Tamoxifen Citrate is (*Z*)-2-[4-(1,2-diphenylbut-1-enyl)-1-phenoxy]ethyldimethylamine citrate.

Tamoxifen Citrate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{26}H_{29}NO, C_6H_8O_7$, calculated on the dried basis.

Category. Antioestrogen.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests C and D may be omitted if tests A and B are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tamoxifen citrate IPRS* or with the reference spectrum of tamoxifen citrate.

B. When examined in the range 220 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution in *methanol* shows absorption maxima at about 237 nm and 275 nm.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 90 volumes of *toluene* and 10 volumes of *triethylamine*.

Test solution. Dissolve 0.1 g of the substance under examination in 10 ml of *methanol*.

Reference solution. A 1.0 per cent w/v solution of *tamoxifen citrate IPRS* in *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

D. To 10 mg add 4 ml of *pyridine* and 2 ml of *acetic anhydride* and shake; a yellow colour is produced. Heat in a water-bath for 2 minutes; a pink to red colour is produced.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

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NOTE — Prepare the solutions immediately before use, protect from light.

Test solution. Dissolve 15 mg of the substance under examination in mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). A 0.0015 per cent w/v solution of *tamoxifen citrate IPRS* in the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A 0.15 per cent w/v solution of *tamoxifen citrate for performance test IPRS* (containing impurities A and F) in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 0.9 g of *sodium dihydrogen orthophosphate* and 4.8 g of *N,N-dimethyloctylamine* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minutes,
- spectrophotometer set at 240 nm,
- injection volume: 10 µl.

Name	Relative retention time
Tamoxifen impurity A[1]	0.8
Tamoxifen impurity F[2]	0.9
Tamoxifen (retention time: about 20 minute)	1.0

[1]2-[4-[(E)-1,2-diphenylbut-1-en-1-yl]phenoxy]-N,N-dimethylethan-1-amine((e)-isomer),

[2]2-[4-[(Z)-1,2-diphenylbut-1-en-1-yl]phenoxy]-N-methylethan-1-amine.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to tamoxifen impurity A and tamoxifen impurity F is not less than 3.0.

Inject reference solution (a) and the test solution. Run the chromatogram twice the retention time of the principal peak for test solution. The area of any peak corresponding to tamoxifen impurity A is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent), the area of any peak corresponding to tamoxifen impurity F is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore the peak due to citrate and the area of any peak less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 1.0 g in 150 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, using *1-naphthol-benzein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.05636 g of $C_{26}H_{29}NO, C_6H_8O_7$.

Storage: Store protected from light and moisture.

Tamoxifen Tablets

Tamoxifen Citrate Tablets

Tamoxifen Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of tamoxifen, $C_{26}H_{29}NO$.

Usual strengths: The equivalent of 10 mg, 20 mg and 40 mg of tamoxifen.

Identification

Tests B and C may be omitted if tests A and D are carried out. Test A may be omitted if tests B, C and D are carried out.

A. To a quantity of the powdered tablets containing 0.1 g of Tamoxifen add 20 ml of *water*, warm, add 2 ml of *5 M sodium hydroxide* and cool. Extract with two quantities, each of 10 ml, of *ether*, filtering after each extraction. Combine the ether extracts and evaporate to dryness in a stream of nitrogen at room temperature. Dry the residue at a pressure not exceeding 0.7 kPa for 30 minutes.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tamoxifen citrate IPRS* treated in the same manner or with the reference spectrum of tamoxifen.

B. When examined in the range 220 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution in *methanol* of the residue obtained in test A shows absorption maxima at about 237 nm and 275 nm.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 100 volumes of *ethyl acetate*, 10 volumes of *methanol* and 1 volume of *strong ammonia solution*.

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Test solution. Shake a quantity of the powdered tablets containing 0.1 g Tamoxifen with 10 ml of *methanol*, filter, evaporate the filtrate to dryness on a water-bath, dry the residue at 100° for 30 minutes and dissolve 10 mg of the residue in 10 ml of *methanol*.

Reference solution. A 0.1 per cent w/v solution of *tamoxifen citrate IPRS* in *methanol*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

D. Dissolve 10 mg of the residue obtained in the preparation of the test solution in test C in 4 ml of *pyridine*, add 2 ml of *acetic anhydride* and heat on a water-bath for 2 minutes; a pink to red colour is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium, 1000 ml of *0.02 M hydrochloric acid*,

Speed and time. 150 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter, dilute if necessary with the dissolution medium. Measure the absorbance of the resulting solution at the maximum at about 275 nm (2.4.7). Calculate the content of $C_{26}H_{29}NO$ in the medium taking 305 as the specific absorbance at 275 nm.

Q. Not less than 75 per cent of the stated amount of $C_{26}H_{29}NO$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use, protect from light.

Test solution. Disperse a quantity of powdered tablets containing 50 mg of tamoxifen in the mobile phase, with the aid of ultrasound for 5 minutes and dilute to 50.0 ml with the mobile phase, centrifuge and filter.

Reference solution (a). A 0.0015 per cent w/v solution of *tamoxifen citrate IPRS* in the mobile phase.

Reference solution (b). A 0.15 per cent w/v solution of *tamoxifen citrate for performance test IPRS* in the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 20.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 0.9 g of *sodium dihydrogen orthophosphate* and 4.8 g of *N,N-dimethyloctylamine* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 20 µl.

Name	Relative retention time
Tamoxifen E- isomer[1]	0.8
Tamoxifen impurity F[2]	0.9
Tamoxifen (retention time: about 20 minutes)	1.0

[1]2-[4-[(E)-1,2-diphenylbut-1-en-1-yl]phenoxy]-N,N-dimethylethan-1-amine((e)-isomer),

[2]2-[4-[(Z)-1,2-diphenylbut-1-en-1-yl]phenoxy]-N-methylethan-1-amine.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to tamoxifen E-isomer and tamoxifen impurity F is not less than 3.0 and between the peaks due to tamoxifen impurity F and tamoxifen is not less than 1.5.

Inject reference solution (a), (c) and the test solution. Run the chromatogram twice the retention time of the principal peak for test solution. The area of any peak corresponding to tamoxifen E-isomer is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent) and the sum of the areas of all the secondary peaks, excluding tamoxifen E-isomer peak, is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore the peak due to citrate and the area of any peak less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Uniformity of content. Determine by liquid chromatography (2.4.14), as described under Assay using the following solution.

Test solution. Disperse one tablet in the mobile phase and dilute with the mobile phase to obtain a solution containing 0.01 per cent w/v of tamoxifen.

Inject the reference solution and the test solution.

Calculate the content of $C_{26}H_{29}NO$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 5 mg of tamoxifen in 35 ml of the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution. A 0.015 per cent w/v solution of *tamoxifen citrate IPRS* in the mobile phase.

Use chromatographic system as described under Related substances.

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Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

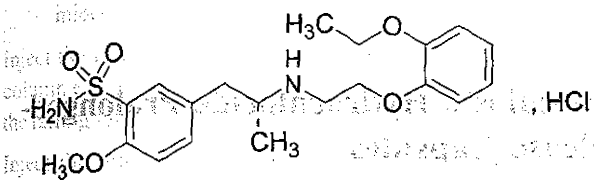
Inject the reference solution and the test solution.

Calculate the content of $C_{26}H_{29}NO$ in the tablets.

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of the equivalent amount of tamoxifen.

Tamsulosin Hydrochloride



$C_{20}H_{28}N_2O_5S,HCl$ Mol. Wt. 445.0

Tamsulosin Hydrochloride is (*R*)-5-(2{[2-(*o*-ethoxyphenoxy) ethyl]aminopropyl)-2-methoxybenzenesulfonamide hydrochloride.

Tamsulosin Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{20}H_{28}N_2O_5S,HCl$, calculated on the dried basis.

Category. Uninary tract analgesic.

Description. A white to off white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tamsulosin hydrochloride IPRS* or with the reference spectrum of tamsulosin hydrochloride.

B. When examined in the range 200 nm to 350 nm (2.4.7), a 0.001 per cent w/v solution in *methanol* shows absorption maxima at about 279 and 225 nm.

C. Dissolve with heating 0.75 g in *water* and dilute to 100.0 ml with *water*. To 5 ml of the solution, cooled in an ice-bath add 3 ml of *dilute nitric acid* and shake. Allow to stand at room temperature for 30 minutes and filter. The filtrate gives reaction (A) of chlorides (2.3.1).

Tests

Specific optical rotation (2.4.22). −21.0° to −17.0°, determined in a 0.75 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

A. *Test solution.* Dissolve 50 mg of the substance under examination in 10 ml of the mobile phase.

Reference solution(a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 4 mg of *2-methoxy-5-[(2R)-2-[(2-(2-methoxyphenoxy)ethyl]amino]propyl]benzenesulphonamide IPRS* (*tamsulosin impurity A IPRS*) and 4 mg of the substance under examination in the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (c). Dissolve 4 mg of *(2R)-N- [2-(2-ethoxyphenoxy)ethyl]-1-(4-methoxyphenyl)propan-2-amine IPRS* (*tamsulosin impurity B IPRS*) and 4 mg of the substance under examination in the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 20.0 ml with the mobile phase

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 600 ml of *acetonitrile* and 1400 ml of a solution prepared by dissolving 3.0 g of *sodium hydroxide* in a mixture of 8.7 ml of *perchloric acid* and 1900 ml of *water*, adjusted to pH 2.0 with *0.5 M sodium hydroxide* and diluting to 2000 ml with *water*,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 10 µl.

Inject reference solution (b). Run the chromatogram 1.5 times the retention time of tamsulosin (retention time = about 6 minutes). The test is not valid unless the resolution between the peaks due to tamsulosin impurity A and tamsulosin is not less than 6.0.

In the chromatogram obtained with the test solution, for each unspecified impurity eluting before tamsulosin, the area of the peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

B. Repeat the chromatographic procedure with the following modifications.

- mobile phase: dissolve 3.0 g of *sodium hydroxide* in a mixture of 8.7 ml of *perchloric acid* and 900 ml of *water*, adjusted to pH 2.0 with *0.5 M sodium hydroxide* and dilute to 2000 ml with *water*; add 2000 ml of *acetonitrile*,
- flow rate: 1 ml per minute,

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Inject reference solution (c). Run the chromatogram 5 times the retention time of tamsulosin (retention time = 2.5 minutes). The test is not valid unless the resolution between the peaks due to tamsulosin and tamsulosin impurity B is not less than 2.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, for each unspecified impurity eluting after tamsulosin, the area of any peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of the impurities eluting before tamsulosin in test A and after tamsulosin in test B is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

***S*-isomer**. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.05 g of the substance under examination in *methanol* and add sufficient *methanol* to produce 25.0 ml.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol*. Dilute 1.0 ml of the solution to 10.0 ml with *methanol*.

Reference solution (b). Dissolve 5.0 mg of *tamsulosin racemate IPRS* in *methanol* and dilute to 25.0 ml with *methanol*. Dilute 2.0 ml of the solution to 10.0 ml with *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with silica gel OD for chiral separations (Such as Chiralpak AD-H) (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 1 volume of *diethylamine*, 150 volumes of *methanol*, 200 volumes of *anhydrous ethanol* and 650 volumes of *hexane*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 10 µl.

The relative retention with reference to tamsulosin (retention time is about 14 minutes) of tamsulosin impurity C is about 0.8.

Inject reference solution (b). Run the chromatogram for about 20 minutes. The test is not valid unless the resolution between tamsulosin and impurity C (5-[(2*S*)-2-[[2-(2-ethoxyphenoxy) ethyl]amino] propyl]-2-methoxybenzenesulphonamide) is not less than 2.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of the peak obtained due to tamsulosin impurity C is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Assay. Dissolve 0.35 g in 5.0 ml of *anhydrous formic acid*, add 75 ml of a mixture of 2 volumes of *acetic anhydride* and 3 volumes of *glacial acetic acid*. Titrate immediately with *0.1M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.0445 g of $C_{20}H_{28}N_2O_5S,HCl$.

Storage. Store protected from light.

Tamsulosin Hydrochloride Prolonged-release Capsules

Tamsulosin Prolonged-release Capsules

Tamsulosin Prolonged-release Capsules manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Tamsulosin Prolonged release Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of Tamsulosin hydrochloride, $C_{20}H_{28}N_2O_5S,HCl$.

Usual strengths. 0.2 mg; 0.4 mg.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. When examined in the range 210 nm to 400 nm (2.4.7), the test solution obtained in the Assay exhibits absorption maximum at 225 nm.

Tests

Dissolution (2.5.2). Complies with the test stated under Capsules.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of mixed contents of the capsules containing 0.8 mg of Tamsulosin Hydrochloride in 10 ml of *1 M methanolic hydrochloric acid* with the aid of ultrasound for 15 minutes and with intermittent shaking and

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filter through a 0.7-µm glass fibre filter. To 1 volume of the filtrate, add 4 volumes of *1 M methanolic hydrochloric acid*.

Reference solution. Dilute 1.0 ml of the test solution to 500.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 300 volumes of *acetonitrile* and 700 volumes of *water* containing 0.44 per cent v/v *perchloric acid* and 0.15 per cent w/v *sodium hydroxide* previously adjusted to pH 2.0 with *1 M sodium hydroxide*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. Run the chromatogram 7 times the retention time of the principal peak. The retention time of tamsulosin is about 6 minutes. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 2.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than 7.5 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent).

Uniformity of content. Complies with the test stated under Capsules.

Determine by liquid chromatography (2.4.14).

Test solution. Disperse the contents of 1 capsule with 10 ml of *1 M methanolic hydrochloric acid* with the aid of ultrasound for 15 minutes and with intermittent shaking, filter through a 0.7-µm glass fibre filter. Dilute 1 volume of the filtrate to 10 volumes with *1 M methanolic hydrochloric acid* and filter through a 0.7-µm glass fibre filter.

Reference solution. A 0.04 per cent w/v solution of *tamsulosin hydrochloride IPRS* in *methanol* with the aid of ultrasound. Dilute 1 volume to 100 volumes with *1 M methanolic hydrochloric acid*.

Use chromatographic system as described under Related substances.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{28}N_2O_5S,HCl$ in the capsule.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Use freshly prepared solutions.

Test solution. Disperse a quantity of the mixed contents of 20 capsules containing 1.6 mg of Tamsulosin Hydrochloride in 50 ml of *1 M methanolic hydrochloric acid* with the aid of ultrasound for 15 minutes and dilute to 100.0 ml with *1 M methanolic hydrochloric acid*, filter using a 0.7-µm glass fibre filter. Dilute 1 volume of the filtrate to 4 volumes with *1 M methanolic hydrochloric acid*.

Reference solution. A 0.04 per cent w/v solution of *tamsulosin hydrochloride IPRS* in *methanol* with the aid of ultrasound. Dilute 1 volume to 100 volumes with *1 M methanolic hydrochloric acid*.

Use chromatographic system as described under Related substances.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{28}N_2O_5S,HCl$ in the capsules.

Tamsulosin Hydrochloride Prolonged-release and Dutasteride Capsules

Tamsulosin Hydrochloride Prolonged-release and Dutasteride Capsules manufactured by different manufacture, whilst complying with the requirements of the monograph are not interchangeable, as the dissolution profile of the products of different manufactures may not be the same.

Tamsulosin hydrochloride Prolonged - release and Dutasteride Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of tamsulosin hydrochloride, $C_{20}H_{28}N_2O_5S,HCl$ and dutasteride, $C_{27}H_{30}F_6N_2O_2$.

Usual strength. Dutasteride, 0.5 mg and Tamsulosin Hydrochloride 0.4 mg.

Identification

A. In the Assay of dutasteride, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. In the Assay of tamsulosin hydrochloride, the principal peak in the chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2). Determine by liquid chromatography (2.4.14).



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For tamsulosin hydrochloride. Complies with the test stated under Tablets.

For Dutasteride —

Apparatus No. 2 (Paddle),

Medium: 500 ml of a buffer solution prepared by dissolving 2.0 g of *sodium chloride* and 7.0 ml *hydrochloric acid* in 800 ml of *water* and add 5.0 g of *sodium lauryl sulphate* and dilute to 1000 ml with *water*,

Speed and time: 75 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14)

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.025 per cent w/v solution of *dutasteride IPRS* in mobile phase. Dilute 2.0 ml of the solution to 500.0 ml with dissolution medium.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 40.0 volumes of a buffer solution prepared by 0.05 M *sodium dihydrogen phosphate monohydrate* and adjusted to pH 5.0 with *dilute sodium hydroxide* and 60 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 200 µl.

Inject the reference solution and the test solution.

Q: Not less than 70 per cent of the stated amount of $C_{27}H_{30}F_6N_2O_2$.

Related substances. Determine by liquid chromatography (2.4.14).

For Dutasteride III and Isomer impurity —

Test solution. Disperse a quantity of the powder containing 2 mg of dutasteride in *acetonitrile* and dilute to 10.0 ml with *acetonitrile*.

Reference solution. A 0.0001 per cent w/v solution of *dutasteride IPRS* in *acetonitrile*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 35°,
- mobile phase: A. a buffer solution prepared by *0.02 M potassium dihydrogen phosphate*, adjusted to pH 3.0 with *dilute orthophosphoric acid*,

  B. 90 volumes of *acetonitrile* and 10 volumes of *water*,
- a gradient programme using the conditions given below;
- flow rate: 1 ml per minute,
- spectrophotometer set at 242 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
20	90	10
30	30	70
40	40	60
60	40	60
61	90	10
70	90	10

Name	Relative retention time
Dutasteride III[1]	0.69
Dutasteride isomer[2]	1.05

[1]3-oxo-4-aza-5a-androst-1-ene-17β-carboxylic acid,

[2]17β-N-[2,5-bis(trifluoromethyl)phenyl]carbomoyl-4-aza-5β-androst-1-ene-3-one.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the test solution. The area of any peak corresponding to dutasteride III and dutasteride isomer is not more than 1.0 per cent, the area of any other secondary peak is not more than 1.0 per cent and the sum of areas of all the secondary peaks is not more than 2.0 per cent, calculated by area normalisation.

For Dutasteride dihydro impurity —

Test solution. Disperse a quantity of the powder containing 3.5 mg of dutasteride in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution. A solution containing 0.05 per cent w/v solution of *dutasteride IPRS* in mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with porous silica (5 µm) (Such as kromasil 100 silica),
- mobile phase: a mixture of 60 volumes of *n-hexane* and 40 volumes of *1,4 dioxane*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

The relative retention time with respect to dutasteride for dutasteridedihydro impurity (*17β-N-{2,5-bis(trifluoromethyl) phenyl}carbomoyl-4-aza-5a-androstane-3-one*) is about 1.98.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 3.0.

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Inject the test solution. The area of any peak corresponding to dutasteride dihydro impurity is not more than 1.0 per cent, the area of any other secondary peak is not more than 1.0 per cent and the sum of areas of all the secondary peaks is not more than 2.0 per cent, calculated by area normalisation.

Uniformity of content. Complies with the test stated under Capsules.

For Dutasteride — Determine by liquid chromatography (2.4.14), solvent mixture and chromatographic system as described under Assay.

Test solution. Transfer the contents of one capsule into a 10.0 ml volumetric flask, add 8 ml of solvent mixture and disperse with the aid of ultrasound for about 30 minutes, cool and dilute with solvent mixture, mix well and filter.

Reference solution. A 0.005 per cent w/v solution of *dutasteride IPRS* in solvent mixture.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{30}F_6N_2O_2$ in the capsule.

For Tamsulosin hydrochloride — Determine by liquid chromatography (2.4.14), Using reference solution, solvent mixture (a), (b) and chromatographic system as described under Assay.

Test solution. Transfer the contents of one capsule into a 10.0 ml volumetric flask, add 8 ml of solvent mixture(a), and disperse with the aid of ultrasound for about 30 minutes, cool and dilute with solvent mixture (a), mix well and filter. Dilute 5.0 ml of the solution to 10 .0 ml with solvent mixture (b).

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{28}N_2O_5S,HCl$ in the capsule.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

For Dutasteride —

Solvent mixture. 90 volumes of *acetonitrile* and 10 volumes of *water*.

Test solution. Weigh and transfer a quantity of the mixed contents of 20 capsules containing about 5.0 mg of Dutasteride to a 20.0 ml volumetric flask and dissolve in solvent mixture. Mix well and filter.

Reference solution. A solution containing 0.025 per cent w/v solution of *dutasteride IPRS* in solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- mobile phase: A. 80 volumes of a buffer solution prepared by *0.02 M disodium hydrogen phosphate dihydrate*, adjusted to pH 5.0 with *dilute ortho phosphoric acid* and 20 volumes of *acetonitrile*,

B. 90 volumes of *acetonitrile* and 10 volumes of *water*,

- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	30	70
10	30	70
12	0	100
35	0	100
38	30	70
45	30	70

Inject the reference solution. The test is not valid unless theoretical plates are not less than 3000, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{30}F_6N_2O_2$ in the Capsules.

For Tamsulosin Hydrochloride — Determine by liquid chromatography (2.4.14).

Solvent mixture (a). A buffer solution prepared by dissolving 4 g of *sodium hydroxide* into a 1000 ml volumetric flask add 100 ml of *water* and dilute to 1000 ml with *methanol*.

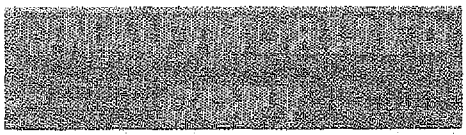
Solvent mixture (b). A solution of 80 volumes of *water* and 20 volumes of *acetonitrile*.

Test solution. Weigh and transfer a quantity of the mixed contents of 20 capsules containing about 5 mg of Tamsulosin Hydrochloride in 50.0 ml volumetric flask add 30 ml of solvent mixture (a), disperse with the aid of ultrasound for 20 minutes, cool and dilute to volume with solvent mixture (a), mix well. centrifuge at 3500 rpm for 15 minutes and use the clear, supernatant liquid. Dilute 5.0 ml of the solution to 25.0 ml with solvent mixture (b).

Reference solution. A 0.002 per cent w/v solution of *tamsulosin hydrochloride IPRS* in solvent mixture (b).

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 70 volumes of a buffer solution prepared by dissolving 1.5 ml of *orthophosphoric acid* in 1000 ml of *water* and 30 volumes of *cetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.



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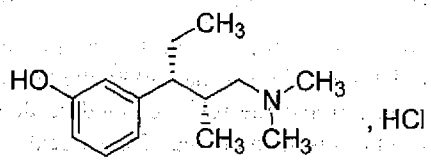
Inject the reference solution . The test is not valid unless the theoretical plates is not less than 2000, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{28}N_2O_5S,HCl$ in the Capsules.

Storage Store protected from light and moisture at a temperature below 30°.

Tapentadol Hydrochloride



$C_{14}H_{23}NO, HCl$ Mol Wt. 257.8

Tapentadol Hydrochloride is (2*R*,3*R*)-3-(1-Dimethylamino-2-methylpentan-3-yl)phenol hydrochloride.

Tapentadol Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{14}H_{23}NO, HCl$, calculated on the dried basis.

Category. Analgesic.

Description. A white to off white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tapentadol hydrochloride IPRS* or with reference spectrum of tapentadol hydrochloride.

B. In the Assay, the principal peak in the chromatogram obtained with test solution corresponds to the peak in the chromatogram obtained with the reference solution.

C. It gives reaction (A) for chlorides (2.3.1).

Tests

pH (2.4.24). 4.0 to 6.0, determined in a 1.0 per cent w/v of solution in *carbon dioxide-free water*.

Specific optical rotation (2.4.22). −28.5° to −24.5°, determined in 1.0 per cent w/v solution in *methanol*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and diluted to 50.0 ml of the mobile phase.

Reference solution. A 0.025 per cent w/v solution of *tapentadol hydrochloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase. a mixture of 80 volumes of buffer solution prepared by dissolving 2.72 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, add 2.0 ml of *triethyl amine*. Adjusted to pH 2.5 with *orthophosphoric acid* and 20 volumes of *methanol*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the test solution. the area of any other secondary peak is not more than 0.5 per cent and sum of the area of all the secondary peaks is not more than 1.0 per cent, calculated by area normalization.

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14).

Use the chromatographic system as described under Related substances.

Test solution. Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.02 per cent w/v solution of *tapentadol hydrochloride IPRS* in the mobile phase.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0 and relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference and the test solution.

Calculate the content of $C_{14}H_{23}NO, HCl$.

Storage. Store protected from moisture , temperature between 15° to 30°.

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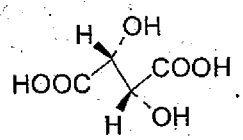
Tartaric Acid

L-Tartaric Acid



$C_4H_6O_6$ Mol. Wt. 150.1

Tartaric Acid is (2*R*,3*R*)-2,3-dihydroxybutanedioic acid.

Tartaric Acid contains not less than 99.5 per cent and not more than 101.0 per cent of $C_4H_6O_6$, calculated on the dried basis.

Category. Pharmaceutical aid (buffering agent).

Description. Colourless crystals or a white or almost white crystalline powder.

Identification

A. Ignite a few mg; it gradually decomposes giving off an odour resembling that of burnt sugar (distinction from citric acid).

B. A 10 per cent w/v solution in *distilled water* (solution A) is strongly acidic (2.4.46).

C. It gives reactions (A) and (B) of tartrates (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and not more intensely coloured than reference solution YS6 (2.4.1).

Specific optical rotation (2.4.22). + 12.0° to + 12.8°, determined in a 20.0 per cent w/v solution.

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Chlorides (2.3.12). 20 ml of solution A complies with the limit test for chlorides (125 ppm).

Sulphates (2.3.17). 10 ml of solution A complies with the limit test for sulphates (150 ppm).

Oxalate. Neutralise 10 ml of solution A with *dilute ammonia solution*, add 0.1 ml of *dilute acetic acid* and 10 ml of *calcium sulphate solution*; no opalescence is produced within 20 minutes.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

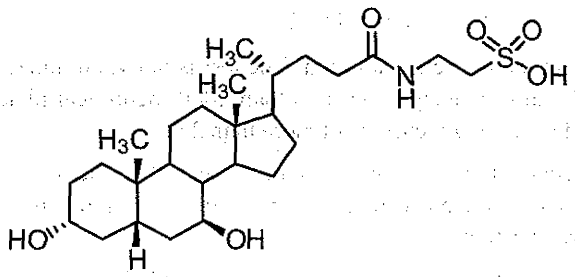
Loss on drying (2.4.19). Not more than 0.2 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 1.0 g in 25 ml of *water* and titrate with *1 M sodium hydroxide* using 0.5 ml of *phenolphthalein solution* as indicator. Carry out a blank titration.

1 ml of *1 M sodium hydroxide* is equivalent to 0.07505 g of $C_4H_6O_6$.

Storage. Store protected from moisture.

Tauroursodeoxycholic Acid



$C_{26}H_{45}NO_6S$ Mol Wt. 499.7

Tauroursodeoxycholic Acid is 3α, 7β-dihydroxy-5β – cholanoyltaurine.

Tauroursodeoxycholic Acid contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{26}H_{45}NO_6S$, calculated on the anhydrous basis.

Category. Use to dissolve cholesterol gallstones.

Description. A white to off white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tauroursodeoxycholic acid IPRS* or with the reference spectrum of tauroursodeoxycholic acid.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). + 42° to + 48°, determined on 1.0 per cent w/v solution in *ethanol* at 20°.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution. A 0.1 per cent w/v solution of *tauroursodeoxycholic acid IPRS* in the mobile phase.

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Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 30 volumes of a solution prepared by dissolving 0.69 g of *sodium dihydrogen orthophosphate* into 1000 ml of *water*, 40 volumes of *methanol*, 30 volumes of *acetonitrile* and 0.1 volume of *triethylamine*, adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 1.2 ml per minute,
- refrective index detector set at a constant temperature,
- injection volume: 100 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates n and the tailing factor is not more than 2.0.

Inject the test solution. The sum of areas of all the secondary peaks is not more than 3.0 per cent, calculated by area normalization.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.5 per cent.

Water (2.3.43). Not more than 8.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution. A 0.1 per cent w/v solution of *tauroursodeoxycholic acid IPRS* in the mobile phase.

Chromatographic system

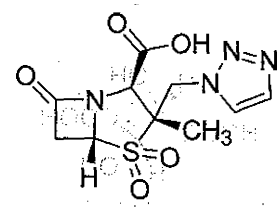
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 30 volumes of a solution prepared by dissolving 0.69 g of *sodium dihydrogen orthophosphate* into 1000 ml of *water*, 40 volumes of *methanol*, 30 volumes of *acetonitrile* and 0.1 volume of *triethylamine*, adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 1.2 ml per minute,
- refractive index detector set at a constant temperature,
- injection volume: 100 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 1500 theoretical plates , the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{26}H_{45}NO_6S$

Tazobactam



$C_{10}H_{12}N_4O_5S$ Mol. Wt. 300.3

$C_{10}H_{12}N_4O_5S,½H_2O$ Mol. Wt. 309.3

Tazobactam is (2*S*,3*S*,5*R*)-3-methyl-7-oxo-3-(1*H*-1,2,3-triazol-1-ylmethyl)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,4,4-dioxide hemihydrate.

Tazobactam contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{10}H_{12}N_4O_5S$, calculated on the anhydrous basis.

Category. Beta-lactamase inhibitor.

Description. A white to pale yellow, non-hygroscopic, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tazobactam IPRS* or with the reference spectrum of tazobactam.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 1.8 to 2.8, determined in a 0.25 per cent w/v solution in *water*.

Specific optical rotation (2.4.22). + 160.0° to + 167°, determined at 20° in a 1.0 per cent w/v solution in *dimethylformamide*.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Use freshly prepared solutions, store and inject at 3°.

Test solution. Use the test solution as given under Assay.

Reference solution. A solution containing 0.0016 per cent w/v of *L-phenylalanine*, 0.0050 per cent w/v of *tazobactam IPRS* and 0.0008 per cent w/v of *tazobactam- related compound A IPRS [(2S,3S)-2-amino-3-methyl-3-sulphino-4-(1H-1,2,3-triazol-1-yl)butyric acid]* in mobile phase.

Use the chromatographic conditions as described under Assay.

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Inject the reference solution. The relative retention time with reference to tazobactam for tazobactam- related compound A is about 0.3 and for *L*-phenylalanine is about 0.7.

The test is not valid unless the resolution between tazobactam and *L*-phenylalanine peaks is not less than 6.0.

Inject the test solution. The area of any peak corresponding to tazobactam- related compound A is not more than 1.0 per cent. The area of any other secondary peak is not more than 0.1 per cent and the sum of areas of all the secondary peaks other than tazobactam- related compound A is not more than 0.3 per cent, calculated by area normalization.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.6 per cent for anhydrous form and between 2.2 per cent to 3.8 per cent for hemihydrate form.

Microbial contamination (2.2.9). The total viable aerobic count does not exceed 10^3 cfu per g and the total combined moulds and yeasts count does not exceed 10^2 cfu per g.

Assay. Determine by liquid chromatography (2.4.14).

NOTE—Use freshly prepared solutions, store and inject at 3°, using a cooled autosampler.

Test solution. Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution. A 0.05 per cent w/v solution of *tazobactam IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- sample temperature: 2° to 4°,
- mobile phase: a mixture of a buffer solution prepared by dissolving 1.32 g of *dibasic ammonium phosphate* in about 750 ml of *water*, adjusted to pH 2.5 with 5 per cent v/v solution of *orthophosphoric acid*, dilute to 1000 ml with *water* and 30 ml of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.8 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{12}N_4O_5S$.

Tazobactam intended for use in the manufacture of parenteral preparations without a further appropriate sterilisation procedure complies with the following additional requirement.

Sterility (2.2.11). Complies with the test for sterility.

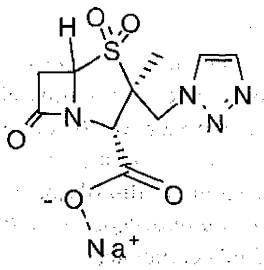
Tazobactam intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.08 Endotoxin Unit per mg of tazobactam.

Storage. Store protected from moisture, preferably at temperature below 30°.

Labelling. The label states whether the material is anhydrous or hemihydrates form.

Tazobactam Sodium



$C_{10}H_{11}N_4NaO_5S$ Mol. Wt. 322.3

Tazobactam Sodium is Sodium (2S,3S,5R)-3-methyl-7-oxo-3-(1*H*-1,2,3-triazol-1-ylmethyl)-4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid,4,4-dioxide.

Tazobactam Sodium contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{10}H_{11}N_4NaO_5S$, calculated on the anhydrous basis.

Category. Beta-lactamase inhibitor.

Description. A white to off-white crystalline hygroscopic powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tazobactam sodium IPRS* or with the reference spectrum of tazobactam sodium.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

C. Gives the reactions of sodium salts (2.3.1).

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Tests

Appearance of solution. A 10.0 per cent w/v solution in *water* is clear (2.4.1) and colourless (2.4.1).

Specific optical rotation (2.4.22). + 140.0° to + 150.0°, determined in a 1.0 per cent w/v solution.

pH (2.4.24). 5.0 to 8.0, determined in a 10.0 per cent w/v solution.

Light absorption (2.4.7). Absorbance of a 10 per cent w/v solution in *water* at about 430 nm is not more than 0.10.

Water (2.3.43). Not more than 5.0 per cent, determined on 0.2 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.01 per cent w/v solution of *tazobactam IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 30 volumes of *methanol*, 20 volumes of *acetonitrile* and 50 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*.
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{11}N_4NaO_5S$ by multiplying the content of $C_{10}H_{12}N_4O_5S$ with 1.073.

Tazobactam Sodium intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

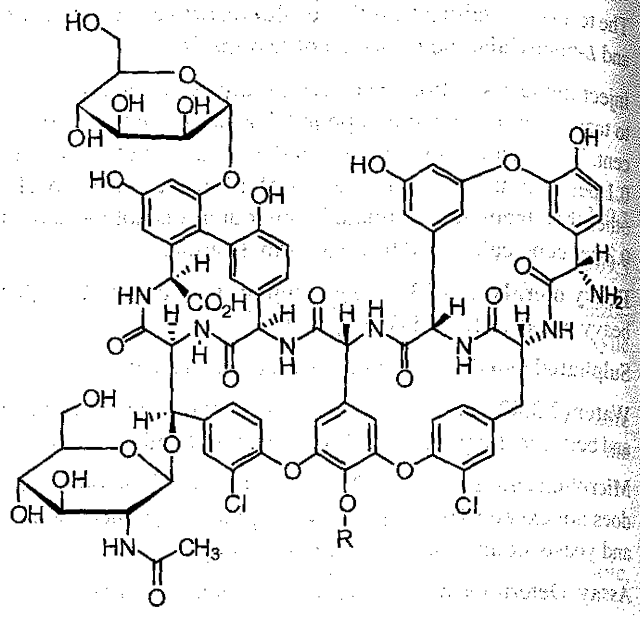
Bacterial endotoxins (2.2.3). Not more than 0.20 Endotoxin Unit per mg of tazobactam sodium.

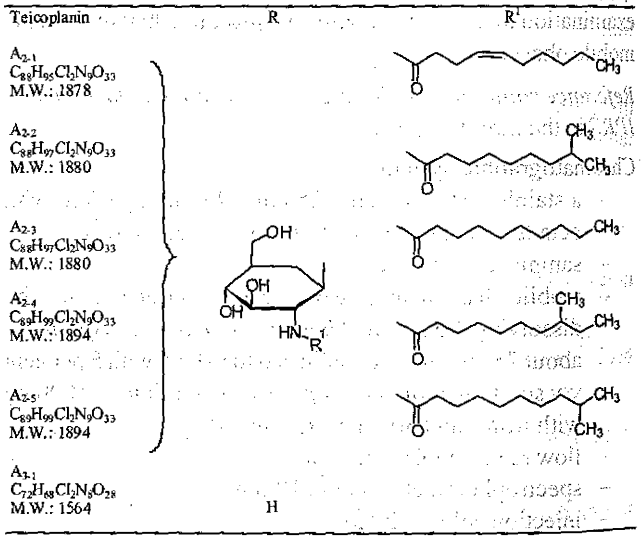
Tazobactam Sodium intended for use in the manufacture of parenteral preparations without a further appropriate sterilisation procedure complies with the following additional requirement.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Teicoplanin





Category. Glycopeptides antibacterial.

Teicoplanin is a mixture of the glycopeptides produced by the growth of certain strains of *Actinoplanes teichomyceticus sp.* fermentation product. The 6 principle contents of the mixture are teicoplanin A_{2-1} to A_{2-5} and teicoplanin A_{3-1}.

Teicoplanin has a potency of not less than 900 Units per mg, calculated on the anhydrous basis and on sodium chloride free substances.

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Description. A yellowish, amorphous powder.

Identification

A. Determine by infrared absorption spectrophotometery (2.4.6). Compare the spectrum with that obtained with *teicoplanin IPRS* or with the reference spectrum of teicoplanin.

B. In the test for Composition and Related substances, the retention time and area of the principal peaks (teicoplanins A_{3-1}, A_{2-1}, A_{2-2}, A_{2-3}, A_{2-4} and A_{2-5}) in the chromatogram obtained with test solution corresponds to the principal peaks in the chromatogram obtained with reference solution (a).

Tests

Appearance of solution (2.4.1). A 8.0 per cent w/v solution in *carbon dioxide- free water* is clear and not more intensely coloured than reference solution BYS3 or BS4.

pH (2.4.24). 6.5 to 7.5, determined in 5.0 per cent w/v solution in *carbon dioxide-free water.*

Composition and Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in *water* and dilute to 50.0 ml with *water.*

Reference solution (a). Dissolve 20 mg of *teicoplanin peak identification IPRS* in *water* and dilute to 10.0 ml with *water.*

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with *water.* Dilute 1.0 ml of the solution to 20.0 ml with *water.*

Reference solution (c). Dissolve 50 mg of *mesityl oxide IPRS* in *water* and dilute to 25.0 ml with *water.* Dilute 1.0 ml of the solution to 10.0 ml with *water.* Dilute 1.0 ml of the solution to 100.0 ml with *water.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with spherical end capped octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 900 volumes of a solution prepared by dissolving 3.0 g of *anhydrous sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.0 with *1 M sodium hydroxide solution* and 100 volumes of *acetonitrile,*

  B. a mixture of 300 volumes of a solution prepared by dissolving 3.0 g of *anhydrous sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.0 with *1 M sodium hydroxide solution* and 700 volumes of *acetonitrile.*
- a gradient programme using the conditions given below,
- flow rate: 2.3 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
30	50	50
31	10	90
35	10	90
38	100	0

Name	Relative retention time
Teicoplanin A_{3-1}	0.43
Teicoplanin A_{2-1}	0.93
Teicoplanin A_{2-2} (retention time about 18 minutes)	1.0
Teicoplanin A_{2-3}	1.04
Teicoplanin A_{2-4}	1.12
Teicoplanin A_{2-5}	1.14
Impurities is more than 1.25	

Inject treference solution (a) The test is not valid unless the resolution between the peak due to teicoplanin A_{2-4} and teicoplanin A_{2-5} is not less than 1.0.

The relative retention time with respect to Teicoplanin A_{2-2} peak for of groups and impurities with reference to teicoplanin A_{2-2} are

- Teicoplanin A_3 group is less than 0.7,
- Teicoplanin A_2 group is more than 0.7 and less than 1.25 and within this group,
- Teicoplanin A_{2-1} group is less than 1.0,
- Teicoplanin A_{2-3} group is more than 1 and less than 1.12,
- Teicoplanin A_{2-4} is about 1.12,
- Teicoplanin A_{2-5} group is more than 1.12 and less than 1.25.

Calculate the content of the different components using the following equations.

$$\text{teicoplanin } A_2 \text{ group} = \frac{S_a}{S_a + 0.83 \times S_b + S_c} \times 100$$

$$\text{teicoplanin } A_{2-2} = \frac{S_2}{S_a + 0.83 \times S_b + S_c} \times 100$$

$$\text{teicoplanin } A_{2-1} \text{ group} = \frac{S_1}{S_a + 0.83 \times S_b + S_c} \times 100$$

$$\text{teicoplanin } A_{2-3} \text{ group} = \frac{S_3}{S_a + 0.83 \times S_b + S_c} \times 100$$

$$\text{teicoplanin } A_{2-4} = \frac{S_4}{S_a + 0.83 \times S_b + S_c} \times 100$$

$$\text{teicoplanin } A_{2-5} \text{ group} = \frac{S_5}{S_a + 0.83 \times S_b + S_c} \times 100$$

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$$\text{teicoplanin } A_3 \text{ group} = \frac{0.83 \times S_b}{S_a + 0.83 \times S_b + S_c} \times 100$$

$$\text{impurities} = \frac{S_C}{S_a + 0.83 \times S_b + S_c} \times 100$$

Where, S_a = sum of the areas of the peaks due to teicoplanin A_2 group in the chromatogram obtained with the test solution;

S_b = sum of the areas of the peaks due to teicoplanin A_3 group in the chromatogram obtained with the test solution; disregard any peak due to mesityl oxide;

S_c = sum of the areas of the peaks with a relative retention more than 1.25;

S_1 = sum of the areas of the peaks due to teicoplanin $A_{2\text{-}1}$ group in the chromatogram obtained with the test solution;

S_2 = area of the peak due to teicoplanin $A_{2\text{-}2}$ in the chromatogram obtained with the test solution;

S_3 = sum of the areas of the peaks due to teicoplanin $A_{2\text{-}3}$ group in the chromatogram obtained with the test solution;

S_4 = area of the peak due to teicoplanin $A_{2\text{-}4}$ in the chromatogram obtained with the test solution;

S_5 = sum of the areas of the peaks due to teicoplanin $A_{2\text{-}5}$ group in the chromatogram obtained with the test solution.

The area of any secondary peak corresponding to teicoplanin A_2 group is not less than 80.0 per cent; teicoplanin $A_{2\text{-}2}$ group is not less than 35.0 per cent and not more than 55.0 per cent. Teicoplanin $A_{2\text{-}1}$ group is not more than 20.0 per cent, teicoplanin teicoplanin $A_{2\text{-}3}$ group is not more than 20.0 per cent, teicoplanin $A_{2\text{-}4}$ group is not more than 20.0 per cent, teicoplanin teicoplanin $A_{2\text{-}5}$ group is not more than 20.0 per cent and teicoplanin A_3 group is not more than 15.0 per cent and the sum of the areas of all other secondary peak other than mesityl oxide with a relative retention time 1.25 times is not more than 5.0 per cent. Ignore the peak due to teicoplanin $A_{2\text{-}2}$ with an area less than 0.5 times of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent).

Impurity A. Determine by liquid chromatography (2.4.14).

Use the Chromatographic system as described under test for Composition and related substances.

Name	Relative retention time
Teicoplann impurity A[1]	0.6
Teicoplann $A_{2\text{-}2}$ (Retention time: about 18 minutes)	1.0

[1]4-methylpent-3-en-2-one (mesityl oxide).

Inject reference solution (c) and the test solution. The area of peak corresponding to impurity A is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent).

Chlorides (2.3.12). Not more than 5.0 per cent expressed as *sodium chloride*, calculated on anhydrous basis, determined by following method.

Dissolve 1.0 g in 300 ml of *water*, stir and acidify with 2 ml of *nitric acid*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (2.4.25).

1 ml of *0.1 M silver nitrate* is equivalent to 0.005844 g of NaCl.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not more than 15.0 per cent, determined on 0.30 g

Bacterial endotoxins (2.2.3). Not more than 0.31 Endotoxin units per mg of teicoplanin.

Assay. Determine by the microbiological assay of antibiotics, Method A (2.2.10).

Storage. Store protected from light, at a temperature of 2° to 8°.

Teicoplanin Injection

Teicoplanin Injection is a sterile material consisting of Teicoplanin with or without auxiliary substances. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of Sterile Water for Injections, immediately before use.

The constituted solution complies with the requirements for the Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Teicoplanin Injection contains not less than 90.0 per cent and not more than 120.0 per cent of the stated amount of Teicoplanin.

The contents of the sealed container comply with requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Usual Strengths. 200 mg; 400 mg.

Description. A white or off white to yellowish powder.

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Identification

A. When examined in the range 200 nm to 400 nm (2.4.7), a 0.005 per cent w/v solution in *water* shows an absorption maximum at about 278 nm.

B. In the composition and related substances, The principal peaks (teicoplanins A_{3-1}, A_{2-1}, A_{2-2}, A_{2-3}, A_{2-4} and A_{2-5}) in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 6.0 to 8.0, determined in 5.0 per cent w/v solution in the carbon dioxide free *water*.

Composition and Related Substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of injection powder containing 400 mg of Teicoplanin, disperse in sufficient *water* with the aid of ultrasound and dilute to 200.0 ml with *water*.

Reference solution. A 0.2 per cent w/v solution of *teicoplanin IPRS* in *water*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS 3V),
- mobile phase: A. a mixture of 850 volumes of a solution prepared by dissolving 3.9 g of *sodium dihydrogen orthophosphate* in 850 ml of *water* and 150 volumes of *acetonitrile*, adjusted to pH 6.0 with *sodium hydroxide* solution.

  B. a mixture of 300 volumes of a solution prepared by dissolving 3.9 g of *sodium dihydrogen orthophosphate* in 300 ml of *water* and 700 volumes of *acetonitrile*, adjusted to pH 6.0 with *sodium hydroxide* solution.
- a gradient programme using the conditions given below,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
19	70	30
22	50	50
24	100	0
30	100	0

Relative retention of groups and impurities with reference to teicoplanin A_{2-2} is.

Name	Relative retention time
Teicoplanin A_3 group	< 0.70
Teicoplanin A_2	> 0.70 and < 1.25 and within this group
Teicoplanin A_{2-1} group	< 1.00
Teicoplanin A_{2-2}	1.00
Teicoplanin A_{2-3} group	> 1.00 and < 1.12
Teicoplanin A_{2-4}	about 1.12
Teicoplanin A_{2-5} group	> 1.12 and < 1.25
Impurities	> 1.25

Relative retention of principal peaks of the groups with reference to teicoplanin A_{2-2} is.

Name	Relative retention time
Teicoplanin A_{3-1}	about 0.43
Teicoplanin A_{2-1}	about 0.93
Teicoplanin A_{2-2}	1.00
Teicoplanin A_{2-3}	about 1.04
Teicoplanin A_{2-5}	about 1.14

Inject the reference solution. The test is not valid unless the resolution between the peak due to teicoplanin A_{2-4} and teicoplanin A_{2-5} is not less than 1.0.

Inject the test solution.

Calculate the percentage content ratio of the different components using the following equations

$$\text{Teicoplanin } A_2 \text{ group} = \frac{S_a}{S_a + 0.83 \times S_b + S_c} \times 100$$

$$\text{Teicoplanin } A_3 \text{ group} = \frac{0.83 \times S_b}{S_a + 0.83 \times S_b + S_c} \times 100$$

$$\text{Impurities} = \frac{S_c}{S_a + 0.83 \times S_b + S_c} \times 100$$

Where, S_a = sum of the areas of the peaks due to teicoplanin A2 group in the chromatogram obtained with the test solution;

S_b = sum of the areas of the peaks due to teicoplanin A3 group in the chromatogram obtained with the test solution;

S_c = sum of the areas of the peaks with a relative retention more than 1.25;

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The area of peaks corresponding to teicoplanin A_2 group is not less than 75.0 per cent, teicoplanin A_3 group is not more than 20.0 per cent and the sum of the areas of all other secondary peaks with a relative retention time more than 1.25 is not more than 5.0 per cent.

Water (2.3.43). Not more than 5.0 per cent, determined on 0.5 g.

Bacterial endotoxins (2.2.3). Not more than 0.30 Endotoxin unit per mg of teicoplanin.

Sterility (2.2.11). Complies with the test for sterility.

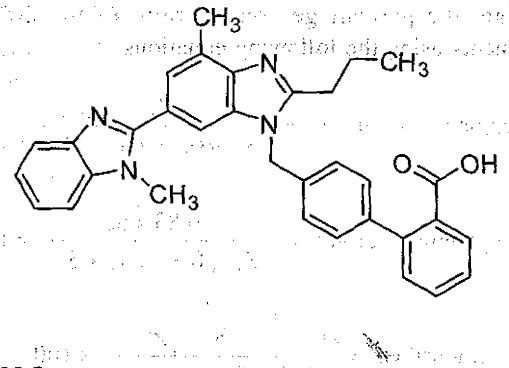
Assay. Determine by the microbiological assay of antibiotics, Method A (2.2.10) on a solution prepared in the following manner.

Reconstitute teicoplanin for injection as directed in the labeling. Withdraw an accurately measured volume of a resultant solution, using a suitable hypodermic needle and syringe, and dilute quantitatively and stepwise with buffer.

The upper fiducial limit of error is not less than 90.0 per cent and the lower fiducial limit of errors is not more than 120 per cent of the estimated potency.

Storage. Store in a cool place.

Telmisartan



$C_{33}H_{30}N_4O_2$ Mol. Wt. 514.6

Telmisartan is 4'-{[4-methyl-6-(1-methyl-1*H*-benzimidazol-2-yl)-2-propyl-1*H*-benzimidazol-1-yl]methyl}-2-biphenylcarboxylic acid.

Telmisartan contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{33}H_{30}N_4O_2$, calculated on the dried basis.

Category. Antihypertensive.

Description. A white to off-white crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *telmisartan IPRS* or with the reference spectrum of telmisartan.

Tests

Appearance of solution. A 5 per cent w/v solution in *1 M sodium hydroxide* is not more intensely coloured than reference solution YS4 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 25 mg of the substance under examination in 5 ml of *methanol*, add 100 µl of a 4 per cent w/v solution of *sodium hydroxide* and dilute to 50.0 ml with *methanol*.

Reference solution. Dilute 1.0 ml of the test solution to 10.0 ml with *methanol*. Dilute 1.0 ml of the solution to 100.0 ml with *methanol*.

Chromatographic system

- a stainless steel column 12.5 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as Thermoquest),
- column temperature: 40°,
- mobile phase: A. dissolve 2.0 g of *potassium dihydrogen phosphate* and 3.8 g of *sodium pentanesulphonate monohydrate* in *water*, adjusted to pH 3.0 with *orthophosphoric acid* and dilute to 1000 ml with *water*,
  B. a mixture of 20 volumes of *methanol* and 80 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
3	70	30
28	20	80
30	70	30

Inject the reference solution. The test is not valid unless the theoretical plates is not less than 2000, tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent) and the sum of areas of all the secondary peaks

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is not more than 10 times the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1 g by drying in oven at 105°.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of buffer solution prepared by diluting 5.0 ml of *triethylamine* to 2000 ml with *water* and 20 volumes of *methanol*.

Test solution. Dissolve 40 mg of substance under examination in 100.0 ml of the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.004 per cent w/v solution of *telmisartan IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (such as Inertsil ODS-3),
- mobile phase: a mixture of 60 volumes of buffer solution prepared by dissolving 2.72 g of *potassium dihydrogen phosphate* in 1000 ml of *water*; add 2 ml of *triethylamine* and adjusted to pH 2.4 with *orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 298 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the theoretical plates is not less than 3000, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{33}H_{30}N_4O_2$.

Storage. Store protected from light and moisture.

Telmisartan Tablets

Telmisartan Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of telmisartan, $C_{33}H_{30}N_4O_2$.

Usual strengths. 20 mg; 40 mg; 80 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *phosphate buffer pH 7.5*, prepared by dissolving 13.61 g of *potassium dihydrogen phosphate* in about 800 ml of *water*, adjusted to pH 7.5 with *2M sodium hydroxide*, and diluted with *water* to 1000 ml,
Speed and time. 75 rpm for 30 minutes.

Withdraw a suitable volume of the medium and filter. Reject the first few ml of the filtrate and dilute a suitable volume of the filtrate with dissolution medium. Measure the absorbance of the resulting solution at the maximum at about 296 nm (2.4.7). Calculate the content of $C_{33}H_{30}N_4O_2$ in the medium from the absorbance obtained from a solution prepared by dissolving 44 mg of *telmisartan IPRS* in 1 ml of *0.1M sodium hydroxide*, and dilute to 100 ml with *methanol*. Further dilute the solution with dissolution medium to obtain a solution with a final concentration of 0.0011per cent of *telmisartan*.

Q. Not less than 75 per cent of the stated amount of $C_{33}H_{30}N_4O_2$.

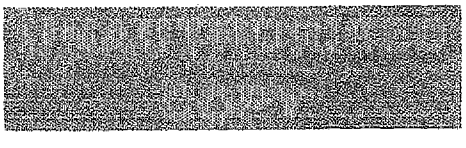
Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of buffer solution prepared by diluting 5.0 ml of *triethylamine* to 2000 ml with *water* and 20 volumes of *methanol*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 100 mg of Telmisartan in 100.0 ml of solvent mixture with the aid of ultrasound for 45 minutes and filter.

Reference solution. A 0.0005 per cent w/v solution of *telmisartan IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS-3),
- mobile phase: A. dissolve 0.5 g of *potassium dihydrogen phosphate* in 1000 ml of *water*; add 2 ml of *triethylamine*, adjusted to pH 3.2 with *orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 298 nm,
- injection volume: 20 µl.



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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	78	22
6	80	20
7	70	30
15	60	40
25	60	40
26	40	60
35	20	80
40	78	22

Inject the reference solution. The test is not valid unless the theoretical plates is not less than 3000, tailing factor is not more than 2.0 and relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent), the sum of the areas of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (2.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of buffer solution prepared by diluting 5.0 ml of *triethylamine* to 2000 ml with *water* and 20 volumes of *methanol*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 40 mg of Telmisartan in 100 ml of the solvent mixture and sonicate for 45 minutes. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.004 per cent w/v solution of *telmisartan IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (such as Inertsil ODS-3),
- mobile phase: a mixture of 60 volumes of buffer solution prepared by dissolving 2.72 g of *potassium dihydrogen phosphate* in 1000 ml of *water*; add 2 ml of *triethylamine* and adjusted to pH 2.4 with *orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 298 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the theoretical plates is not less than 3000, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{33}H_{30}N_4O_2$ in the tablets.

Telmisartan and Amlodipine Tablets

Telmisartan and Amlodipine Besylate Tablets; Telmisartan and Amlodipine Besilate Tablets

Telmisartan and Amlodipine Tablets contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of telmisartan, $C_{33}H_{30}N_4O_2$ and amlodipine, $C_{20}H_{25}ClN_2O_5$.

Usual strengths. Telmisartan, 40 mg and amlodipine, 5 mg; Telmisartan, 80 mg and amlodipine, 10 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (c).

Tests

Dissolution (2.5.2).

For Amlodipine —

Apparatus No. 2 (Paddle),
Medium. 500 ml *0.01 M hydrochloric acid*,
Speed and time.75 rpm and 20 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution.A 0.001 per cent w/v solution of *amlodipine besylate IPRS* in the dissolution medium.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5µm),
- sample temperature: 10°,
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 3.43 g *monobasic sodium phosphate dihydrate* and 2 ml of *triethylamine* in 1000 ml of *water* and adjusted to pH 6.0 with *orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,

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– spectrophotometer set at 257 nm,
– injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{25}ClN_2O_5$.

Q. Not less than 80 per cent of the stated amount of $C_{20}H_{25}ClN_2O_5$.

For Telmisartan —

Apparatus No. 2 (Paddle),

Medium, 900 ml of *phosphate buffer pH 7.5*, prepared by dissolving 6.8 g of *monobasic potassium phosphate* and 1.52 g *sodium hydroxide* in 1000 ml of *water*, adjusted to pH 7.5 with *dilute sodium hydroxide*,

Speed and time. 75 rpm and 20 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Solvent mixture. Add 5 volumes of *triethylamine* in 500 volumes of *water* and further add 500 volumes of *acetonitrile* and mix.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.1 per cent w/v solution of *telmisartan IPRS* in solvent mixture. Dilute 5.0 ml of the solution to 100.0 ml with the dissolution medium.

Use chromatographic system as described under dissolution of Amlodipine.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{33}H_{30}N_4O_2$.

Q. Not less than 80 per cent of the stated amount of $C_{33}H_{30}N_4O_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 10 volumes of *0.1M hydrochloric acid* and 90 volumes of *methanol*.

Test solution. Weigh and powder 20 tablets into fine powder. Transfer the powder containing 25 mg of Amlodipine into 100.0 ml volumetric flask and dissolve in about 70.0 ml solvent mixture and sonicate for 45 minutes. After sonication bring the solution into room temperature and dilute to volume up to the mark with solvent mixture.

Reference solution (a). A 0.004 per cent w/v solution of *telmisartan IPRS* in solvent mixture.

Reference solution (b). A solution of *amlodipine besylate IPRS* containing 0.00125 per cent w/v of amlodipine in solvent mixture.

Reference solution (c). Transfer 5ml of reference solution (a) and 2 ml of reference solution (b) in 100.0 ml volumetirc flask and dilute to volume upto the mark with solvent mixture and mix.

Chromatographic system

– a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
– mobile phase: A. a mixture of 85 volumes of a buffer solution prepared by dissolving 1.36 g *potassium dihydrogen orthophosphate* in 1000.0 ml of *water* and adjusted to pH 3.2 with *orthophosphoric acid* and 15 volumes of *acetonitrile*.

B. a mixture of 15 volumes of a buffer solution prepared by dissolving 1.36 g *potassium dihydrogen orthophosphate* in 1000.0 ml of *water* and adjusted to pH 3.2 with *orthophosphoric acid* and 85 volumes of *acetonitrile*.
– a gradient programme using the conditions given below,
– flow rate: 1.5 ml per minute,
– spectrophotometer set at 237 nm,
– injection volume: 25 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
7	85	15
15	90	10
20	75	25
35	60	40
40	55	45
45	45	55
50	35	65
55	85	15
60	85	15

Inject reference solution (c). The test is not valid unless the column efficiency for both the peak is not less than 1500 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent.

The relative retention time with reference to amlodipine for Impurity D (3-ethyl 5-methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methylpyridine-3,5-dicarboxylate) is 0.5. Multiply the peak area of impurity D by correction factor 2.58.

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution, the area of any

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peak due to impurity D of amlodipine is not more than the 10 times the area of the amlodipine principal peak in reference solution (c) (1.0 per cent). The area of any secondary peak due to amlodipine and telmisartan is not more than the 5 times the area of the telmisartan principal peak in reference solution (c) (0.5 per cent) and the sum of the areas of the all secondary peaks excluding Impurity D is not more than 20 times the area of the principal peak of telmisartan in the chromatogram obtained with reference solution (c) (2.0 per cent). Ignore any peaks with an area less than 0.5 times of telmisartan peak in chromatogram obtained with reference solution (c) (0.05 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14).

Use chromatographic condition, reference solution, solvent mixture and system suitability as described under Assay.

Test solution. Transfer 1 tablet into 100.0 ml volumetric flask add about 5.0 ml of mobile phase and sonicate to disperse whole tablet completely, add about 70.0 ml of solvent mixture and sonicate for 90 minutes. After sonication bring the solution into room temperature and dilute up to the mark with solvent mixture, mix and filter. Dilute 5.0 ml of the solution to 25.0 ml with solvent mixture and mix.

Inject reference solution (c) and test solution.

Calculate the content of $C_{20}H_{25}ClN_2O_5$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 10 volumes of *0.01 M hydrochloric acid* and 90 volumes of *methanol*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 5 mg of Amlodipine in 50.0 ml volumetric flask, add about 5ml of mobile phase and sonicate add 30.0 ml of solvent mixture and sonicate. After sonication dilute up to the mark with solvent mixture and mix. Dilute 5.0 ml of the solution to 50.0 ml with solvent mixture and mix.

Reference solution (a). A 0.08 per cent w/v solution of *telmisartan IPRS* in solvent mixture.

Reference solution (b). A solution of *amlodipine besylate IPRS* containing 0.02 per cent w/v solution of *amlodipine IPRS* in solvent mixture.

Reference solution (c). Dilute reference solution (a) and (b) with the solvent mixture to obtain a solution having a known concentration similar to the test solution.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolve 2.72 g *potassium dihydrogen orthophosphate* in 1000 ml of *water* and adjusted to pH 3.0 with *orthophosphoric acid* and 40 volumes of *acetonitrile*.
- flow rate: 1 ml per minute,
- spectrophotometer set at 237 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the column efficiency for both the analyte peak is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (c) and the test solution.

Calculate the content of $C_{33}H_{30}N_4O_2$ and $C_{20}H_{25}ClN_2O_5$ in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of amlodipine.

Telmisartan and Hydrochlorothiazide Tablets

Telmisartan and Hydrochlorothiazide Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of telmisartan $C_{33}H_{30}N_4O_2$ and not less than 90.0 per cent and not more than 107.5 per cent of the stated amount of hydrochlorothiazide $C_7H_8ClN_3O_4S_2$.

Usual strengths. Telmisartan, 40 mg and Hydrochlorothiazide, 12.5 mg; Telmisartan, 80 mg and Hydrochlorothiazide, 12.5 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with reference solution (c).

Tests

Dissolution (2.5.2).

For Telmisartan —

Apparatus No. 2 (Paddle),
Medium. 900 ml of a *phosphate buffer pH 7.5*, prepared by dissolving 13.61 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 7.5 with *2 M sodium hydroxide*,
Speed and time. 75 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

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For Hydrochlorothiazide —

Apparatus No. 1 (Basket),

Medium. 900 ml of *0.1 M hydrochloric acid,*

Speed and time. 100 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution (a). Dissolve a quantity of *telmisartan IPRS* in minimum quantity of *methanol* and dilute with dissolution medium to obtain a solution having a known concentration similar to the test solution.

Reference solution (b). Dissolve a quantity of *hydrochlorthiazide IPRS* in minimum quantity of *methanol* and dilute with dissolution medium to obtain a solution having a known concentration similar to the test solution.

Chromatographic system

- a stainless steel column 6 cm x 3.0 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. a buffer solution prepared by dissolving 5.0 g of *ammonium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *diluted phosphoroic acid*,
  B. *acetonitrile,*
- a gradient programme using the conditions given below,
- spectrophotometer set at 270 nm for hydrochlorothiazide and 298 nm for telmisartan,
- injection volume: 4 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)	Flow rate (ml per min.)
0	85	15	0.6
1.5	85	15	0.6
1.51	60	40	0.6
5.0	60	40	0.6
5.01	20	80	1.0
6.2	20	80	1.0
6.21	85	15	0.6
9.7	85	15	0.6

The relative retention time with reference to telmisartan for hydrochlorothiazide is about 0.33.

Inject reference solution (a) and (b). The test is not valid unless the tailing factor for each peak is not more than 2.5 and the relative standard deviation for replicate injections for each peak is not more than 2.0 per cent.

Inject reference solution (a) and (b) and the test solution.

Q. Not less than 80 per cent of the stated amount of $C_{33}H_{30}N_4O_2$ and $C_7H_8ClN_3O_4S_2$.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Reference solution (d). A 0.0025 per cent w/v solution of *4-Amino-6-chloro-1,3-benzenedisulphonamide IPRS* (*benzothiadiazine impurity A IPRS*) in the solvent mixture and further dilute with a solution prepared by diluting equal volumes of mobile phase A and mobile phase B to obtain a final concentration of 0.00005 per cent w/v for telmisartan 40 mg and hydrochlorothiazide 12.5 mg and 0.000025 per cent w/v for telmisartan 80 mg and hydrochlorothiazide 12.5 mg.

Reference solution (e). A solution containing 0.016 per cent w/v of *telmisartan IPRS* and 0.005 per cent w/v *hydrochlorothiazide IPRS* in the solvent mixture. Dilute 1.0 ml of the solution and 2.0 ml of 0.0025 per cent w/v solution of *benzothiadiazine impurity A* RS in the solvent mixture to 100.0 ml with the solvent mixture and further dilute 1.0 ml of the solution to 5.0 ml with a solution prepared by diluting equal volumes of mobile phase A and mobile phase B.

Inject reference solution (c) and (e). The test is not valid unless the resolution between the peaks due to hydrochlorothiazide and benzothiadiazine impurity A in the chromatogram obtained with reference solution (c) is not less than 2.0 and the relative standard deviation for replicate injections for both peaks of telmisartan and hydrochlorothiazide in thechromatogram obtained with reference solution (c) is not more than 2.0 per cent. The signal to noise ratio for the telmisartan, hydrochlorothiazide and benzothiadiazine impurity A peaks in the chromatogram obtained with reference solution (e) is not less than 3.0.

Inject reference solution (c), (d) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to benzothiadiazine impurity A is not more than 6.4 times the area of the benzothiadiazine impurity A peak in the chromatogram obtained with reference solution (d) (1.0 per cent),the area of any individual secondary peak due to telmisartan is not more than 0.005 times the area of the telmisartan peak in the chromatogram obtained with reference solution (c) (0.5 per cent), the area of any individual secondary peak due to hydrochlorothiazide is not more than 0.002 times the area of the hydrochlorothiazide peak in the chromatogram obtained with reference solution (c) (0.2 per cent) and the sum of areas of all the secondary peaks due to telmisartan is not more than 0.005 times the area of the telmisartan peak in the chromatogram obtained with reference solution (c) (0.5 per cent) and the sum of areas of all the secondary peaks due to hydrochlorothiazide is not more than 0.02 times the area of the

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hydrochlorothiazide peak in the chromatogram obtained with reference solution (c) (2.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 0.005 M methanolic solution of sodium hydroxide.

NOTE — To prevent heat from degrading the sample, do not extend the sonication time and also maintain the bath temperature at a temperature not exceeding 22° by adding ice.

Test solution. Weigh and disperse 10 intact tablets in a suitable volumetric flask, add *0.1 M Sodium hydroxide solution,* not exceeding 5 per cent of the total volume and shake until the tablets are completely dispersed. Add *methanol,* not exceeding 80 per cent of the total volume, mix with the aid of ultrasound for 10 minutes, stir vigorously for 30 minutes, cool and dilute to volume with *methanol* to obtain a solution containing 0.16 per cent w/v of telmisartan. Centrifuge the solution at 4000 rpm and use clear supernatant liquid. Dilute 1.0 ml of the solution to 5.0 ml with a solution prepared by diluting equal volumes of mobile phase A and mobile phase B.

Reference solution (a). A 0.32 per cent w/v solution of *telmisartan IPRS* in the solvent mixture.

Reference solution (b). A 0.05 per cent w/v solution of *hydrochlorothiazide IPRS* in the solvent mixture.

Reference solution (c). Dilute a suitable volume of reference solution (a), reference solution (b) and 0.0025 per cent w/v solution of *benzothiadiazine impurity A* in solvent mixture in equal volumes of mobile phase A and mobile phase B to obtain a solution having similar concentration to the test solution and 0.00005 per cent w/v solution of *benzothiadiazine impurity A.*

Chromatographic system

- a stainless steel column 12.5 cm x 4.0 mm packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. a buffer solution prepared by dissolving 2.0 g of *ammonium dihydrogen orthophosphate* in 1000 ml of *water,* adjusted to pH 3.0 with *diluted phosphoric acid,*
  B. a mixture of equal volumes of *methanol* and *acetonitrile,*
- flow rate: 1.2 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 270 nm for hydrochlorothiazide and 298 nm for telmisartan,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
3.5	85	15
3.51	45	55
7.7	45	55
7.71	20	80
12.0	20	80
12.1	85	15
15.5	85	15

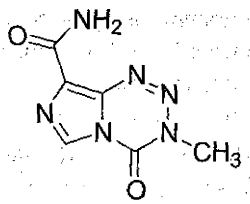
Inject reference solution (c). The test is not valid unless the resolution between the peaks due to hydrochlorothiazide and benzothiadiazine impurity A is not less than 2.0 and the relative standard deviation for replicate injections for both peaks of telmisartan and hydrochlorothiazide is not more than 2.0 per cent.

Inject reference solution (c) and the test solution.

Calculate the content of $C_{33}H_{30}N_4O_2$ and $C_7H_8ClN_3O_4S_2$ in the tablet.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Temozolomide



$C_6H_6N_6O_2$ Mol. Wt. 194.2

Temozolomide is 3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxamide.

Temozolomide contains not less than 98.0 per cent and not more than 102.0 per cent of $C_6H_6N_6O_2$, calculated on anhydrous basis.

Category. Anticancer.

Description. A white to light pink or light tan powder.

NOTE— Temozolomide is cytotoxic; extra care required to prevent inhaling particles and exposing the skin to it.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *temozolomide IPRS* or with the reference spectrum of temozolomide.

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B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Shake the solutions to dissolve. Do not sonicate.

Test solution. Dissolve 100 mg of the substance under examination in *dimethylsulphoxide* and dilute to 100.0 ml with *dimethylsulphoxide. (NOTE – Use freshly opened bottle)*

Reference solution (a). A 0.0001 per cent w/v solution of *temozolomide IPRS* in *dimethylsulphoxide.*

Reference solution (b). Mix 5 ml of *0.1 M hydrochloric acid* and 5 ml of 0.1 per cent w/v solution of *temozolomide IPRS in dimethylsulphoxide.* Heat the mixture in a water-bath for 1hour. [*The preparation forms 2-azahypoxanthine, temozolomide acid and aminoimidazolecarboxamide.*]

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase : a mixture of 96 volumes of 0.5 per cent v /v solution of *glacial acetic acid* in *water* and 4 volumes of *methanol* containing 0.094 per cent w/v of *sodium 1-hexanesulphonate,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 10 µl.

Name	Relative retention time	Correction factor
2-Azahypoxanthine[1]	0.42	0.63
Temozolomide related compound A[2]	0.53	—
Temozolomide acid[3]	0.84	—
Temozolomide	1.0	—
Aminoimidazolecarboxamide[4]	1.37	0.4
Cyanotemozol omide[5,6]	2.3	—

[1]4a,5-dihydro-4H -imidazo[4,5-d][1,2,3]triazin-4-one,

[2]4-diazo-4H -imidazole-5-carboxamide,

[3]3-methyl-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine -8-carboxylic acid,

[4]5-aminoimidazole-4-carboxamide. Two peaks may be observed, use the sum of the peak areas for calculation,

[5]3-methyl-4-oxo-3,4-dihydroimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carbonitrile,

[6]If possible from the manufacturing process.

Inject reference solution (b).The test is not valid unless the resolution between the peaks due to temozolomide acid and temozolomide is not less than 1.5.

Inject reference solution (a) and the test solution. Run the chromatogram 3.2 times the retention time of the principal peak. The area of any peak due to 2-azahypoxanthine is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any peak due to temozolomide related compound A is not more than 5 times the area of principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent), the area of any peak corresponding to temozolomide acid and aminoimidazolecarboxamide, each of, is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the area of any peak corresponding to cyanotemozol omide is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent).

Heavy metals (2.3.13). 0.67 g complies with the limit test for heavy metals, Method B (30 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.4 per cent, using method 3.

Assay. Determine by liquid chromatography (2.4.14) as described under Related substances using following modifications.

Reference solution. A 0.1 per cent w/v solution of *temozolomide IPRS* in *dimethylsulphoxide.*

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.9 and the relative standard deviation of replicate injections is not more than 1.5.

Inject the reference solution and the test solution.

Calculate the content of $C_6H_6N_6O_2$.

Storage. Store protected from light and moisture.

Temozolomide Capsules

Temozolomide Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of temozolomide, $C_6H_6N_6O_2$.

CAUTION —Temozolomide is cytotoxic; extra care required to prevent inhaling particles and exposing the skin to it.

Usual strengths. 20 mg; 100 mg; 250 mg.

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Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution.

Tests

Other tests. Comply with tests stated under capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of mixed contents of 20 capsules containing 20 mg of Temozolomide in 100.0 ml of mobile phase.

Reference solution. A 0.02 per cent w/v solution of *temozolomide IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil BDS),
- mobile phase: a mixture of 90 volumes of 0.5 per cent v/v *acetic acid* and 10 ml volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 329 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency is not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_6H_6N_6O_2$ in the capsules.

Storage. Store protected from light and moisture.

Temozolomide Injection

Temozolomide Injection is a sterile, lyophilized material consisting of Temozolomide with or without excipients. It is filled in a sealed container.

Temozolomide Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of temozolomide, $C_6H_6N_6O_2$.

NOTE — Temozolomide is cytotoxic; extra care required to prevent inhaling particles and exposing the skin to it.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Usual strength. 100 mg.

Storage. The reconstituted solution stand at room temperature, must be used within 14 hours, including infusion time.

The content of the sealed container comply with the requirements stated under Parenteral Preparation (Powder for Injection) and with the following requirement.

Identification

A. Disperse a quantity of injection containing 50 mg of temozolomide in *methylene chloride* with the aid of ultrasound for 30 minutes and filter. Evaporate the filtrate under a stream of nitrogen to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *temozolomide IPRS* treated in similar manner or with the reference spectrum of temozolomide.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

pH (2.4.28). 3.0 to 4.5, determined on a freshly constituted vial of temozolomide injection, using 40 ml *carbon- dioxide free water.*

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Reconstitute 5 vials with each of 40 ml of *water*, with the aid of ultrasound, combine and dilute to 250.0 ml with water. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (a). A 0.01 per cent w/v solution of *temozolomide IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Transfer 50 ml of *0.1 M hydrochloric acid* and 50 ml of reference solution (a) to a suitable glass container. Heat the container at 80° for 4 hours. Dilute 10.0 ml of the solution to 25.0 ml with the mobile phase. *(NOTE — the preparation forms 2-azahypoxanthine, temozolomide acid and aminoimidazole carboxamide).*

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- sample temperature: 2° to 8°,
- mobile phase: a mixture of 96 volumes of 0.5 per cent v/v solution of *glacial acetic acid* in *water* and 4 volumes of *methanol* containing 0.094 per cent w/v of *sodium 1-hexanesulphonate*,

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– flow rate: 1 ml per minute,
– spectrophotometer set at 270 nm,
– injection volume: 75 µl.

Name	Relative retention time	Correction factor
2-Azahypoxanthine[1]	0.4	0.63
Temozolomide related compound A[2]	0.5	—
Temozolomide acid[3]	0.9	1.32
Temozolomide	1.0	—
Aminoimidazolecarboxamide[4]	1.4	0.29
Cyanotemozolomide[5]	2.3	

[1]4a,5-dihydro-4H -imidazo[4,5-d][1,2,3]triazin-4-one,

[2]4-diazo-4H -imidazole-5-carboxamide, (This is a process impurity and include for peak identification only),

[3]3-methyl-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine -8-carboxylic acid,

[4]5-aminoimidazole-4-carboxamide,

[5]3-methyl-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carbonitrile (This is a process impurity and include for peak identification only).

Inject reference solution (c) and (d). The test is not valid unless the resolution between the peaks due to temozolomide and aminoimidazole carboxamide is not less than 2.5 in the chromatogram obtained with reference solution (c) and the signal to noise ratio is not less than 10 in the chromatogram obtained with reference solution (d).

Inject reference solution (b) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak for the test solution. The area of any peak corresponding to 2-azahypoxanthine is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent), the area of any peak corresponding to temozolamide acid is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of areas of all the secondary peaks other than peaks corresponding to temozolamide related compound A and cyanotemozolamide is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Bacterial endotoxins (2.2.3). Not more than 0.88 Endotoxin Units per mg of temozolomide.

Assay. Determine by liquid chromatography (2.4.14) as described under Related substances with the following modifications.

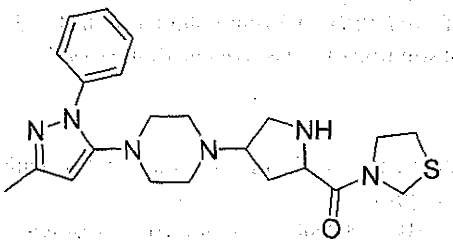
Inject reference solution (a). The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation of replicate injections is not more than 1.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_6H_6N_6O_2$ in the injection.

Storage. Store protected from light and moisture, at a temperature 2° to 8°.

Teneligliptin Hydrobromide Hydrate



$C_{22}H_{30}N_6OS, 2½ HBr, xH_2O$ Mol. Wt.628.9

Teneligliptin Hydrobromide Hydrate is {(2*S*,4*S*)-4-[4-(3-methyl-1-phenyl-1*H*-pyrazol-5-yl)piperazine-1-yl]pyrrolidine-2-yl} (1,3-thiazolidine-3-yl) methanone hemipentahydrobromide hydrate.

Teneligliptin Hydrobromide Hydrate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{22}H_{30}N_6OS$, 2½ HBr, calculated on the anhydrous basis.

Category. Antidiabetic.

Description. An off white to cream coloured powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *teneligliptin hydrobromide hydrate IPRS* or with the reference spectrum of teneligliptin hydrobromide hydrate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Hydrobromic acid content. Not less than 28.0 per cent w/w and not more than 34.0 per cent w/w, calculated on the anhydrous basis, determine by liquid chromatography

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(2.4.14), as described under Assay with following modifications:

Reference solution. A 0.003 per cent w/v solution of *hydrobromic acid IPRS* in the solvent mixture.

Calculate the content of HBr.

Enantiomeric purity. Not more than 0.5 per cent. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 50 mg of the substance under examination in 25.0 ml of *ethanol.*

Reference solution (a). A 0.02 per cent w/v solution of *R-isomer IPRS [{(2R,4R)-4-[4-(3-Methyl-1-phenyl-1H-pyrazol-5-yl)piperazine-1-yl]pyrrolidine-2-yl}(1,3-thiazolidine-3-yl)methanone hemipentahydrobromide hydrate IPRS]* in *ethanol.*

Reference solution (b). Weigh about 100 mg of *teneligliptin hydrobromide hydrate IPRS* to a 50-ml volumetric flask, add 5.0 ml of reference solution (a), dissolve and dilute to volume with *ethanol.*

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with chiral Pak IC (5 µm),
- mobile phase: a 0.1 per cent w/v solution of *diethylamine* in *ethanol.*
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

The relative retention time of R- isomer peak is about 0.61 with respect to teneligliptin peak.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to R-isomer and teneligliptin is not less than 3.0.

Inject the test solution and measure the area of the *R*-isomer.

Calculate the content of the *R*-isomer by area normalization.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of *water* and 20 volumes of *acetonitrile.*

Test solution. Dissolve about 50 mg of substance under examination in 50.0 ml of the solvent mixture.

Reference solution (a). A 0.01 per cent w/v solution each of *teneligliptin impurity A IPRS, impurity B IPRS* and *impurity C IPRS* in the solvent mixture.

Reference solution (b). Weigh and transfer about 50 mg of *teneligliptin hydrobromide hydrate IPRS* to a 50-ml volumetric flask, add 5.0 ml of reference solution (a), dissolve and dilute to volume with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with phenyl group bonded to porous silica (5 µm) (Such as Inertsil Ph-3),
- mobile phase: A. a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* and 1g of *1-octane sulphonic acid sodium salt* in 1000 ml of *water*, adjusted to pH 2.7± 0.03 with *dilute orthophosphoric acid,*

  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	75	25
7	75	25
35	60	40
45	50	50
50	75	25
60	75	25

Name	Relative retention time	Correction factor
Hydrobromic acid	0.09	—
Impurity A[1]	0.46	0.58
Impurity C[2]	0.58	1.70
Impurity B[3]	1.96	0.81

[1]1-3-methyl-1-phenyl-5-pyrazolyl) piperazine,

[2]tert-butyl(2*S*)-4-hydroxy-2-(thiazolidine-3-carbonyl) pyrrolidine-1-carboxylate,

[3]tert-butyl(2*S*,4*S*)-4-(4-(3-methyl-1-phenyl-1*H*-pyrazol-5-yl) piperazine-1-yl)-2-(thiazolidine-3-carbonyl) pyrrolidine-1-carboxylate.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to impurity C and teneligliptin is not less than 4.0.

Inject the test solution. The area of the peak due to impurity A, impurity B and impurity C is not more than 0.5 per cent. The area of any other secondary peak is not more than 0.3 per cent and the sum of the areas of all the secondary peaks is not more than 1.0 per cent, calculated by area normalisation. Ignore the peak due to hydrobromic acid.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). 2.8 per cent to 6.0 per cent, determined on 0.25 g.

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Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 60 volumes of *water* and 40 volumes of *acetonitrile.*

Test solution. Dissolve about 50 mg of substance under examination in 50.0 ml of the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *teneligliptin hydrobromide hydrate IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm packed with phenyl group bonded to porous silica (5 µm) (Such as Inertsil Ph-3),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* and 1g of *1-octane sulphonic acid sodium salt* into 1000 ml of *water*; adjusted to pH 2.7± 0.03 with *dilute orthophosphoric acid* and 40 volumes of *acetonitrile,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2500 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{30}N_6OS$, 2½ HBr.

Teneligliptin Tablets

Teneligliptin Hydrobromide Hydrate Tablets

Teneligliptin Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of teneligliptin, $C_{22}H_{30}N_6OS$.

Usual strength. 20 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *water,*

Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.0033 per cent w/v solution of *teneligliptin hydrobromide hydrate IPRS* in the dissolution medium.

Use chromatographic system as described under Assay.

Inject the reference solution. The test is not valid unless the theoretical plates is not less than 1000, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{30}N_6OS$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{22}H_{30}N_6OS$.

Related substances. Determine by liquid chromatography (2.4.14).

Solution A. Dissolve 1 g of *1-octane sulphonic acid sodium salt anhydrous* in 900 ml of *water.* Adjusted to pH 3.1 with *dilute orthophosphoric acid* and dilute to 1000 ml with *water.*

Solution B. A mixture of 50 volumes of solution A, 10 volumes of *water* and 40 volumes of *acetonitrile.*

Test solution. Weigh and transfer intact tablets containing about 100 mg of teneligliptin in 250-ml volumetric flask. Add about 200 ml of solution B and sonicate for 15 minutes with intermittent swirling. Cool and dilute to volume with solution B, filter.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Peerless Basic),
- mobile phase: A. a mixture of 82 volumes of solution A and 18 volumes of *acetonitrile,*
  B: a mixture of 50 volumes of solution A and 50 volumes of *acetonitrile,*
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 248 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	52	48
5	52	48
30	0	100
31	52	48
40	52	48

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The relative retention time with reference to teneligliptin for teneligliptin impurity A [1-(3-methyl -1-phenyl-5-pyrazolyl) piperazine] is about 0.52.

Inject the test solution. The area of peak corresponding to teneligliptin impurity A, multiplied with the correction factor 0.59 is not more than 1.0 per cent. The area of any other secondary peak is not more than 0.5 per cent and the sum of the areas of all the secondary peaks is not more than 2.0 per cent, calculated by area normalisation. Ignore any peak due to hydrobromic acid obtained with the test solution at relative retention time about 0.09.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 60 volumes of *water* and 40 volumes of *acetonitrile*.

Test solution. Weigh and transfer intact tablets containing about 100 mg of Teneligliptin in 500-ml volumetric flask. Add about 10 ml of *water* and sonicate. Add about 350 ml of the solvent mixture and sonicate for 15 minutes with intermittent swirling. Cool and dilute to volume with the solvent mixture, filter. Dilute 5.0 ml of the solution to 25.0 ml with the solvent mixture.

Reference solution. A 0.0059 per cent w/v solution of *teneligliptin hydrobromide hydrate IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Eurosphere II 100-5),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* and 1g of *1-octane sulphonic acid sodium salt monohydrate* into 1000 ml of *water*, adjusted to pH 2.7 with *orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 246 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the theoretical plates is not less than 1000, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{30}N_6OS$ in the tablets.

Labelling. The label states the strength in terms of the equivalent amount of teneligliptin.

Teneligliptin and Metformin Hydrochloride Prolonged-release Tablets

Teneligliptin and Metformin Hydrochloride Sustained-release Tablets; Teneligliptin and Metformin Hydrochloride Extended-release Tablets

Teneligliptin and Metformin Hydrochloride Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Teneligliptin and Metformin Hydrochloride Prolonged-release Tablets contain teneligliptin hydrobromide hydrate equivalent to not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of teneligliptin, $C_{22}H_{30}N_6OS$ and metformin hydrochloride, $C_4H_{11}N_5$ HCl.

Usual strengths. Teneligliptin, 20 mg and Metformin hydrochloride, 500 mg; Teneligliptin, 20 mg and Metformin hydrochloride, 1000 mg.

Identification

A. In the Assay for teneligliptin, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. In the Assay for metformin hydrochloride, the absorbance maxima obtained with the test solution corresponds to the absorbance maxima at same wavelength obtained with the reference solution.

Tests

Dissolution (2.5.2).

For Teneligliptin —

Apparatus No. 2 (Paddle),

Medium. 1000 ml of phosphate buffer pH 6.8 prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 6.8 with *1M sodium hydroxide*,

Speed and time. 75 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14)

Solvent mixture. Equal volumes of *isopropyl alcohol* and *water*.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.02 per cent w/v solution of *teneligliptin hydrobromide hydrate IPRS* in the solvent

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mixture. Dilute a suitable volume of the solution with the dissolution medium to obtain a solution having similar concentration as that of the test solution.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil BDS),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 3.48 g of *dipotassium hydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 7.2 with *orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{30}N_6OS$ in the medium.

Q: Not less than 70 per cent of the stated amount of $C_{22}H_{30}N_6OS$.

For Metformin Hydrochloride — Complies with the test stated under Tablets.

Related substances. Determine by liquid chromatography (2.4.14).

For Teneligliptin —

Solvent mixture. 70 volumes of *water* and 30 volumes of *acetonitrile*.

Test solution. Disperse a quantity of the powdered tablets containing 50 mg of Teneligliptin in 70 ml of *water*, with the aid of ultrasound for 25 minutes and dilute to 100.0 ml with *water*. Centrifuge the solution at 3000 rpm for 10 minutes. Use the supernatant liquid.

Reference solution. A solution containing 0.05 per cent w/v, each of, *teneligliptin impurity A IPRS, teneligliptin impurity B IPRS* and *teneligliptin hydrobromide hydrate IPRS* in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS-3V),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 2.16 g of *octane sulphonic acid sodium salt* in 1000 ml of *water*, adjusted to pH 3.5 with *dilute orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to teneligliptin impurity A and B, each of, is not more than 0.5 times the area of the corresponding peaks in the chromatogram obtained with the reference solution (0.5 per cent), the area of any other secondary peak is not more than 0.3 times the area of the principal peak in the chromatogram obtained with the reference solution (0.3 per cent) and the sum of the areas of all the secondary peaks is not more than 1.5 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent). Ignore the peak due to metformin hydrochloride and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

For Metformin hydrochloride —

Test solution. Disperse a quantity of the powdered tablets containing 0.5 g of Metformin Hydrochloride in 70 ml of *water*, with the aid of ultrasound for 20 minutes and dilute to 100.0 ml with *water*.

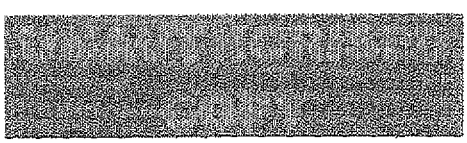
Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *water*. Dilute 1.0 ml of the solution to 10.0 ml with *water*.

Reference solution (b). A 0.0001 per cent w/v solution of *dicyandiamide IPRS* in *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a buffer solution prepared by dissolving 0.87 g of *sodium pentane-1 sulphonic acid* and 1.2 g of *sodium chloride* in 1000 ml of *water*, adjusted to pH 3.5 with *dilute orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 218 nm,
- injection volume: 20 µl.

Inject reference solution (a), (b) and the test solution. Run the chromatogram 3 times the retention time of the principal peak for test solution. The area of any peak corresponding to dicyandiamide is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent) and the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent). Ignore any peak due to teneligliptin.



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Uniformity of content. Complies with the test stated under Tablets.

For Teneligliptin — Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Disperse 1 tablet in sufficient quantity of the solvent mixture, with the aid of ultrasound for 20 minutes with intermittent shaking and dilute with the solvent mixture to obtain a solution containing 0.02 per cent w/v of Teneligliptin. Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{30}N_6OS$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14), as described under dissolution for Teneligliptin with following modifications.

For Teneligliptin —

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 20 mg of Teneligliptin in 70 ml of the solvent mixture, with the aid of ultrasound for 25 minutes and dilute to 100.0 ml with the solvent mixture.

Reference solution. A 0.02 per cent w/v solution of *teneligliptin hydrobromide hydrate IPRS* in the solvent mixture.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the contents of $C_{22}H_{30}N_6OS$ in the tablets.

For Metformin hydrochloride —

Test solution. To a quantity of the powdered tablets containing 0.5 g of Metformin Hydrochloride, add 10 g of *sodium dihydrogen orthophosphate* and 80 ml of *water*, stir the solution on magnetic stirrer for 1 hour with intermittent shaking and dilute to 1000.0 ml with *water*. Dilute 2.0 ml of the solution to 100.0 ml with *water*.

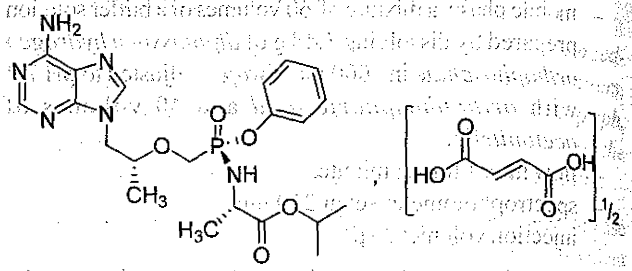
Reference solution. To 100 mg of *metformin hydrochloride IPRS*, add 1 g of *sodium dihydrogen orthophosphate* and 80 ml of *water*, sonicate for 2 minutes and dilute to 100.0 ml with *water*. Dilute 1.0 ml of the solution to 100.0 ml with *water*.

Measure the absorbance of the reference solution and the test solution at 233 nm (2.4.7). Calculate the content of $C_4H_{11}N_5$, HCl in tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of teneligliptin and metformin hydrochloride.

Tenofovir Alafenamide Fumarate



$C_{23}H_{31}N_6O_7P$ Mol. Wt. 534.5

Tenofovir Alafenamide Fumarate is propan-2-yl N-[(S)-({[(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl] oxy}methyl) (phenoxy) phosphoryl]-*L*-alaninate hemi(2E)-but-2-enedioate.

Tenofovir Alafenamide Fumarate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{23}H_{31}N_6O_7P$, calculated on the anhydrous basis.

Category. Antiviral.

Description. A white to off- white powder or light tan powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tenofovir alafenamide fumarate IPRS* or with the reference spectrum of tenofovir alafenamide fumarate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 70 volumes of *tetrahydrofuran* and 30 volumes of *acetonitrile*.

Buffer solution. Dissolve 0.7 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 6.0 with *glacial acetic acid*.

Test solution. Dissolve 25 mg of the substance under examination in mobile phase A and dilute to 50.0 ml with mobile phase A.

Reference solution. A 0.005 per cent w/v solution of *tenofovir alafenamide fumarate IPRS* in mobile phase A. Dilute 1.0 ml of the solution to 100.0 ml with mobile phase A.

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Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm) (Such as X-bridge shield RP-18),
- sample temperature: 5°,
- mobile phase: A. a mixture of 99 volumes of the buffer solution and 1 volume of the solvent mixture,
  B. a mixture of 50 volumes of the buffer solution and 50 volumes of the solvent mixture,
- flow rate: 0.8 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 260 nm,
- injection volume: 15 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
1	100	0
5	90	10
8	78	22
12	75	25
15	73	27
30	71	29
40	71	29
45	40	60
55	40	60
56	100	0
70	100	0

Name	Relative retention time	Correction factor
Fumaric Acid	0.06	—
Tenofovir impurity	0.15	—
PMPA anhydro impurity[1]	0.20	0.60
Monophenyl PMPA[2]	0.30	0.77
PMPA monoamidate[3]	0.31	—
Methyl impurity[4]	0.50	—
Ethyl impurity[5]	0.67	—
PMPA bisamidate[6]	0.79	—
Diastereomer-2 or 3	0.90	—
Diastereomer-1[7]	0.96	—
Tenofovir alafenamide	1.00	—
n-propyl impurity[8]	1.09	—

[1]({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)({[2R)-1-1(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)diphosphoric acid,

[2]phenyl hydrogen ({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)phosphonate hydrate or [(R)-2(phenyl phosphonomethoxy)-propyl] adenine monohydrate or Mono Phenyl PMPA mono hydrate,

[3]P-({[(2R)-1-(-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)-N-[(2S)-1-oxo-1-(propan-2-yloxyl)propan-2-yl]phosphonamidic acid lithium salt,

[4]methyl N-[(S)-({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)(phenoxy)phosphoryl]-L-alaninate(2E)-but-2-enedioate or Methyl analog impurity,

[5]ethyl N-[(S)-({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)(phenoxy) phosphoryl]-L-alaninate(2E)-but-2-enedioate or ethyl analog impurity,

[6]propan-2-yl(2S,6S)-4-({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)-2,6,9-trimethyl-7-oxo-8-oxa-3,5-diaza-4-phosphadecan-1-oate-4-oxide,(2E)-but-2-enedioate,

[7]propan-2-yl N-[({[1-(6-amino-9Hpurin-9-yl) propan-2-yl]oxy}methyl)(phenoxy)phosphoryl]alaninate(2E)-but-2-enedioate,

[8]propyl N-[(S)-({[(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl]oxy}methyl)(phenoxy) phosphoryl]-L-alaninate(2E)-but-2-enedioate.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 10000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solutions and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to PMPA anhydro impurity is not more than 5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent), the area of any peak corresponding to monophenyl PMPA, PMPA monoamidate, methyl impurity, ethyl impurity, n-propyl impurity and PMPA bisamidate, each of, is not more than 1.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.15 per cent), the area of any peak corresponding to diastereomer-1 is not more than 10 times the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 20 times the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent). Ignore peak due to tenofovir impurity, fumaric acid and diastereomer-2 or 3.

Fumaric acid and tenofovir impurity. 10.3 per cent to 11.4 per cent of fumaric acid and not more than 1.0 per cent of tenofovir impurity.

Determine by liquid chromatography (2.4.14).

Solvent mixture. 95 volumes of mobile phase A and 5 volumes of *acetonitrile*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

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Reference solution (a). A 0.05 per cent w/v solution of *fumaric acid IPRS* in the solvent mixture.

Reference solution (b). A 0.005 per cent w/v solution of *tenofovir impurity IPRS* in the solvent mixture.

Reference solution (c). Dilute 5.0 ml each of reference solution (a) and (b) to 50.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, stable bond aqueous column (5 µm) (Such as zorbax SB-aq),
- column temperature: 35°,
- sample temperature: 4°,
- mobile phase: A. a buffer solution prepared by dissolving 1.4 ml of *triethylamine* in 1000 ml of *water*, adjusted to pH 3.5 with *orthophosphoric acid*,
  B. a mixture of 90 volumes of *acetonitrile* and 10 volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
10	95	5
15	30	70
20	30	70
22	95	5
30	95	5

Name	Relative retention time
Fumaric acid[1]	0.2
Tenofovir impurity[2]	0.43
Tenofovir alafenamide (Retention time: about 17 minutes)	1.0

[1](2E)-but-2-enedioic acid,

[2]({[1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy}methyl) phosphonic acid.

Inject reference solution (c). The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 for fumaric acid and tenofovir peak and the relative standard deviation for replicate injections is not more than 1.0 per cent for fumaric acid peak and not more than 5.0 per cent for tenofovir impurity peak.

Inject reference solution (c) and the test solution.

Calculate the content of fumaric acid and tenofovir impurity.

Enantiomeric purity. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in *ethanol* and dilute to 25.0 ml with *ethanol*.

Reference solution. A 0.001 per cent w/v solution of *tenofovir alafenamide fumarate IPRS* in *ethanol*.

Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with chiral Pak AD-H (5 µm),
- column temperature: 15°,
- sample temperature: 5°,
- mobile phase: a mixture of 60 volumes of *n-hexane* and 25 volumes of *isopropyl alcohol*, 15 volumes of *ethanol* and 0.1 volume of *methanesulphonic acid*,
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 10 µl.

Name	Relative retention time
Diastereomer-3[1]	0.58
Diastereomer-2[2]	0.65
Enantiomer Impurity[3]	0.85
Tenofovir alafenamide	1.0

[1]Propan-2-yl N[({[1-(6-amino-9H-purin-9yl)propan-2-yl]oxy}methyl) (phenoxy) phosphoryl] alaninate (2E)-but-2-enedioate,

[2]Propan-2-yl N[({[1-(6-amino-9H-purin-9yl)propan-2-yl]oxy}methyl) (phenoxy) phosphoryl] alaninate (2E)-but-2-enedioate,

[3]Propan-2-yl N[(R)-({[(2S)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl) (phenoxy) phosphoryl]-D-alaninate(2E)-but-2-enedioate.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to diastereomer-3, diastereomer-2 and enantiomer peak, each of, is not more than 0.15 times the area of the principal peak in the chromatogram obtained with the reference solution (0.15 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 50 volumes of *water* and 50 volumes of *methanol*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the same solvent.

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Reference solution. A 0.05 per cent w/v solution of *tenofovir alafenamide fumarate IPRS* in the solvent mixture

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with phenyl group bonded to porous silica (5 µm) (Such as X-Bridge Phenyl),
- sample temperature: 5°,
- mobile phase: a mixture of 85 volumes of a buffer solution prepared by dissolving 3.4 g of *tetrabutyl ammonium hydrogen sulphate* in 1000 ml of *water* and add 5 ml of *triethylamine*, adjusted to pH 2.0 with *orthophosphoric acid*, 5 volumes of *acetonitrile*, 5 volumes of *methanol* and 5 volumes of *tetrahydrofuran*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency in not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{31}N_6O_7P$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Tenofovir Alafenamide Tablets

Tenofovir Alafenamide Fumarate Tablets

Tenofovir Alafenamide Tablets contain Tenofovir Alafenamide Fumarate equivalent to not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of tenofovir alafenamide, $C_{21}H_{29}N_6O_5P$.

Usual strength. 25 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *phosphate buffer pH 6.8*,

Speed and time. 50 rpm for 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, diluted if necessary, with the dissolution medium.

Reference solution. A 0.031 per cent w/v solution of *tenofovir alafenamide fumarate IPRS* in *water*. Dilute 1.0 ml of the solution to 10.0 ml with the dissolution medium.

Chromatographic system
- a stainless steel column 5 cm × 2.1 mm, packed with octadecylsilane bonded to porous silica (1.7 µm) (Such askinetex XB, 100 Å),
- column temperature: 35°,
- mobile phase: A. a 0.1 per cent v/v solution of *trifluoroacetic acid*,
  B. *acetonitrile*,
- flow rate: 0.8 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 260 nm,
- injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	96.5	3.5
0.1	96.5	3.5
0.8	10	90
1.2	10	90
1.3	96.5	3.5

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{29}N_6O_5P$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{21}H_{29}N_6O_5P$.

Related substances. Determine by liquid chromatography (2.4.14).

Solution A. 70 volumes of *tetrahydrofuran* and 30 volumes of *acetonitrile*.

Test solution. Disperse a quantity of powdered tablets containing 100 mg of Tenofovir Alafenamide in 5 ml of *water*, with the aid of ultrasound and dilute to 100.0 ml with *methanol*. Dilute 5.0 ml of the solution to 50.0 ml with equal volumes of *water* and *methanol*.

Reference solution. A 0.011 per cent w/v solution of *tenofovir alafenamide fumarate IPRS* in *methanol*. Dilute 1.0 ml of the solution to 100.0 ml with equal volumes of *water* and *methanol*.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as Ace 5),
- sample temperature: 5°,

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– mobile phase: A, a mixture of 99 volumes of solution prepared by dissolving 2.31 g of *ammonium acetate* in 1000 ml of *water* and adjusted to pH 6.0 with *dilute glacial acetic acid* and 1 volume of Solution A,
B, a mixture of 50 volumes of solution prepared by dissolving 2.31 g of *ammonium acetate* in 1000 ml of *water* and adjusted to pH 6.0 with *dilute glacial acetic acid* and 50 volumes of Solution A,
– flow rate: 0.8 ml per minute,
– a gradient programme using the conditions given below,
– spectrophotometer set at 260 nm,
– injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
15	100	0
22	95	5
33	95	5
38	85	15
43	75	25
48	80	20
58	60	40
63	60	40
68	40	60
73	20	80
85	20	80
90	100	0
100	100	0

Name	Relative retention time
Tenofovir impurity	0.14
PMPA anhydro impurity[1]	0.38
Mono phenyl PMPA impurity[2]	0.61
PMPA monoamidate impurity[3]	0.65
Methyl analog impurity[4]	0.83
Ethyl analog impurity[5]	0.91
PMPA bisamidate impurity[6]	0.96
Diastereomer-2 impurity[7]	0.98
Diastereomer-3 impurity[8]	0.98
Tenofovir alafenamide	1.0
n-propyl impurity[9]	1.01

[1]([[(2R)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy}methyl)({[2R)-1-1(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy}methyl)diphosphoric acid,

[2]phenyl hydrogen ({[(2R)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy}methyl)phosphonate hydrate or [(*R*)-2(phenyl phosphonomethoxy)-propyl], adenine monohydrate,

[3]P-({[(2R)-1-(-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)-N-[(2*S*)-1-oxo-1-(propan-2-yloxyl)propan-2-yl]phosphonamidic acid lithium salt,

[4]methyl *N*-[(*S*)-({[(2*R*)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy}methyl)(phenoxy)phosphoryl]-*L*-alaninate(2*E*)-but-2-enedioate or 9-{(*R*)-2-[[(*S*)-{[(*S*)-1-(methoxycarbonyl) ethyl] amino}phenoxyphosphinyl] methoxy] propyl} adenine fumarate,

[5]ethyl *N*-[(*S*)-({[(2*R*)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy}methyl)(phenoxy)phosphoryl]-*L*-alaninate(2*E*)-but-2-enedioate,

[6]propan-2-yl(2*S*,6*S*)-4-({[(2*R*)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy}methyl)-2,6,9-trimethyl-7-oxo-8-oxa-3,5-diaza-4-phosphadecan-1-oate-4-oxide,(2E)-but-2-enedioate,

[7]propan-2-yl *N*-[({[1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy}methyl)(phenoxy)phosphoryl]-*L*-alaninate(2*E*)-but-2-enedioate,

[8]propyl *N*-[(*S*)-({[(2*R*)-1-(6-amino-9*H*-purin-9-yl) propan-2-yl]oxy}methyl)(phenoxy) phosphoryl]-*D*-alaninate(2*E*)-but-2-enedioate,

[9]propyl *N*-[(*S*)-({[(2*R*)-1-(6-amino-9*H*-purin-9-yl) propan-2-yl]oxy}methyl)(phenoxy) phosphoryl]-*L*-alaninate(2*E*)-but-2-enedioate,

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of tenofovir impurity peak is not more than 1.25 times the area of the peak in the chromatogram obtained with the reference solution (1.25 per cent), the area of monophenyl PMPA and PMPA monoamidate impurity peak is not more than 0.5 times the area of the peak in the chromatogram obtained with the reference solution (0.5 per cent) , the area of PMPA anhydro impurity peak is not more than 0.8 times the area of the peak in the chromatogram obtained with the reference solution (0.8 per cent) and the area of any other secondary peak is not more than 0.2 times the area obtained with the reference solution (0.2 per cent) and the sum of areas of all the secondary peaks is not more than 3.5 times the area of the peak in the chromatogram obtained with the reference solution (3.5 per cent). Ignore peak due to fumaric acid.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 70 volumes of *terhahydrofuran* and 30 volumes of *acetonitrile*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 250 mg of Tenofovir Alafenamide in 5 ml of *water*, with the aid of ultrasound and dilute to 250.0 ml with *methanol*. Dilute 5.0 ml of the solution to 50.0 ml with equal volumes of *water* and *methanol*.

Reference solution. A 0.11 per cent w/v solution of *tenofovir alafenamide fumarate IPRS* in *methanol*. Dilute 5.0 ml of the solution to 50.0 ml with equal volumes of *water* and *methanol*.

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Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as Ace 5),
- sample temperature: 8°,
- mobile phase: A. a mixture of 99 volumes of solution prepared by dissolving 2.31 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 6.0 with *dilute glacial acetic acid* and 1 volume of the solvent mixture,

  B. a mixture of 50 volumes of solution prepared by dissolving 2.31 g of *ammonium acetate* in 1000 ml of *water* and adjusted to pH 6.0 with *dilute glacial acetic acid* and 50 volumes of solvent mixture,
- flow rate: 0.8 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 260 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
5	70	30
8	65	35
15	60	40
20	60	40
25	70	30
35	70	30

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

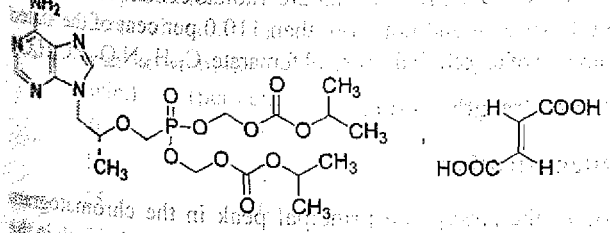
Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{29}N_6O_5P$ in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of tenofovir alafenamide.

Tenofovir Disoproxil Fumarate



$C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$ Mol. Wt. 635.5

Tenofovir Disoproxil Fumarate salt of bis(isopropyloxycarbonyloxymethyl ester of (*R*)-9-(2-phosphonomethoxypropyl)adenine with fumaric acid.

Tenofovir Disoproxil Fumarate contain not less than 97.0 per cent and not more than 102. 0 per cent of $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$, calculated on the anhydrous basis.

Category. Antiviral.

Description. A white to off- white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tenofovir disoproxil fumarate IPRS* or with the reference spectrum of tenofovir disoproxil fumarate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Buffer solution. Dissolve 3.5 g of *potassium dihydrogen phosphate* and 1.7 g of *tertabutyl ammonium hydrogen sulphate* in 800 ml of *water*, adjusted to pH 6.0 with *1M sodium hydroxide* and dilute to 1000 ml with *water*.

Test solution. Dissolve 10 mg of the substance under examination in *water* and dilute to 10.0 ml with *water*.

Reference solution (a). A 0.0005 per cent w/v solution of *tenofovir disoproxil fumarate IPRS* in *water*.

Reference solution (b). Dissolve 10 mg of *fumaric acid* in 50.0 ml of *water*.

Reference solution (c). Heat the test solution in a boiling water-bath for 20 minutes.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- sample temperature: 6°,
- mobile phase: A. a mixture of 2 volumes of *acetonitrile*, 20 volumes of the buffer solution and 78 volumes of *water*.

  B. a mixture of 65 volumes of *acetonitrile*, 20 volumes of the buffer solution and 15 volumes of *water*,
- flow rate: 1 ml per minutes,
- spectrophotometer set at 260 nm,
- injection volume: 20 µl.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	81	19
5	81	19
40	49	51
60	0	100
65	0	100
70	81	19
80	81	19

The relative retention time with reference to tenofovir disoproxil fumarate for tenofovir monoester is about 0.5.

Inject reference solution (c). The test is not valid unless the resolution between the tenofovir disoproxil fumarate peak and tenofovir monoester peak is not less than 25.0.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with test solution, the area of tenofovir monoester (impurity A) is not more than twice the area multiplied with correction factor of 0.62 of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent), the area of any other secondary peak is not more than the area of principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent). Ignore any peak corresponding to the peak in the chromatogram obtained with reference solution (b) and any peak having area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Fumaric Acid. 17.5 per cent to 19.0 per cent.

Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in 100 ml of the mobile phase.

Reference solution. Dissolve 25 mg of the *fumaric acid* in 50 ml of the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 40 volumes of *acetonitrile* and 60 volumes of a buffer solution prepared by adding 0.1 per cent v/v of *triethylamine* in *0.05M sodium dihydrogen phosphate*, adjusted to pH 2.3 with *orthophosphoric acid* and filtered,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency is not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of fumaric acid.

Water (2.3.43). Not more than 1.0 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use and carry out the test protected from light.

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.1 per cent w/v solution of *tenofovir disoproxil fumarate IPRS* in the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 40 volumes of *acetonitrile* and 60 volumes of a buffer solution prepared by adding 1 ml *triethylamine* to *0.05M sodium dihydrogen phosphate*, adjusted to pH 2.3 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency in not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$.

Storage. Store protected from light in a refrigerator (2° to 8°).

Tenofovir Disoproxil Fumarate Tablets

Tenofovir Disoproxil Fumarate Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of tenofovir disoproxil fumarate, $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$.

Usual strength. 300 mg.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

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B. When examined in the range 200 nm to 400 nm (2.4.7), a 0.001 per cent w/v solution in *methanol* shows an absorption maximum at the same wavelength as the reference solution.

Tests

Dissolution (2.5.2).
Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 50 rpm and 45 minutes

Withdraw a suitable volume of the medium and filter promptly. Use the filtrate, dilute if necessary, with the same solvent. Measure the absorbance (2.4.7) of the resulting solution at the maximum at about 260 nm. Calculate the content of $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$ in the medium from the absorbance obtained from a solution of known concentration of *tenofovir disoproxil fumarate IPRS*.

Q. Not less than 80 per cent of the stated amount of $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powder containing 100 mg of Tenofovir Disoproxil Fumarate and disperse in 50 ml of *methanol* and filter.

Reference solution (a). A 0.2 per cent w/v solution of *tenofovir disoproxil fumarate IPRS* in *methanol*.

Reference solution (b). Dilute 1 ml of reference solution (a) to 100 ml with *methanol*.

Reference solution (c). Dissolve 10 mg of *fumaric acid* in 50 ml of *methanol*.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. dissolve 1.9 g of *ammonium acetate* in 1000 ml of *water* and adjusted to pH 3.8 with *glacial acetic acid*,

  B. *methanol*,
- flow rate: 1.5 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 260 nm,
- injection volume: 10 µl.

Time (in min.)	mobile phase A (per cent v/v)	mobile phase B (per cent v/v)
0	95	5
10	50	50
25	50	50
50	20	80
60	95	5

Inject reference solution (a). The test is not valid unless the tailing factor is not more than 2.0 and the column efficiency in not less than 2000 theoretical plates.

Inject reference solution (b), (c) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 3.5 times the area of the peak in the chromatogram obtained with reference solution (b) (3.5 per cent) and the sum of areas of all the secondary peaks is not more than 6 times the area of the peak in the chromatogram obtained with the reference solution (b) (6.0 per cent). Ignore the peak corresponding to the peak in the chromatogram obtained in the reference solution (c).

Other tests. Comply with the tests stated under Tablets.

Water (2.3.43). Not more than 5.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Solvent mixture. Equal volumes of *water* and *methanol*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 300 mg of Tenofovir Disoproxil Fumarate in 100 ml of solvent mixture and filter. Dilute 5.0 ml of the filtrate to 50.0 ml with the mobile phase.

Reference solution. A 0.120 per cent w/v solution of *tenofovir disoproxil fumarate IPRS* in the solvent mixture. Dilute 5.0 ml of the solution to 20.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS 3V),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 7.8 g of *sodium dihydrogen orthophosphate* in 1000 ml of *water*, adding 1 ml of *triethylamine* and adjusted to pH 2.3 with *orthophosphoric acid (10 per cent v/v)*, and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency in not less than 2000 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

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Tenofovir and Emtricitabine Tablets

Tenofovir Disoproxil Fumarate and Emtricitabine Tablets

Tenofovir and Emtricitabine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of tenofovir disoproxil fumarate, $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$ and emtricitabine, $C_8H_{10}FN_3O_3S$.

Usual strength. Tenofovir disoproxil fumarate 300 mg and Emtricitabine 200 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle)

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14)

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A solution containing 0.32 per cent w/v of *tenofovir disoproxil fumarate IPRS* and 0.24 per cent w/v of *emtricitabine IPRS* in *methanol*. Dilute 5 ml of the solution to 50 ml with the dissolution medium.

Use the chromatographic system given under Assay.

Inject the reference solution and the test solution.

Calculate the contents of $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$ and $C_8H_{10}FN_3O_3S$ in the medium.

Q. Not less than 75 per cent of the stated amounts of $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$ and $C_8H_{10}FN_3O_3S$.

Related substances. Determine by liquid chromatography (2.4.14).

For Emtricitabine —

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powder containing 50 mg of Emtricitabine, disperse in 5 ml of *methanol*, dilute to 50 ml with mobile phase A and filter.

Reference solution (a). A 0.1 per cent w/v solution of *emtricitabine IPRS* in mobile phase A.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with mobile phase A.

Chromatographic system,

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: A. a mixture of 95 volumes of a buffer solution prepared by dissolving 1.9 g of ammonium *acetate* in 1000 ml of *water*, adjusted to pH 3.8 with *glacial acetic acid*, and 5 volumes of *methanol*,
  B. a filtered mixture of 30 volumes of the buffer solution and 70 volumes of *methanol*,
- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 277 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
30	100	0
35	0	100
55	0	100
60	100	0
75	100	0

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 500 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and the sum of areas of all the secondary peaks is not more than 3 times the area of the peak in the chromatogram obtained with reference solution (b) (3.0 per cent).

For Tenofovir Disoproxil Fumarate —

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of Tenofovir Disoproxil Fumarate and disperse in 50 ml of *methanol*.

Reference solution (a). A 0.1 per cent w/v solution of *tenofovir disoproxil fumarate IPRS* in *methanol*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *methanol*.

Reference solution (c). Dissolve 10 mg of *fumaric acid* in 50 ml of *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution prepared by dissolving 1.9 g of *ammonium acetate* in 1000 ml of *water* and adjusted to pH 3.8 with *glacial acetic acid*,

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B. *methanol*,

– flow rate: 1.5 ml per minute,
– a gradient programme using the conditions given below,
– spectrophotometer set at 260 nm,
– injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
10	50	50
25	50	50
50	20	80
60	95	5
75	95	5

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b), (c) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 3.5 times the area of the peak in the chromatogram obtained with reference solution (b) (3.5 per cent) and the sum of areas of all the secondary peaks is not more than 6 times the area of the peak in the chromatogram obtained with reference solution (b) (6.0 per cent). Ignore the peak corresponding to the peak in the chromatogram obtained in reference solution (c).

Other tests. Comply with the tests stated under the Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 30 volumes of *water* and 70 volumes of *methanol*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 100 mg of Tenofovir Disoproxil Fumarate in 10 ml of *water* and dilute to 250.0 ml with *methanol*. Dilute 5.0 ml of the solution to 25.0 ml with the solvent mixture.

Reference solution. A solution containing 0.025 per cent w/v of *emtricitabine IPRS* and 0.04 per cent w/v of *tenofovir disoproxil fumarate IPRS* in *methanol*. Dilute 5.0 ml of the solution to 25.0 ml with the solvent mixture.

Chromatographic system

– a stainless steel column 5 cm x 4.6 mm packed with octylsilane bonded to porous silica (3 µm),
– column temperature: 40°,
– mobile phase: A. a buffer solution prepared by dissolving 1.35 g of *monobasic potassium phosphate* in 1000 ml of *water* and adjusted to pH 3.0 with *orthophosphoric acid*,

B. a mixture of 20 volumes of the buffer solution and 80 volumes of *acetonitrile*,

– flow rate: 1.5 ml per minute,
– a gradient programme using the conditions given below,
– spectrophotometer set at 260 nm,
– injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	94	6
3	94	6
5	50	50
8	35	65
9	94	6
12	94	6

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0, the column efficiency in not less than 2000 theoretical plates for the peak due to tenofovir disoproxil, 500 theoretical plates for the peak due to emtricitabine and the relative standard deviation for replicate injections is not more than 2.0 per cent for each component.

Inject the reference solution and the test solution.

Calculate the contents of $C_{19}H_{30}N_5O_{10}P$, $C_4H_4O_4$ and $C_8H_{10}FN_3O_3S$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Tenofovir Disoproxil Fumarate, Lamivudine and Efavirenz Tablets

Tenofovir Disoproxil Fumarate, Lamivudine and Efavirenz Tablets contain not less than 90.0 per cent and not more than 110.0 per cent each of the stated amounts of tenofovir disoproxil fumarate $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$, lamivudine $C_8H_{11}N_3O_3S$ and efavirenz $C_{14}H_9ClF_3NO_2$.

Usual strength. Tenofovir Disoproxil Fumarate 300 mg, Lamivudine 300 mg and Efavirenz 600 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

For lamivudine and tenofovir disoproxil fumarate—

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 75 rpm and 45 minutes.

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Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A solution containing 0.03 per cent w/v each of *lamivudine IPRS* and *tenofovir disoproxil fumarate IPRS* prepared by dissolving in a mixture of equal volumes of *methanol* and *water* and diluted with the dissolution medium.

Use chromatographic system as described under Assay.

The retention time of tenofovir disoproxil is about 4.5 minutes and lamivudine is about 1.3 minutes.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates for tenofovir disoproxil and 800 theoretical plates for lamivudine peak. The tailing factor for both lamivudine and tenofovir disoproxil peaks is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of lamivudine, $C_8H_{11}N_3O_3S$ and tenofovir disoproxil fumarate, $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$ in the medium.

Q. Not less than 75 per cent of the stated amount of lamivudine, $C_8H_{11}N_3O_3S$ and tenofovir disoproxil fumarate, $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$.

For efavirenz—

Apparatus No. 2 (Paddle),
Medium. 1000 ml of 2.0 per cent w/v solution of *sodium lauryl sulphate solution,*
Speed and time. 75 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.
Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.06 per cent w/v solution of *efavirenz IPRS* prepared by dissolving in *methanol* and diluting with the dissolution medium.

Chromatographic system
- a stainless steel column 5.0 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: a mixture of 30 volumes of buffer solution prepared by dissolving 2.16 g of *sodium octanesulphonate* in 1000 ml of 0.1 per cent v/v of *triethylamine*, adjusted to pH 2.0 with *orthophosphoric acid* and 70 volumes of *methanol,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 5 µl.

Inject the reference solution. The test is not valid unless the column efficiency for efavirenz is not less than 1000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of efavirenz, $C_{14}H_9ClF_3NO_2$ in the medium.

Q. Not less than 75 per cent of the stated amount of efavirenz $C_{14}H_9ClF_3NO_2$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 50 volumes of *methanol* and 50 volumes of *water.*

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 6 g of Efavirenz, 3 g of Lamivudine and 3 g of Tenofovir Disoproxil Fumarate and transfer into 1000-ml volumetric flask. Add about 300 ml of *water*, disperse with the aid of ultrasound and add 400 ml of *methanol* and sonicate for 30 minutes with intermittent swirling, dilute to volume with *methanol*, filter. Dilute 5.0 ml of the filtrate to 50.0 ml with the solvent mixture.

Reference solution. A solution containing 0.03 per cent w/v each of *lamuvidine IPRS* and *tenofovir disoproxil fumarate IPRS* and 0.06 per cent w/v of *efavirenz IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 5.0 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: A. dissolve 2.16 g of *sodium octanesulphonate* in 1000 ml of 0.1 per cent v/v *triethylamine*, adjusted to pH 2.0 with *orthophosphoric acid*,

  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	60	40
2.0	60	40
3.0	25	75
4.5	25	75
5.0	60	40
6.5	60	40

The retention time of tenofovir Disoproxil is about 4.5 minutes, efavirenz is about 5.0 minutes and lamivudine is about 1.3 minutes.

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Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates for tenofovir disoproxil, 800 theoretical plates for lamivudine and 1000 theoretical plates for efavirenz. The tailing factor for each peak is not more than 2.0 and the relative standard deviation for replicate injections of each peak is not more than 2.0 per cent.

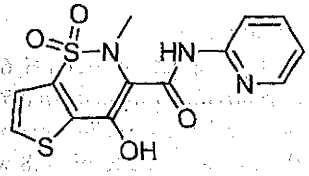
Inject the reference solution and the test solution.

Calculate the content of efavirenz $C_{14}H_9ClF_3NO_2$, lamuvidine $C_8H_{11}N_3O_3S$, and tenofovir disoproxil fumarate $C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$ in the tablets.

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of the amount of lamivudine, tenofovir disoproxil fumarate and efavirenz.

Tenoxicam



$C_{13}H_{11}N_3O_4S_2$ Mol. Wt. 337.4

Tenoxicam is 4-Hydroxy-2-methyl-N-(pyridin-2-yl)-2H-thieno[2,3-e]1,2-thiazine-3-carboxamide1,1-dioxide.

Tenoxicam contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{13}H_{11}N_3O_4S_2$, calculated on the anhydrous basis.

Category. Analgesic; antiinflammatory.

Description. A yellow crystalline powder. It shows polymorphism (2.5.11).

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tenoxicam IPRS* or with the reference spectrum of tenoxicam.

If the spectra obtained in solid state show difference, dissolve the substance under examination and reference substance separately in minimum volume of *methylene chloride*. Evaporate to dryness and record new spectra using the residues.

Tests

Appearance of solution. A 0.5 per cent w/v solution in *methylene chloride* is clear (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Carry out the test protect from light.

Solvent mixture. Equal volumes of *acetonitrile* and *water*, adjusted to pH 3.2 with *dilute orthophosphoric acid*.

Test solution. Dissolve 35 mg of the substance under examination in the solvent mixture with the aid of ultrasound and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution (b). Dissolve the content of a vial of *tenoxicam impurity mixture IPRS* (containing impurities B, G and H) in 1.0 ml of the test solution.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with diisopropylcyanosilane bonded to porous silica (3.5 µm),
- column temperature: 35°,
- mobile phase: A. a mixture of 25 volumes of *methanol*, 75 volumes of *water*, adjusted to pH 3.2 with *dilute orthophosphoric acid*,
  B. a mixture of 25 volumes of *water* and 75 volumes of *methanol*, adjusted to pH 3.2 with *dilute orthophosphoric acid*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	96	4
5	96	4
16	76	24
25	76	24
26	96	4
30	96	4

Name	Relative retention time	Correction factor
Tenoxicam impurity A[1]	0.10	0.2
Tenoxicam impurity G[2]	0.85	—
Tenoxicam impurity H[3]	0.90	—
Tenoxicam (Retention time: about 12 minutes)	1.0	—
Tenoxicam impurity B[4]	1.30	2.0

[1]Pyridin-2-amine,

[2]4-hydroxy-2-methyl-2*H*-thieno[2,3-e]1,2-thiazine-3-carboxamide1,1-dioxide,

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[3-[(methylamino)sulfonyl]thiophene-2-carboxylic acid, [4]methyl 4-hydroxy-2-methyl-2*H*-thieno[2,3-e]1,2-thiazine-3-carboxylate 1,1-dioxide.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to tenoxicam and tenoxicam impurity H (or G if peaks are inverted) is not less than 1.3 and the resolution between the peaks due to tenoxicam impurity G and tenoxicam impurity H is not less than 1.3.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution the area of any peak corresponding to tenoxicam impurity A multiplied with correction factor 0.2 and tenoxicam impurity B multiplied with correction factor 2.0, each of, is not more than 1.5 times the area of principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent) the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.4.43). Not more than 0.5 per cent, determined on 1.0 g.

Assay. Dissolve 0.25 g of the substance under examination in 5 ml of *anhydrous formic acid* and add 70 ml of *anhydrous acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03374 g of $C_{13}H_{11}N_3O_4S_2$.

Storage. Store protected from light.

Tenoxicam Tablets

Tenoxicam Tablets contain not less than 92.5 per cent and not more than 105.0 per cent of the stated amount of tenoxicam, $C_{13}H_{11}N_3O_4S_2$.

Usual strength. 20 mg

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 4 volumes of *anhydrous formic acid*, 30 volumes of *acetone* and 70 volumes of *dichloromethane*.

Test solution. Disperse a quantity of the powdered tablets containing 20 mg of Tenoxicam in 20 ml of *dichloromethane*, with the aid of ultrasound for 15 minutes, centrifuge and use the supernatant liquid.

Reference solution. A 0.1 per cent w/v solution of *tenoxicam IPRS* in *dichloromethane*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to the spot in the chromatograms obtained with the reference solution.

Tests

Dissolution (2.5.2)

Apparatus No. 2 (Paddle),
Medium. 900 ml of phosphate buffer pH 6.8 prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 500 ml of *water*, add 2 ml of *1M sodium hydroxide* and dilute with *water* to 1000 ml. Adjusted to pH 6.8 with *1M sodium hydroxide* or 10 per cent w/v solution of *orthophosphoric acid* as required.
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of medium and filter. Reject the first few ml of the filtrate and dilute a suitable volume of filtrate with dissolution medium if necessary. Measure the absorbance of filtered solution at maximum at about 368 nm (2.4.7). Calculate the content of tenoxicam, $C_{13}H_{11}N_3O_4S_2$ in the medium from the absorbance obtained from a solution of known concentration of *tenoxicam IPRS* in dissolution medium.

Q. Not less than 70 per cent of the stated amount of $C_{13}H_{11}N_3O_4S_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *acetonitrile* and *water*.
Test solution. Weigh a quantity of the powdered tablets containing 100 mg of Tenoxicam, disperse in 50 ml of the solvent mixture with the aid of ultrasound for 70 minutes with intermittent shaking and dilute to 100.0 ml with the solvent mixture. Allow to stand for 10 minutes, dilute 5.0 ml of the clear supernatant liquid to 20.0 ml with the mobile phase and filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

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Reference solution (b). A 0.0625 per cent w/v solution of *2-pyridylamine* in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Chromatographic system

– a stainless steel column 25 cm × 4.0 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as Nucleosil C8),

– mobile phase: a mixture of 7 volumes of 0.012 per cent w/v solution of *sodium dodecyl sulphate* in *methanol* and 10 volumes of *0.05 M potassium dihydrogen orthophosphate* and adjusted to pH 2.8 with *orthophosphoric acid*,

– flow rate: 0.7 ml per minute,

– spectrophotometer set at 254 nm and 290 nm,

– injection volume: 20 µl.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to 2-pyridylamine is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent) at 290 nm, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than 4 times the area of principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent) at 254 nm.

Other tests. Comply with tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14), as stated under Related substances with the following modifications.

Test solution. Disperse 10 intact tablets in 200.0 ml of the solvent mixture, with the aid of ultrasound for 70 minutes with intermittent shaking. Allow to stand for 10 minutes, dilute a volume of clear supernatant liquid with the mobile phase to obtain a solution containing 0.025 per cent w/v of Tenoxicam and filter.

Reference solution. A 0.1 per cent w/v solution of *tenoxicam IPRS* in the solvent mixture. Dilute 5.0 ml of the solution to 20.0 ml with the mobile phase.

Chromatographic system

– spectrophotometer set at 290 nm.

Inject the reference solution. The test is not valid unless the column efficiency in not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

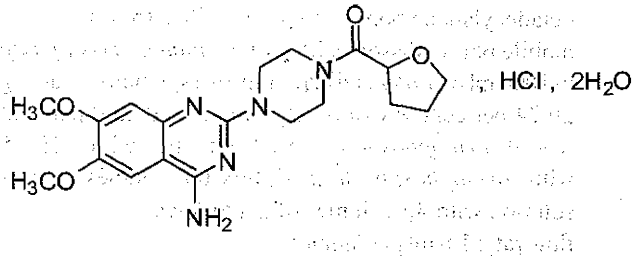
Inject the reference solution and the test solution.

Calculate the content of $C_{13}H_{11}N_3O_4S_2$ in the tablets.

Storage. Store protected from light.

Terazosin Hydrochloride

Terazosin Hydrochloride Dihydrate



$C_{19}H_{25}N_5O_4,HCl,2H_2O$ Mol. Wt. 459.9

Terazosin Hydrochloride is (*RS*)-6,7-dimethoxy-2-[4-(tetrahydrofuran-2-carbonyl)piperazin-1-yl]quinazolin-4-ylamine hydrochloride dihydrate.

Terazosin Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{19}H_{25}N_5O_4,HCl$, calculated on the anhydrous basis.

Category. Antihypertensive.

Description. A white or slightly yellow, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *terazosin hydrochloride IPRS* or with the reference spectrum of terazosin hydrochloride.

B. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 1.0 per cent w/v solution in *carbon-dioxide free water* is clear (2.4.1) and not more intensely coloured than reference solution YS7 (2.4.1).

pH (2.4.24). 3.0 to 5.0, determined in 2.0 per cent w/v solution in *carbon-dioxide free water*.

Impurities N and O. Determine by liquid chromatography (2.4.14).

Solvent mixture. 20 volumes of *acetonitrile* and 80 volumes of *water*.

Test solution. Dissolve 50 mg of the substance under examination in 50 ml of the solvent mixture.

Reference solution (a). Dissolve 5 mg each of *2-chloro-6,7-dimethoxyquinazolin-4-amine (terazosin impurity A IPRS)* and *1-[[(2RS)-tetrahydrofuran-2-yl]carbonyl]piperazine IPRS (terazosin impurity N) IPRS* in *acetonitrile*, add 5 ml of the test solution and dilute to 50 ml with *acetonitrile*. Dilute 10.0 ml of the solution to 100.0 ml with the solvent mixture.

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Reference solution (b). Dilute 10.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 2.8 g of *sodium laurylsulphate* in 1000 ml of *water* and add 11 ml of a solution containing 20.24 per cent w/v of *triethylamine* and 23.0 per cent w/v of *orthophosphoric acid* and adjusted to pH 2.5 with *orthophosphoric acid*; mix 60 volumes of this solution with 40 volumes of *acetonitrile,*
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject reference solution (b). The relative retention time with reference to terazosin for 1,4-bis[(tetrahydrofuran-2-yl)carbonyl]piperazine (terazosin impurity O) is about 0.2, for terazosin impurity N is about 0.3 and for terazosin impurity A is about 0.4. The test is not valid unless the resolution between the peaks due to terazosin impurity A and N is not less than 1.5.

Inject reference solution (b) and the test solution. Run the chromatogram 4 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of each peak corresponding to terazosin impurity N and terazosin impurity O is not more than the area of the peak due to terazosin in the chromatogram obtained with reference solution (b) (0.1 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 100 ml of the mobile phase.

Reference solution (a). Dilute 2.0 ml of the test solution to 100 ml with the mobile phase. Dilute 5.0 ml of the solution to 100 ml with the mobile phase.

Reference solution (b). Dissolve the contents of a vial of *terazosin for system suitability IPRS* (containing terazosin impurity A, terazosin impurity B (1-(4-hydroxy-6,7-dimethoxyquinazolin-2-yl)-4-[[(2*RS*)-tetrahydrofuran-2-yl] carbonyl]piperazine), terazosin impurity C (6,7-dimethoxy-2-(piperazin-1-yl)quinazolin-4-amine), terazosin impurity J (1-(4-amino-6,7-dimethoxyquinazolin-2-yl)-4-[(2*RS*)-2-hydroxy-pentanoyl]piperazine), terazosin impurity K (prazosin) and terazosin impurity M (1,4-bis(furan-2-ylcarbonyl) piperazine) in 10 ml of the mobile phase.

Reference solution (c). Dissolve 5 mg of *1-(furan-2-ylcarbonyl)piperazine IPRS (terazosin impurity L IPRS)* in 100 ml of the mobile phase. Dilute 1.0 ml of the solution to 100 ml with the mobile phase.

Reference solution (d). To 5 mg of *2,2-(piperazine-1,4-diyl)bis(6,7-dimethoxyquinazolin-4-amine) IPRS (terazosin impurity E IPRS)* add 70 ml of *methanol* and 30 ml of *water.* Allow to stand for at least 1 hour to dissolve the substance.

Chrmatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: mix 2 volumes of *triethylamine*, 350 volumes of *acetonitrile*, and 1650 volumes of a solution containing 0.6 per cent w/v of *sodium citrate* and 1.425 per cent w/v of *anhydrous citric acid,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to terazosin impurity B and terazosin impurity J is not less than 1.5.

Inject reference solution (a), (c) and the test solution. Run the chromatogram 4 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of secondary peak corresponding to terazosin impurity A, C, E and K, each of, is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent), the area of secondary peak corresponding to terazosin impurity L is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent), the area of secondary peak corresponding to terazosin impurity B, J, M, each of, is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent). Multiply the peak areas of the impuirites by the correction factor for calculating the contents, for impuirty C is 0.7 and for impuirty M is 1.6, the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all other secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). 7.0 to 8.6 per cent, determined on 0.2 g.

Assay. Dissolve 0.3 g in a mixture of 5 ml of *0.01 M hydrochloric acid* and 50 ml of *methanol*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

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1 ml of *0.1 M sodium hydroxide* is equivalent to 0.04239 g of $C_{19}H_{26}ClN_5O_4$.

Storage. Store protected from light.

Terazosin Tablets

Terazosin Hydrochloride Tablets

Terazosin Tablets contain Terazosin Hydrochloride.

Terazosin Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of terazosin, $C_{19}H_{25}N_5O_4$.

Usual strengths. 1 mg; 2 mg; 4 mg; 5 mg.

Identification

A. Disperse a quantity of the powdered tablets containing 10 mg of terazosin in 50 ml of *0.1 M hydrochloric acid* with the aid of ultrasound for 10 minutes and dilute to 100.0 ml with *0.1 M hydrochloric acid*. Dilute 5.0 ml of the solution to 100.0 ml with *0.1 M hydrochloric acid* and filter. The filtrate when examined in the range from 200 nm to 400 nm (2.4.7) shows an absorption maximum similar to that obtained with the same concentration of *terazosin hydrochloride IPRS* in *0.1 M hydrochloric acid*.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium: 900 ml of *water*,

Speed and time: 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the resulting solution at the maximum at about 245 nm (2.4.7). Calculate the content of $C_{19}H_{25}N_5O_4$ in the medium from the absorbance obtained from a solution of known concentration of *terazosin hydrochloride IPRS*, prepared by dissolving in the dissolution medium to get similar concentration as that of the test solution.

Q: Not less than 75 per cent of the stated amount of $C_{19}H_{25}N_5O_4$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing 15 mg of terazosin in 25 ml of the mobile phase with the aid of ultrasound for 10 minutes and with mechanical shaking for about 20 minutes and dilute to 50.0 ml with the mobile phase, filter through 0.45 µm nylon or teflon filter.

Reference solution (a). A solution of *terazosin hydrochloride IPRS* in the mobile phase to obtain 0.0003 per cent w/v of terazosin.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 6 volumes of *acetonitrile* and 19 volumes of buffer solution prepared by dissolving 4.1 g of *monobasic potassium phosphate* and 1.1 g of *sodium 1-heptanesulphonate monohydrate* in 950 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and dilute to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 246 nm,
- injection volume: 10 µl.

Name	Relative retention time	Relative response factor
Piperazinyl-ADMQ[1]	0.52	1.1
Terazosin Hydrochloride	1.0	—
Chloro ADMQ[2]	1.37	1.2
Bis-ADMQ-piperazine[3]	3.85	1.0

[1] *N*-(4-Amino-6,7 dimethoxy-2-quinazolinyl)piperazine,

[2] 2-Chloro-4-amino-6,7 dimethoxy-2-quinazoline,

[3] *N,N*-Bis-(4-amino-6,7 dimethoxy-2-quinazolinyl)piperazine.

Inject reference solution (a) and (b). The test is not valid unless the tailing factor is not more than 2.0 in the chromatogram obtained with reference solution (a) and signal-to-noise ratio is not less than 10.0 in the chromatogram obtained with reference solution (b).

Inject reference solution (a) and the test solution. Run the test solution 4.5 times the retention time of the principal peak. The area of any peak corresponding to piperazinyl-ADMQ, chloro ADMQ and bis-ADMQ piperazine, each of, is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent), the area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 1.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.2 per cent).

Uniformity of content. Determine by liquid chromatography (2.4.14), as described under Assay using following modification.

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Test solution. Transfer one tablet to 20 ml of solvent mixture, disperse with the aid of ultrasound for about 20 minutes, cool and dilute, with sufficient of the solvent mixture, to produce a solution containing 0.005 percent w/v tearzosin, mix and shake well, filter.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Use an all-glass syringe.

Solvent mixture. 40 volumes of *0.01 M methanolic hydrochloric acid* and 60 volumes of *water.*

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 10 mg of terazosin in 100 ml of the solvent mixture with the aid of ultrasound for 10 minutes and mechanical shaking for further 10 minutes and dilute to 200.0 ml with the solvent mixture and filter.

Reference solution. A 0.0055 per cent w/v solution of *terazosin hydrochloride IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 70 volumes of *acetonitrile* and 30 volumes of *water*, add 1 volume of *glacial acetic acid*, filter and pipet 0.2 volume of *diethylamine* into the solution,
- flow rate: 2.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 25 µl.

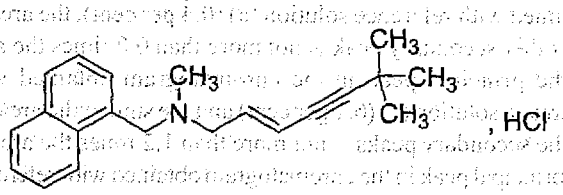
Inject the reference solution. The test is not valid unless the tailing factor is not morre than 2.0 and the relative standard deviation for replicate injections is not more than 2.0

Inject the reference solution and the test solution. Run the chromatogram twice the retention time of the principal peak.

Calculate the content of $C_{19}H_{25}N_3O_4$ in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Terbinafine Hydrochloride



$C_{21}H_{25}N,HCl$ Mol. Wt. 327.9

Terbinafine Hydrochloride is *N*-(6, 6-dimethyl-2-hepten-4-ynyl)-N-methyl-,(E)-,hydrochloride.

Terbinafine Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{21}H_{25}N,HCl$, calculated on the dried basis.

Category. Antifungal.

Description. A white, crystalline powder; hygroscopic.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *terbinafine hydrochloride IPRS* or with the reference spectrum of terbinafine hydrochloride.

B. It gives reaction (A) of chloride (2.3.1).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Carry out the test protected from light.

Solvent mixture. Equal volumes of *acetonitrile* and *water.*

Solution A. 300 volumes of *methanol* A and 200 volumes of *acetonitrile.*

Test solution. Dissolve 50 mg of the substance under examination in 100.0 ml of *solvent mixture.*

Reference solution (a). A solution containing 0.00005 per cent w/v each of *terbinafine hydrochloride IPRS, terbinafine related compound A IPRS, terbinafine related compound B IPRS, terbinafine related compound C IPRS and terbinafine related compound D IPRS;* in the solvent mixture.

Reference solution (b). A 0.000025 per cent w/v solution of *terbinafine hydrochloride IPRS* from reference solution (a) in the solvent mixture.

Reference solution (c). A 0.1 per cent w/v solution of *terbinafine hydrochloride IPRS* and 0.0005 per cent w/v solution of *terbinafine related compound B IPRS* in the solvent mixture.

Reference solution (d). A 0.05 per cent w/v solution of *terbinafine hydrochloride IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 3.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- buffer solution: A buffer solution prepared by dissolving 2.0 ml of *triethylamine* in 1000 ml of *water*, adjusted to pH 7.5 with *dilute acetic acid,*
- mobile phase: A. 70 volumes of solution A and 30 volumes of buffer solution,
  B. 95 volumes of solution A and 5 volumes of buffer solution,
- flow rate: 0.8 ml per minute,

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– spectrophotometer set at 280 nm,
– injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
4	100	0
25	0	100
30	0	100
30.1	100	0
38	100	0

Name	Relative retention time	Correction factor
Terbinafine related compound A[1]	0.1	—
Terbinafine related compound C[2]	0.92	—
Terbinafine related compound B[3]	0.94	—
Terbinafine	1	—
Terbinafine related compound D[4]	1.1	—
Terbinafine Diamer[5]	1.7	0.4
Individual impurity	—	1.0
Total impurity	—	—

[1]*N*-Methyl-1-(naphthalene-1-yl)methanamine also known as *N*-methyl-*C*-(naphthalene-2-yl)methanamine.

[2]trans-Isoterbinafine or (*E*)-*N*,6,6-trimethyl-*N*-(napthalen-1-ylmethyl)hept-2-en-4-yn-1-amine.

[3]cis-Terbinafine or (z)-*N*,6,6-trimethyl-*N*-(naphthalene-1-ylmethyl)hept-2-en-4-yn-1-amine.

[4]4-Methylterbinafine or (E)-N,6,6-trimethyl-*N*-((4-methylnaphthalen-1-yl)methyl)hept-2-en-4-yn-1-amine.

[5](2*E*,4*E*)-4-(4,4-Dimethylpent-2-ynylidene)-N^1, N^5-dimethyl-N^1, N^5-bis(naphthalene-1-ylmethyl)pent-2-ene-1,5-diamine.

Inject reference solution (c). The test is not valid unless the resolution between terbinafine releted compound B and terbinafine peaks is not less than 2.0.

Inject reference solution (a). The relative standard deviation of replicate injections is not more than 10.0 per cent for peak due to terbinafine hydrochloride.

Inject reference solution (b). The signal-to- noise ratio is not less than 10.0 for peak due to terbinafine hydrochloride.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of impurity due to terbinafine related compound A is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the area of impurity due to terbinafine related compound C is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the area of impurity due to terbinafine related compound B is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the area of impurity due to terbinafine related compound D is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the area of impurity due to terbinafine diamer is not more than the, 0.5 times the area of peak due to terbinafine in the chromatogram obtained with reference solution (a) (0.05 per cent) , the area of any other secondary peak is not more than the area of the peak due to terbinafine hydrochloride in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than three times the area of the peak due to terbinafine hydrochloride in the chromatogram obtained with reference solution (a) (0.3 per cent). Disregard any peak which is 0.5 times the area of the peak due to terbinafine hydrochloride in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Determine by liquid chromatography (2.4.14) as described under Related substances.

Inject reference solution (d). The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (d) and the test solution.

Calculate the content of $C_{21}H_{25}N,HCl$.

Storage. Store protected from light and moisture.

Terbinafine Cream

Terbinafine Hydrochloride Cream

Terbinafine Cream contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of terbinafine hydrochloride, $C_{21}H_{25}N,HCl$ in a suitable cream base.

Usual strength. 1 per cent w/w.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

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Test solution. Shake a quantity of the cream containing about 25 mg of Terbinafine Hydrochloride with 70.0 ml of the mobile phase, warm on a water-bath at 55° for 10 minutes. Cool to room temperature. Sonicate with intermittent swirling to obtain uniform dispersion and dilute to 100.0 ml with mobile phase and filter.

Reference solution. A 0.000125 per cent w/v solution of *terbinafine hydrochloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 28 volumes of *acetonitrile*, 10 volumes of *methanol*, 62 volumes of *0.05 M phosphate buffer* prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml *water*, 0.1 volume of *orthophosphoric acid* and 0.1 volume of *triethylamine*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 224 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 4000 theoretical plates, the tailing factor is not more than 2.0, and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not greater than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (0.3 per cent) and the sum of areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (0.5 per cent).

Other tests. Comply with the tests stated under Creams.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the cream containing about 25 mg of Terbinafine Hydrochloride with 70 ml of the *methanol*, warm on a water-bath at 55° for 10 minutes with intermittent swirling. Sonicate for 15 minutes with intermittent swirling to obtain a uniform dispersion. Cool to room temperature and dilute to 100.0 ml with *methanol* and filter. Further dilute 5.0 ml to 25.0 ml with mobile phase.

Reference solution. Dissolve 25 mg of *terbinafine hydrochloride IPRS* in 30 ml of *methanol* and dilute to 50.0 ml with the *methanol*. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 500 volumes of *water*, 400 volumes of *acetonitrile*, 100 volumes of *methanol*, 1 volume of *orthophosphoric acid* and 1 volume of *triethylamine*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 282 nm,
- injection volume: 20 µl.

Inject the reference solution, the test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{25}N$,HCl in the cream.

Storage. Store at a temperature not exceeding 30°. The cream should not be allowed to freeze.

Terbinafine Tablets

Terbinafine Hydrochloride Tablets

Terbinafine Hydrochloride Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of terbinafine, $C_{21}H_{25}N$.

Usual strength. 250 mg.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 500 ml of a *citrate buffer pH 3.0*, prepared by dissolving 7.9 g of citric acid anhydrous and 2.3 g *sodium citrate* in *water*.

Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with *citrate buffer* if necessary, at the maximum at about 283 nm (2.4.7). Calculate the content of terbinafine, $C_{21}H_{25}N$, in the medium from the absorbance obtained by repeating the determination using a solution of known concentration of *terbinafine hydrochloride IPRS* in dissolution medium.

Q. Not less than 80 per cent of the stated amount of $C_{21}H_{25}N$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets containing 20 mg of terbinafine with 50 ml of the mobile phase

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and mix with the aid of ultrasound for 20 minutes, dilute to 100.0 ml with mobile phase filter and use the filtrate.

Reference solution. A solution of *terbinafine hydrochloride IPRS* in the mobile phase containing 0.00007 per cent w/v of terbinafine.

Chromatographic system
- a stainless steel column 15 cm x 3.9 mm, packed with octaylsilane chemically bonded to porous silica (5 µm),
- mobile phase: 60 volumes of a buffer solution prepared by dissolving 0.85 g of *monobasic potassium phosphate* and 1.0 g of *sodium 1-decanesulfonate* in 1000 ml of *water* and adjusted to pH 3.0 with *orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 50 µl.

Name	Relative retention time	Correction factor
N-Methyl-1-(naphthalene-1-yl) methanamine	0.14	0.67
Terbinafine	1.0	—
Unknown	—	1.0
Total impurity	—	—

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 10.0 per cent.

Inject the reference solution and the test solution. In the chromatogram with the test solution, the area of impurity due to *N*-Methyl-1-(naphthalene-1-yl) is not more than 0.57 times the area of the principal peak in the chromatogram obtained with reference solution (0.2 per cent), the area of any secondary peak is not more than 0.57 times the area of the principal peak in the chromatogram obtained with reference solution (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 2 times the area of the peak in the chromatogram obtained with reference solution (0.7 per cent).

Limit of Terbinafine Dimer. Determine by liquid chromatography (2.4.14).

Solvent mixture. 40 volumes of *acetonitrile* and 10 volumes of *water*.

Test solution. Shake a quantity of the powdered tablets containing 250 mg of terbinafine with 50 ml of the solvent mixture and mix with the aid of ultrasound for 20 minutes, dilute to 100.0 ml with solvent mixture and filter.

Reference solution. A solution of *terbinafine hydrochloride IPRS* in the solvent mixture containing 0.00014 per cent w/v of terbinafine.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 52°,
- mobile phase: A. 1.0 ml of *triethylamine* in 1000.0 ml of *water*,

  B. 1.0 ml of *triethylamine* in a 1000.0 ml of a mixture of 19 volumes of *acetonitrile* and 1 volume of *water*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 100 µl,

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	40	60
8	30	70
18	30	70
24	16	84
26	5	95
30	0	100
37	40	60
45	40	60

Name	Relative retention time	Correction factor
Terbinafine	1.0	—
Terbinafine dimer[1]	2.0	0.48

[1](2*E* ,4*E*)-4-(4,4-Dimethylpent-2-ynylidene)-N^1, N^5-dimethyl-N^1, N^5-bis(naphthalen-1-ylmethyl)pent-2-ene-1,5-diamine.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 10.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of impurity due to terbinafine diamer is not more than 0.9 times the area of the principal peak in the chromatogram obtained with reference solution (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Shake a quantity of the powder containing 20 mg of Terbinafine with 50 ml of the mobile phase and mix with the aid of ultrasound for 20 minutes, dilute to 100.0 ml with the mobile phase and filter.

Reference solution. A 0.022 per cent w/v solution of *terbinafine hydrochloride IPRS* in the mobile phase.

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Chromatographic system
- a stainless steel column 15 cm x 3.9 mm, packed with octaylsilane bonded to porous silica (5 µm),
- mobile phase: 60 volumes of a buffer solution prepared by dissolving 0.85 g of *monobasic potassium phosphate* and 1.0 g of *sodium 1-decanesulfonate* in 1000 ml of *water* and adjusted to pH 3.0 with *orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 5 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

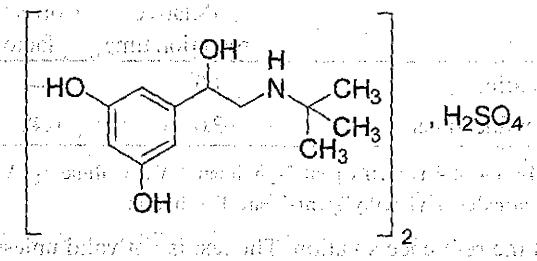
Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{25}N$ in the tablets.

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of the equivalent amount of terbinafine.

Terbutaline Sulphate



$(C_{12}H_{19}NO_3)_2,H_2SO_4$ Mol. Wt. 548.7

Terbutaline Sulphate is (*RS*)-2-(*tert*-butylamino)-1-(3,5-dihydroxyphenyl)ethanol sulphate.

Terbutaline Sulphate contains not less than 98.0 per cent and not more than 101.0 per cent of $(C_{12}H_{19}NO_3)_2,H_2SO_4$, calculated on the dried basis.

Category. Beta-adrenoceptor agonist.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *terbutaline sulphate IPRS* or with the reference spectrum of terbutaline sulphate.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.007 per cent w/v solution in *0.1 M hydrochloric acid* shows absorption maxima at about 276 nm and 280 nm, which may be fused; absorbance at both 276 nm and 280 nm, 0.46 to 0.49.

C. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

D. A 2.0 per cent w/v solution in *carbon dioxide-free water* gives reaction (A) of sulphates (2.3.1).

Tests

Appearance of solution. A 2.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1); absorbance of a 2-cm layer at 400 nm, not more than 0.11 (2.4.7).

Acidity. Dissolve 0.2 g in 10 ml of *carbon dioxide-free water* and titrate with *0.01 M sodium hydroxide*, using *methyl red solution* as indicator. Not more than 1.2 ml of *0.01 M sodium hydroxide* is required to change the colour of the solution to yellow.

tert-Butylamino-3,5-dihydroxyacetophenone. Absorbance of a 2 per cent w/v solution in *0.01 M hydrochloric acid* at about 330 nm, not more than 0.50 (2.4.7).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 75 mg of the substance under examination 50.0 ml of the mobile phase.

Reference solution (a). Dissolve 7.5 mg of *1-(3,5-dihydroxyphenyl)-2-[(1,1-dimethylethyl)amino]ethanone IPRS (terbutaline impurity C IPRS)* and 22.5 mg of *terbutaline sulphate IPRS* in 50.0 ml of the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 20.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 4.23 g of *sodium hexanesulphonate* in 770 ml of *0.05 M ammonium formate solution* prepared by dissolving 3.15 g of *ammonium formate* in about 980 ml of *water*, adjusted to pH 3.0 by *anhydrous formic acid* and dilute to 1000 ml with *water* and add 230 ml of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 276 nm,
- injection volume: 20 µl.

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Inject reference solution (a). The test is not valid unless the resolution between the peaks due to terbutaline impurity C and terbutaline is not less than 2.0.

Inject reference solution (a), (b) and the test solution. Run the chromatogram 6 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to terbutaline impurity C is not more than twice the area of the peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any other secondary peaks is not more than area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of areas of all the secondary peaks other than terbutaline impurity C is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

Heavy metals (2.3.13). Mix 1.6 g with 0.6 g of *anhydrous sodium sulphate* and ignite without melting the sodium sulphate. Cool, add 3 ml of *2 M hydrochloric acid*, boil and dilute to 50 ml with *water*. Cool and filter, 25 ml of the filtrate complies with the limit test for heavy metals, Method A (25 ppm).

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 0.5 g in 60 ml of *anhydrous glacial acetic acid* with the aid of heat. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.05487 g of $(C_{12}H_{19}NO_3)_2, H_2SO_4$.

Storage. Store protected from light and moisture.

Terbutaline Inhalation

Terbutaline Sulphate Inhalation; Terbutaline Inhalation Aerosol; Terbutaline Sulphate Inhalation Aerosol

Terbutaline Inhalation is a suspension of Terbutaline Sulphate, as a superfine powder, in a suitable liquid in a suitable pressurised container. It may contain suitable pharmaceutical aids such as surfactants, stabilising agents, etc.

Terbutaline Inhalation delivers not less than 75.0 per cent and not more than 125.0 per cent of the stated amount of terbutaline sulphate $(C_{12}H_{19}NO_3)_2,H_2SO_4$, per inhalation, by actuation of the valve.

Usual strength. 250 µg in each metered dose.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 65 volumes of *2-propanol*, 25 volumes of *cyclohexane* and 5 volumes of *formic acid*.

Test solution. Remove the actuator from the pressurised container, shake the container for about 30 seconds and place it in an inverted position in a small beaker containing 5 ml of *water*. Discharge a sufficient number of deliveries containing 5 mg of Terbutaline Sulphate, under the surface of the solvent.

Reference solution. A 0.1 per cent w/v solution of *terbutaline sulphate IPRS* in *water*.

Apply to the plate 2 µl of each solution. After development, dry the plate in air, spray with a 2 per cent w/v solution of *4-aminoantipyrine* in *methanol*. Examine in daylight and under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to spot in the chromatogram obtained with reference solution.

Tests

Other tests. Comply with the tests stated under Inhalation Preparations (Pressurised metered-dose Preparations).

Follow the procedure described under Assay wherever the amount of active substance is to be determined in any test.

Assay. Carry out the test for Content of active ingredient delivered per actuation stated under Inhalation Preparations (Pressurised metered-dose Preparations).

Use 35 ml of *water* and finally dilute to 50.0 ml with *water*. Dilute a suitable volume of the solution with *water* to produce a solution containing 50 µg of Terbutaline Sulphate per ml. To 10.0 ml of the solution in a 50-ml volumetric flask add 35 ml of *buffer solution pH 9.5* and 1.0 ml of *4-aminoantipyrine solution*. Mix, add 1.0 ml of *potassium ferricyanide solution* with vigorous swirling of the flask, dilute to volume with *water* and mix. Exactly 75 seconds after the addition of the *potassium ferricyanide solution* measure the absorbance of the resulting solution at the maximum at about 550 nm (2.4.7), using as the blank a solution prepared in the same manner using 10.0 ml of *water* in place of the solution of the substance under examination.

Calculate the content of $(C_{12}H_{19}NO_3)_2,H_2SO_4$ in the solution from the absorbance obtained by repeating the operation using a solution containing 100 µg of *terbutaline sulphate IPRS* in place of the solution of the substance under examination.

Calculate the amount of $(C_{12}H_{19}NO_3)_2,H_2SO_4$ delivered per actuation of the valve.

Determine the content of active ingredient a second and third time by repeating the procedure on the middle ten and on the last ten successive combined actuations of the valve. For each of the three determinations the average content of $(C_{12}H_{19}NO_3)_2,H_2SO_4$ delivered per actuation of the valve meets the requirements.

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Storage. Store protected from moisture at a temperature not exceeding 30°.

Labelling. The label states the amount of active ingredient delivered per inhalation.

Terbutaline Injection

Terbutaline Sulphate Injection

Terbutaline Injection is a sterile solution of Terbutaline Sulphate in Water for Injections.

Terbutaline Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of terbutaline sulphate $(C_{12}H_{19}NO_3)_2, H_2SO_4$.

Usual strength. 500 µg per ml.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 65 volumes of *2-propanol*, 25 volumes of *cyclohexane* and 5 volumes of *formic acid*.

Test solution. Use the injection.

Reference solution. A 0.1 per cent w/v solution of *terbutaline sulphate IPRS* in *saline solution*.

Apply to the plate 2 µl of each solution. After development, dry the plate in air, spray with a 2 per cent w/v solution of *4-aminoantipyrine* in *methanol*. Dry the plate in air and spray with a freshly prepared 8.0 per cent w/v solution of *potassium ferricyanide* in a mixture of 4 volumes of *strong ammonia solution* and 1 volume of *water*. The principal spot in the chromatogram obtained with the test solution corresponds to spot in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 3.0 to 5.0.

Bacterial endotoxins (2.2.3). Not more than 1250.0 Endotoxin Units per mg of terbutaline sulphate.

Other tests. Comply with the tests stated under Parenteral preparations (Injections).

Assay. Measure a volume containing about 5 mg of Terbutaline Sulphate and add sufficient *water* to produce 50.0 ml. To 5.0 ml add 35 ml of a buffer solution prepared by dissolving 36.3 g of *tris (hydroxymethyl) aminomethane* in 900 ml of *water*, adjusted to pH between 9.4 and 9.6 and adding sufficient *water* to produce 1000 ml. Add 1.0 ml of a freshly prepared 2.0 per cent w/v solution of *4-aminoantipyrine*, mix and add 1.0 ml of a freshly prepared 8.0 per cent w/v solution of *potassium ferricyanide* with vigorous swirling and sufficient of the buffer solution to produce 50.0 ml. Exactly 75 seconds after the addition of the *potassium ferricyanide solution* measure the absorbance of the resulting solution at the maximum at about 550 nm (2.4.7), using *water* as the blank.

Calculate the content of $(C_{12}H_{19}NO_3)_2, H_2SO_4$ from the absorbance obtained by repeating the operation using a 0.01 per cent w/v solution of *terbutaline sulphate IPRS* and beginning at the words "To 5.0 ml add 35 ml.....".

Storage. Store protected from light, in single dose containers.

Labelling. The label states that the injection should not be used if the solution is discoloured.

Terbutaline Tablets

Terbutaline Sulphate Tablets

Terbutaline Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of terbutaline sulphate $(C_{12}H_{19}NO_3)_2, H_2SO_4$.

Usual strengths. 2.5 mg; 5 mg.

Identification

A. Shake a quantity of the powdered tablets containing 20 mg of Terbutaline Sulphate with 50 ml of *0.1 M sodium hydroxide* for 10 minutes, dilute to 100 ml with *0.1 M sodium hydroxide* and filter. Dilute 20 ml of the filtrate to 50 ml with *0.1 M sodium hydroxide*.

When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows an absorption maximum at about 296 nm.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 65 volumes of *2-propanol*, 25 volumes of *cyclohexane* and 5 volumes of *formic acid*.

Test solution. Shake a quantity of the powdered tablets containing 10 mg of Terbutaline Sulphate with 4 ml of a mixture of equal volumes of *ethanol (95 per cent)* and *water* for 10 minutes, centrifuge and use the clear supernatant liquid.

Reference solution (a). A 0.25 per cent w/v solution of *terbutaline sulphate IPRS* in *water*.

Reference solution (b). A mixture of equal volumes of the test solution and reference solution (a).

Apply to the plate 2 µl of each solution. After development, dry the plate in air, allow to stand for a few minutes in an atmosphere saturated with *diethylamine* and spray with *diazotised nitroaniline solution*. The principal spot in the chromatogram obtained with the test solution corresponds to

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that in the chromatogram obtained with reference solution (a) and the principal spot in the chromatogram obtained with reference solution (b) appears as a single compact spot.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium. 900 ml of *water*,

Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Carry out the method as described under Assay beginning at the words "To 5.0 ml, add 35 ml of a buffer solution....". Calculate the content of $(C_{12}H_{19}NO_3)_2,H_2SO_4$ in the medium from the absorbance obtained from a solution of known concentration of *terbutaline sulphate IPRS* prepared in the same manner.

Q: Not less than 70 per cent of the stated amount of $(C_{12}H_{19}NO_3)_2.H_2SO_4$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing 75 mg of Terbutaline Sulphate with 35 ml of the mobile phase for 15 minutes and dilute to 50.0 ml with the mobile phase and filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.00045 per cent w/v of *terbutaline sulphate IPRS* and 0.00015 per cent w/v of *terbutaline impurity C IPRS (1-(3,5-dihydroxyphenyl)-2-[(1,1-dimethylethyl)amino]ethanone IPRS)* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with base-deactivated octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil BDS C18),
- mobile phase: dissolve 4.23 g of *sodium hexane sulphonate* in 770 ml of the 0.05 M ammonium formate solution prepared by dissolving 3.15 g of *ammonium formate* in 980 ml of *water*, adjusted to pH 3.0 by the adding about 8 ml of *anhydrous formic acid* and dilute to 1000 ml with *water* and 230 ml of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 276 nm,
- injection volume: 20 µl.

The retention time of terbutaline impurity C is about 9 minutes and of terbutaline sulphate is about 11 minutes.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to terbutaline sulphate and terbutaline impurity C is not less than 2.0.

Inject reference solution (a), (b) and the test solution. Run the chromatogram 6 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to terbutaline impurity C is not more than twice the area of the peak due to terbutaline impurity C in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent) and any peaks with a retention time of less than 2.5 minutes.

Uniformity of content. Complies with the test stated under Tablets.

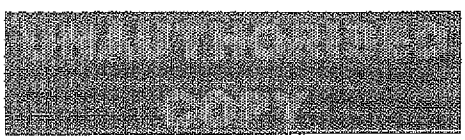
Powder one tablet, transfer to a 25-ml volumetric flask, add 15 ml of *0.01 M hydrochloric acid* and shake for 10 minutes. Dilute to volume with *0.01 M hydrochloric acid* and filter, rejecting the first 5 ml of the filtrate. Dilute, if necessary, a suitable volume of the filtrate with *0.01 M hydrochloric acid* to produce a solution containing 0.01 per cent w/v solution of Terbutaline Sulphate. Carry out the method as described under Assay beginning at the words "To 5.0 ml add 35 ml of a buffer solution....". Calculate the content of $(C_{12}H_{19}NO_3)_2$, H_2SO_4 in the tablet from the absorbance obtained by carrying out the Assay simultaneously using *terbutaline sulphate IPRS*.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 5 mg of Terbutaline Sulphate, transfer to a 50-ml volumetric flask, add 30 ml of *0.01 M hydrochloric acid* and shake for 10 minutes. Dilute to volume with *0.01 M hydrochloric acid* and filter, rejecting the first 5 ml of the filtrate. To 5.0 ml add 35 ml of a buffer solution prepared by dissolving 36.3 g of *tris (hydroxymethyl) aminomethane* in 900 ml of *water*, adjusted to pH between 9.4 and 9.6 and adding sufficient *water* to produce 1000 ml. Add 1.0 ml of a freshly prepared 2.0 per cent w/v solution of *4-aminoantipyrine*, mix and add 1.0 ml of a freshly prepared 8.0 per cent w/v solution of *potassium ferricyanide* with vigorous swirling and sufficient of the buffer solution to produce 50.0 ml. Exactly 75 seconds after the addition of the *potassium ferricyanide solution* measure the absorbance of the resulting solution at the maximum at about 550 nm (2.4.7), using *water* as the blank.

Calculate the content of $(C_{12}H_{19}NO_3)_2$, H_2SO_4 from the absorbance obtained by carrying out the Assay simultaneously using *terbutaline sulphate IPRS*.

Storage. Store protected from light and moisture.



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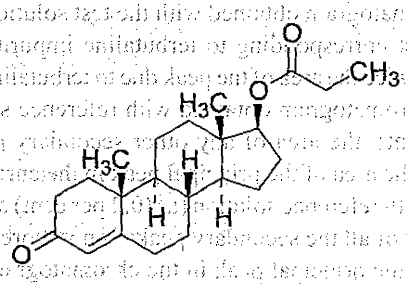
Testosterone Propionate



$C_{22}H_{32}O_3$ Mol. Wt. 344.5

Testosterone Propionate is 3-oxoandrost-4-en-17β-yl propionate.

Testosterone Propionate contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{22}H_{32}O_3$, calculated on the dried basis.

Category. Androgen; anabolic steroid.

Description. A white or almost white powder or colourless crystals.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *testosterone propionate IPRS* or with the reference spectrum of testosterone propionate.

B. In the test for Related substances the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to testosterone propionate in the chromatogram obtained with reference solution (a).

C. Melting range. 119° to 123° (2.4.21).

Tests

Specific optical rotation (2.4.22). +83.0° to +90.0°, determined in a 1.0 per cent w/v solution in *ethanol*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in *methanol* and dilute to 50 ml with the *methanol*.

Reference solution (a). Dissolve 2 mg of *testosterone propionate IPRS* and 2 mg of *testosterone acetate IPRS* in *methanol* and dilute to 50 ml with the *methanol*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 20 volumes of *water* and 80 volumes of *methanol*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention time of about four impurities with reference to testosterone propionate (retention time, about 9 minutes) range from 0.5 to about 1.4.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to testosterone propionate and testosterone acetate is not less than 4.0.

Inject reference solution (b) and the test solutions. Run the chromatogram twice the retention time for testo-sterone propionate. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Assay. Dissolve 25 mg in sufficient *ethanol* to produce 250.0 ml and mix. Dilute 5.0 ml to 50.0 ml with the *ethanol* and measure the absorbance of the resulting solution at the maximum at about 241 nm (2.4.7).

Calculate the content of $C_{22}H_{32}O_3$ taking 490 as the specific absorbance at 241 nm.

Storage. Store protected from light and moisture.

Testosterone Propionate Injection

Testosterone Propionate Injection is a sterile solution of Testosterone Propionate in Ethyl Oleate or any other suitable ester, in a suitable fixed oil or in any mixture of these.

Testosterone Propionate Injection contains not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of testosterone propionate, $C_{22}H_{32}O_3$.

Usual strength. 25 mg per ml; 50 mg per ml.

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Identification

Dissolve a volume containing 50 mg of Testosterone Propionate in 8 ml of *light petroleum (40° to 60°)* and extract with three quantities, each of 8 ml, of a mixture of 7 volumes of *glacial acetic acid* and 3 volumes of *water*. Wash the combined extracts with 10 ml of *light petroleum (40° to 60°)*, dilute with *water* until the solution becomes turbid, allow to stand for 2 hours in ice and filter. The precipitate, after washing with *water* and drying at 105°, complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *testosterone propionate IPRS* or with the reference spectrum of testosterone propionate.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. A mixture of 90 volumes of *light petroleum (40° to 60°)* and 10 volumes of *liquid paraffin*.

Mobile phase. A mixture of 30 volumes of *water* and 20 volumes of *glacial acetic acid*.

Test solution. Dissolve 25 mg of the residue in 10 ml of the solvent mixture.

Reference solution (a). A 0.25 per cent w/v solution of *testosterone propionate IPRS* in the solvent mixture.

Reference solution (b). Mix equal volumes of the test solution and reference solution (a).

Place the dry plate in a tank containing a shallow layer of the solvent mixture, allow the solvent mixture to ascend to the top, remove the plate from the tank and allow the solvent to evaporate. Use within 2 hours, with the flow of the mobile phase in the direction in which the aforementioned treatment was done.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of warm air, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulphuric acid (20 per cent v/v)*. Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot A.

Tests

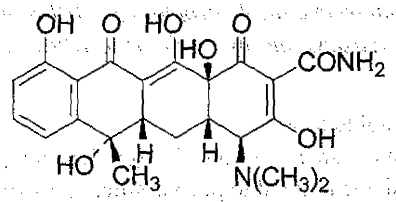
Other tests. Comply with the tests stated under Parenteral preparations (Injections).

Assay. To an measured volume containing about 0.1 g of Testosterone Propionate add sufficient *chloroform* to produce 100.0 ml and mix. Dilute 3.0 ml to 50.0 ml with *chloroform* and to 5.0 ml of the solution add 10 ml of *isoniazid solution* and sufficient *methanol* to produce 20.0 ml. Allow to stand for 45 minutes and measure the absorbance of the resulting solution at the maximum at about 380 nm (2.4.7), using as the blank a solution prepared by treating 5 ml of *chloroform* in the same manner.

Calculate the content of $C_{22}H_{32}O_3$ from the absorbance obtained by repeating the operation using a 0.006 per cent w/v solution of *testosterone propionate IPRS* in *chloroform* and beginning at the words "to 5.0 ml of the solution.....".

Storage. Store protected from light.

Tetracycline



$C_{22}H_{24}N_2O_8$ Mol. Wt. 444.5

Tetracycline is (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxonaphthacene-2-carboxamide. It contains a variable quantity of water.

Tetracycline contains not less than 88.0 per cent of $C_{22}H_{24}N_2O_8$, calculated on the dried basis.

Category. Antibacterial.

Description. A yellow, crystalline powder.

Identification

A. In the test for Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to tetracycline hydrochloride in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 ml of *sulphuric acid*; a reddish violet colour develops. Add the solution to 2.5 ml of *water*; the colour changes to yellow.

C. Dissolve about 10 mg in a mixture of 1 ml of *2 M nitric acid* and 5 ml of *water*, shake well and add 1 ml of *silver nitrate solution*. Any opalescence produced is not more intense than that in a solution prepared in the same manner omitting the substance under examination.

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Tests

pH (2.4.24). 3.5 to 6.0, determined in a 1.0 per cent w/v suspension in *carbon dioxide-free water*.

Specific optical rotation (2.4.22). −280° to −260°, determined at 20° in a 1.0 per cent w/v solution in *0.1 M hydrochloric acid*.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 68 ml of *0.1 M ammonium oxalate* and 27 ml of *dimethylformamide*.

Test solution. Dissolve 0.1 g of the substance under examination in 100 ml of the solvent mixture.

Reference solution (a). A 0.0025 per cent w/v solution of *4-epitetracycline hydrochloride IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.0025 per cent w/v of *4-epitetracycline hydrochloride IPRS* and 0.01 per cent w/v of *tetracycline hydrochloride IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 to 10 µm),
- mobile phase: a mixture of 680 volumes of *0.1 M ammonium oxalate*, 270 volumes of *dimethylformamide* and 50 volumes of *0.2 M dibasic ammonium phosphate*, the pH of the mixture being adjusted, if necessary, to 7.6 to 7.7 with *3 M ammonia* or *3 M phosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to 4-epitetracycline is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) and the sum of areas of all the secondary peaks is not more than 5.0 per cent.

Heavy metals (2.3.13). 0.5 g complies with the limit test for heavy metals, Method B (50 ppm). Use 2.5 ml of *lead standard solution (10 ppm Pb)* to prepare the standard.

Sulphated ash (2.3.18). Not more than 0.5 per cent.

Loss on drying (2.4.19). Not more than 13.0 per cent, determined on 0.5 g by drying in an oven at 105°.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 68 ml of *0.1 M ammonium oxalate* and 27 ml of *dimethylformamide*.

Test solution. Dissolve 50 mg of the substance under examination in 100.0 ml of solvent mixture.

Reference solution (a). A 0.05 per cent w/v solution of *tetracycline hydrochloride IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.0025 per cent w/v of *4-epitetracycline hydrochloride IPRS* and 0.01 per cent w/v of *tetracycline hydrochloride IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 to 10 µm),
- mobile phase: a mixture of 680 ml of *0.1 M ammonium oxalate*, 270 ml of *dimethylformamide* and 50 ml of *0.2 M dibasic ammonium phosphate*, the pH of the mixture being adjusted, if necessary, to 7.6 to 7.7 with *3 M ammonia* or *3 M phosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 1.5.

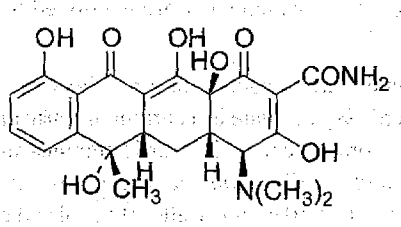
Inject reference solution (a) and the test solution.

Calculate the content of $C_{22}H_{24}N_2O_8$.

1 mg of $C_{22}H_{24}N_2O_8$, HCl is equivalent to 0.92 mg of $C_{22}H_{24}N_2O_8$.

Storage. Store protected from light and moisture.

Tetracycline Hydrochloride



, HCl

$C_{22}H_{24}N_2O_8$,HCl Mol. Wt. 480.9

Tetracycline Hydrochloride is (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-naphthacene-2-carboxamide hydrochloride.

Tetracycline Hydrochloride contains not less than 95.0 per cent and not more than 100.5 per cent of $C_{22}H_{24}N_2O_8$, HCl, calculated on the dried basis.

Category. Antibacterial.

Description. A yellow, crystalline powder.

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Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to tetracycline hydrochloride in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 ml of *sulphuric acid*; a reddish violet colour develops. Add the solution to 2.5 ml of *water*; the colour changes to yellow.

C. It gives reaction (A) of chlorides (2.3.1).

Tests

pH (2.4.24). 1.8 to 3.0, determined in a 1.0 per cent w/v suspension in *carbon dioxide-free water*.

Specific optical rotation (2.4.22). −255° to −239°, determined at 20° in a 1.0 per cent w/v solution in *0.1 M hydrochloric acid*.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 68 ml of *0.1 M ammonium oxalate* and 27 ml of *dimethylformamide*.

Test solution. Dissolve 0.1 g of the substance under examination in 100 ml of solvent mixture.

Reference solution (a). A 0.0025 per cent w/v solution of *4-epitetracycline hydrochloride IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.0025 per cent w/v of *4-epitetracycline hydrochloride IPRS* and 0.01 per cent w/v of *tetracycline hydrochloride IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 to 10 µm),
- mobile phase: 680 volumes of *0.1 M ammonium oxalate*, 270 volumes of *dimethylformamide* and 50 volumes of *0.2 M dibasic ammonium phosphate*, the pH of the mixture being adjusted, if necessary, to 7.6 to 7.7 with *3 M ammonia* or *3 M phosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to 4-epitetracycline is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) and the sum of areas of all the secondary peaks is not more than 5.0 per cent.

Heavy metals (2.3.13). 0.5 g complies with the limit test for heavy metals, Method B (50 ppm). Use 2.5 ml of *lead standard solution (10 ppm Pb)* to prepare the standard.

Sulphated ash (2.3.18). Not more than 0.5 per cent.

Loss on drying (2.4.19). Not more than 2.0 per cent, determined on 1.0 g by drying in an oven at 60° over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa for 3 hours.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 68 ml of *0.1 M ammonium oxalate* and 27 ml of *dimethylformamide*.

Test solution. Dissolve 50 mg of the substance under examination in 50 ml of the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution (a). A 0.05 per cent w/v solution of *tetracycline hydrochloride IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.0025 per cent w/v of *4-epitetracycline hydrochloride IPRS* and 0.01 per cent w/v of *tetracycline hydrochloride IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 to 10 µm),
- mobile phase: a mixture of 680 volumes of *0.1 M ammonium oxalate*, 270 volumes of *dimethylformamide* and 50 volumes of *0.2 M dibasic ammonium phosphate*, the pH of the mixture being adjusted, if necessary, to 7.6 to 7.7 with *3 M ammonia* or *3 M phosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 1.5.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{22}H_{24}N_2O_8$, HCl.

Tetracycline Hydrochloride intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per mg.

Tetracycline Hydrochloride intended for use in the manufacture of parenteral preparations without a further appropriate sterilisation procedure complies with the following additional requirement.

Sterility (2.2.11). Complies with the test for sterility.

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Storage. Store protected from light and moisture. If it is intended for use in the manufacture of parenteral preparations, the container should be sterile, tamper-evident and sealed so as to exclude microorganisms.

Labelling. The label states whether or not the material is intended for use in the manufacture of parenteral preparations.

Tetracycline Capsules

Tetracycline Hydrochloride Capsules

Tetracycline Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of tetracycline hydrochloride, $C_{22}H_{24}N_2O_8$, HCl.

Usual strengths. 250 mg; 500 mg.

Identification

A. In the test for Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to tetracycline hydrochloride in the chromatogram obtained with reference solution (a).

B. To a quantity of the mixed contents of 20 capsules containing about 10 mg of Tetracycline Hydrochloride, add 20 ml of warm *ethanol (95 per cent)*, allow to stand for 20 minutes, filter and evaporate the filtrate to dryness on a water-bath. To 0.5 mg of the residue add *2 ml of sulphuric acid*; a reddish violet colour develops. Add the solution to 2.5 ml of *water*; the colour changes to yellow.

C. The residue obtained in test B gives reaction (A) of chlorides (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water* freshly prepared by distillation,
Speed and time. 75 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter through a membrane filter disc having an average pore diameter not greater than 1.0 µm, rejecting the first few ml of the filtrate. Measure the absorbance of the resulting solution, suitably diluted if necessary, at the maximum at about 276 nm (2.4.7). Calculate the content of $C_{22}H_{24}N_2O_8$, HCl in the medium from the absorbance obtained from a solution of known concentration of *tetracycline hydrochloride IPRS*.

Q. Not less than 70 per cent of the stated amount of $C_{22}H_{24}N_2O_8$, HCl.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the mixed contents of 20 capsules containing 25 mg of Tetracycline Hydrochloride with 80 ml of *0.01 M methanolic hydrochloric acid* for 10 minutes, dilute to 100 ml with the same solvent, mix and filter if necessary.

Reference solution (a). A 0.002 per cent w/v solution of *4-epitetracycline hydrochloride IPRS* in *0.01 M methanolic hydrochloric acid*.

Reference solution (b). A solution containing 0.0015 per cent w/v each of *4-epitetracycline hydrochloride IPRS* and *tetracycline hydrochloride IPRS* in *0.01 M methanolic hydrochloric acid*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- column. temperature 40°,
- mobile phase: a mixture of 5 volumes of *dimethylformamide* and 95 volumes of *0.1 M oxalic acid*, the pH of the mixture being adjusted to 3.9 with *triethylamine*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks in the chromatogram obtained with reference solution (b) is not less than 2.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to 4-epitetracycline hydrochloride is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) and the sum of areas of all the secondary peaks is not more than 10.0 per cent.

Loss on drying (2.4.19). Not more than 3.0 per cent, determined on 1.0 g of the contents of the capsules by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 3 hours.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the mixed contents of 20 capsules containing 50 mg of Tetracycline Hydrochloride, shake with about 50 ml of *0.01 M methanolic hydrochloric acid* and dilute with the same solvent to produce 100.0 ml, mix and filter. Discard the first few ml of the filtrate.

Reference solution (a). A 0.05 per cent w/v solution of *tetracycline hydrochloride IPRS* in *0.01 M methanolic hydrochloric acid*.

Reference solution (b). A solution containing 0.0025 per cent w/v of *4-epitetracycline hydrochloride IPRS* and 0.01 per

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cent w/v of *tetracycline hydrochloride IPRS* in *0.01 M methanolic hydrochloric acid*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 to10 µm),
- mobile phase: a mixture of 68 volumes of *0.1 M ammonium oxalate* and 27 volumes of *dimethylformamide* and 5 volumes of *0.2 M dibasic ammonium phosphate*, the pH of the mixture being adjusted, if necessary, to 7.6 to 7.7 with *3 M ammonia* or *3 M phosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks in the chromatogram obtained with reference solution (b) is not less than 1.5.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{22}H_{24}N_2O_8$, HCl in the capsules.

Storage. Store protected from light and moisture.

Tetracycline Ointment

Tetracycline Hydrochloride Eye Ointment

Tetracycline Ointment contains not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of tetracycline hydrochloride, $C_{22}H_{24}N_2O_8$, HCl.

Usual strengths: 0.5 per cent w/w; 1 per cent w/w.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Water (2.3.43). Not more than 0.5 per cent, determined on 2.0 g dissolved in a mixture of 2 volumes of *carbon tetrachloride*, 2 volumes of *chloroform* and 1 volume of *methanol*.

Other tests. Comply with the tests stated under Eye Ointments.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 68 volumes of *0.1 M ammonium oxalate* and 27 volumes of *dimethylformamide*.

Test solution. Weigh a quantity containing about 25 mg of Tetracycline Hydrochloride and transfer to a separating funnel with the aid of 15 ml of *cyclohexane*. Add 15 ml of solvent mixture and shake well. Collect the lower layer in a 50-ml volumetric flask. Repeat the extraction with two further quantities, each of 15 ml, of the solvent mixture, combining the extracts in the same 50-ml volumetric flask. Add sufficient solvent mixture to produce 50.0 ml, mix and filter.

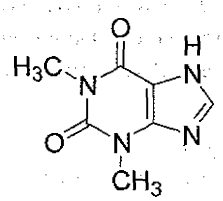
Reference solution. A 0.05 per cent w/v solution of *tetracycline hydrochloride IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 to10 µm),
- mobile phase: a mixture of 68 volumes of *0.1 M ammonium oxalate* and 27 volumes of *dimethylformamide* and 5 volumes of *0.2 M dibasic ammonium phosphate*, the pH of the mixture being adjusted, if necessary, to 7.6 to 7.7 with *3 M ammonia* or *3 M phosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Calculate the content of $C_{22}H_{24}N_2O_8$, HCl in the eye ointment.

Storage. Store protected from moisture.

Theophylline



$C_7H_8N_4O_2$ Mol. Wt. 180.2 (anhydrous)
$C_7H_8N_4O_2,H_2O$ Mol. Wt. 198.2 (hydrate)

Theophylline is 1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione. It contains one molecule of water or is anhydrous.

Theophylline contains not less than 98.0 per cent and not more than 101.0 per cent of $C_7H_8N_4O_2$, calculated on the dried basis.

Category. Xanthine bronchodilator.

Description. A white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and D may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *theophylline IPRS* or with the reference spectrum of theophylline.

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B. Dissolve about 10 mg in 10 ml of *water*, add 0.5 ml of a 5 per cent w/v solution of *mercuric acetate* and allow to stand; a white, crystalline precipitate is produced.

C. The melting range, after drying at 105°, 270° to 274° (2.4.21).

D. It gives the reaction of xanthines (2.3.1).

Tests

Appearance of solution. Dissolve 0.5 g in 75 ml of *carbon dioxide-free water* with heating; the resulting solution (solution A), is clear (2.4.1), and colourless (2.4.1).

Acidity. To 50 ml of solution A add 0.1 ml of *methyl red solution*. The solution is red and not more than 1.0 ml of *0.01 M sodium hydroxide* is required to change the colour of the solution to yellow.

Light absorption (2.4.7). Absorbance of a 0.001 per cent w/v solution in *0.1 M hydrochloric acid* at the maximum at about 270 nm, not less than 0.53.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 40 mg of the substance under examination in 20.0 ml of the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of *theobromine* in the mobile phase, add 5 ml of the test solution and dilute to 100 ml with the mobile phase. Dilute 5 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4 mm, packed with octadecylsilane bonded to porous silica (7 µm),
- mobile phase: a mixture of 7 volumes of *acetonitrile* and 93 volumes of 0.14 per cent w/v solution of *sodium acetate* containing 0.5 per cent v/v solution of *glacial acetic acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 272 nm,
- injection volume: 20 µl.

The relative retention time with reference to theophylline for theophylline impurity C is about 0.3, for theophylline impurity B is about 0.4, for theophylline impurity D is about 0.5 and for theophylline impurity A is about 2.5.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to theobromine and theophylline is not less than 2.0.

Inject reference solution (a) and the test solution. Run the chromatogram 3.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13).1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent (for the anhydrous form) and 8.0 per cent to 9.5 per cent (for the hydrated form), determined on 1.0 g by drying in an oven at 105°.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of substance under examination in 1 ml of *methanol* and 50 ml of *water*, with the aid of ultrasound and dilute to 100.0 ml with *water*. Dilute 5.0 ml of the solution to 50.0 ml with *water*.

Reference solution. A 0.01 per cent w/v solution of *theophylline IPRS* in *water*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 25 volumes of *methanol* and 75 volumes of *water*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of $C_7H_8N_4O_2$.

Storage. Store protected from moisture.

Theophylline Injection

Theophylline in Dextrose Injection

Theophylline Injection is a sterile solution of Theophylline and Dextrose in Water for Injections.

Theophylline Injection contains not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of anhydrous theophylline, $C_7H_8N_4O_2$, and not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of dextrose, $C_6H_{12}O_6, H_2O$.

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Usual strengths. Anhydrous theophylline, 0.4, 0.8, 1.6, 2, 3.2, 4 mg per ml in 5 per cent w/v Dextrose.

Identification

A. When examined in the range 230 nm to 360 nm (2.4.7), the solution obtained in the Assay for theophylline shows an absorption maximum at about 274 nm.

B. Add 0.2 ml of the injection to 5 ml of *potassium cupri-tartrate solution* and heat to boiling; a red to orange precipitate is formed.

Tests

pH (2.4.24). 3.5 to 6.5.

5-Hydroxymethylfurfural and Related substances. Use a glass chromatographic column (66 cm x 11 mm) with a sealed-in, coarse-porosity sintered disc or a glass wool plug and fitted with a stopcock. Mix 8 g of a 20- to 50-mesh styrenedivinylbenzene anion-exchange resin in the hydroxide form with 25 ml of *water*, allow to settle and decant the supernatant liquid until a slurry of resin remains. Pour the slurry into the column and allow to settle as a homogeneous bed having a bed volume of about 15 ml. Wash the resin bed at a flow rate of about 3 ml per minute with 100 ml of a 5.7 per cent w/v solution of *ammonium carbonate* followed by washing with *water* until the eluate has a pH of 7.

Dilute an measured volume of the injection containing 1.0 g of Dextrose, $C_6H_{12}O_6,H_2O$ to 250.0 ml with *water*. Pass this solution through the resin bed in the column at a flow rate of about 3.5 ml per minute, discarding the first 50 ml of the eluate. Measure the absorbance of the eluate at 284 nm, using *water* as the blank; the absorbance is not more than 0.25 (2.4.7).

Bacterial endotoxins (2.2.3). Not more than 1.0 Endotoxin Unit per mg of anhydrous theophylline.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *For theophylline* — Determine by liquid chromatography (2.4.14).

Test solution. Dilute a suitable volume of the injection with sufficient water to produce a solution containing 0.01 per cent w/v of anhydrous theophylline.

Reference solution. A 0.01 per cent w/v solution of *theophylline IPRS* in *water*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 25 volumes of *methanol* and 75 volumes of *water*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of $C_7H_8N_4O_2$, in the injection.

For dextrose — Transfer an measured volume of the injection containing about 5 g of Dextrose to a 100-ml volumetric flask, add 0.2 ml of *6 M ammonia*, dilute to volume with *water* and mix. Determine the optical rotation of the resulting solution in a suitable polarimeter tube at 25° (2.4.22). The observed rotation, in degrees multiplied by $1.0425A$, represents the weight, in g, of $C_6H_{12}O_6,H_2O$ in the volume of the injection taken for the assay, where A is the ratio of 200 divided by the length, in mm, of the polarimeter tube employed.

Storage. Store protected from light, in single dose containers.

Labelling. The label states the strength in terms of the amounts of anhydrous theophylline and Dextrose monohydrate.

Theophylline Prolonged-release Tablets

Theophylline Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Theophylline Prolonged-release Tablets contain Theophylline or Theophylline Hydrate.

Theophylline Prolonged-release Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of theophylline, $C_7H_8N_4O_2$.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Extract a quantity of the powdered tablets containing 0.2 g of Theophylline with 10 ml of a mixture of 60 volumes of *chloroform* and 40 volumes of *methanol*, filter and evaporate the filtrate to dryness. It gives the reaction of xanthenes (2.3.1).

Tests

Dissolution (2.5.2). Complies with the test stated under Tablets.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel F254*.

Mobile phase. A mixture of 10 volumes of *ammonia*, 30 volumes of *acetone*, 30 volumes of *chloroform* and 40 volumes of *butan-1-ol*.

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Solvent mixture. 60 volumes of *chloroform* and 40 volumes of *methanol*.

Test solution. Disperse a quantity of the powdered tablets containing 0.2 g of theophylline with 10 ml of the solvent mixture with the aid of ultrasound and centrifuge.

Reference solution. Dilute 1.0 ml of the test solution to 200.0 ml with the solvent mixture.

Apply 10 µl of each solution. After removal of the plate, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the principal spot in the chromatogram obtained with the reference solution (0.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.1 g of theophylline in 1 ml of *methanol* and 50 ml of *water* with the aid of ultrasound and dilute to 100.0 ml with *water*. Dilute 5.0 ml of the solution to 50.0 ml with *water*.

Reference solution. A 0.01 per cent w/v solution of *theophylline IPRS* in *water*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 25 volumes of *methanol* and 75 volumes of *water*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

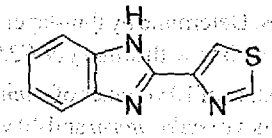
Inject the reference solution and the test solution.

Calculate the content of $C_7H_8N_4O_2$ in the tablets.

Labelling. When the active ingredient is Theophylline Hydrate the quantity is stated in terms of the equivalent amount of theophylline.

Thiabendazole

Tiabendazole



$C_{10}H_7N_3S$ Mol. Wt. 201.3

Thiabendazole is 2-(1,3-thiazol-4-yl)-1*H*-benzimidazole.

Thiabendazole contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{10}H_7N_3S$, calculated on the anhydrous basis.

Category. Anthelmintic.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *thiabendazole IPRS* or with the reference spectrum of thiabendazole.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.0005 per cent w/v solution in *0.1 M hydrochloric acid* shows absorption maxima at about 243 nm and 302 nm; ratio of the absorbance at the maximum at about 302 nm to that at about 243 nm, 1.8 to 2.1.

C. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (c).

D. Dissolve 5 mg in 5 ml of *0.1 M hydrochloric acid*, add 3 mg of *4-phenylenediamine dihydrochloride* and shake until dissolved. Add 0.1 g of *zinc powder*, mix, allow to stand for 2 minutes and add 10 ml of *ferric ammonium sulphate solution*; a deep blue or bluish violet colour is produced.

Tests

Appearance of solution. Add 5 ml of *methanol* to 0.5 g in a flask fitted with a ground-glass stopper, stir for 5 minutes, with a magnetic stirrer and filter through a sintered-glass filter (1.6 µm to 4 µm). The solution is not more intensely coloured than reference solution BS6 (2.4.1).

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Mobile phase. A mixture of 62.5 volumes of *toluene*, 25 volumes of *glacial acetic acid*, 10 volumes of *acetone* and 2.5 volumes of *water*.

Test solution (a). Dissolve 0.1 g of the substance under examination in 10.0 ml of *methanol*.

Test solution (b). Dilute 2.0 ml of test solution (a) to 20.0 ml with *methanol*.

Reference solution (a). Dilute 1.0 ml of test solution (b) to 10.0 ml with *methanol*.

Reference solution (b). Dilute 1.0 ml of test solution (b) to 25.0 ml with *methanol*.

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Reference solution (c). Dissolve 20 mg of *thiabendazole IPRS* in 20.0 ml of *methanol*.

Apply to the plate 20 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b)

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). Not more than 0.5 per cent, determined on 1.0 g.

Assay. Dissolve 0.15 g in 30 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02013 g of $C_{10}H_7N_3S$.

Storage. Store protected from light and moisture.

Thiabendazole Tablets

Tiabendazole Tablets

Thiabendazole Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of thiabendazole, $C_{10}H_7N_3S$.

Usual strength. 500 mg.

Identification

A. When examined in the range 230 nm to 360 nm (2.4.7), the final solution obtained in the Assay shows an absorption maximum at about 302 nm.

B. Dissolve a quantity of the powdered tablets containing 30 mg of Thiabendazole in 5 ml of *0.1 M hydrochloric acid*, add 3 mg of *4-phenylenediamine dihydrochloride* and shake until dissolved. Add 0.1 g of *zinc powder*, mix, allow to stand for 2 minutes and add 10 ml of *ferric ammonium sulphate solution*; a deep bluish violet colour is produced.

Tests

Other tests. Comply with the tests stated under Tablets.

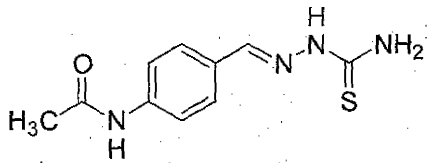
Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 0.1 g of Thiabendazole in 75 ml of *0.1 M hydrochloric acid*, warm on a water-bath for 15 minutes, shaking occasionally, cool, dilute to 100.0 ml with *0.1 M hydrochloric acid* and filter. Dilute 5.0 ml of the filtrate to 1000.0 ml with *0.1 M hydrochloric acid* and measure the absorbance of the resulting solution at the maximum at about 302 nm (2.4.7).

Calculate the content of $C_{10}H_7N_3S$ in the tablets taking 1230 as the specific absorbance at 302 nm.

Storage. Store protected from light and moisture.

Labelling. The label states that the tablets should be chewed before swallowing.

Thiacetazone



$C_{10}H_{12}N_4OS$ Mol. Wt. 236.3

Thiacetazone is 4-acetamidobenzaldehyde thiosemicarbazone.

Thiacetazone contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{10}H_{12}N_4OS$, calculated on the dried basis.

Category. Antitubercular.

Description. Pale yellow crystals or a crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *thiacetazone IPRS* or with the reference spectrum of thiacetazone.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.0003 per cent w/v solution in *ethanol (95 per cent)* shows an absorption maximum at about 328 nm absorbance at about 328 nm, about 0.55.

C. Boil 10 mg with 5 ml of *1 M hydrochloric acid* for 3 minutes, cool and add sufficient *water* to produce 200 ml. Mix 5 ml of the solution with 0.25 ml of *sodium nitrite solution* and add the mixture to 0.5 ml of *2-naphthol solution*; a red colour is produced.

Tests

Thiosemicarbazide. To 2.0 g, finely powdered, add sufficient *water* to produce 50 ml, shake, allow to stand for 1 hour with occasional shaking and filter, discarding the first few ml of the

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filtrate. Acidify 25 ml of the clear filtrate with *dilute sulphuric acid*, add 0.1 ml of *o-phenanthroline-ferrous complex solution* and titrate with *0.1 M ceric ammonium sulphate* to a blue end-point which persists for 1 minute; not more than 0.8 ml is required.

4-acetamidobenzalazine. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. Ethyl acetate.

Test solution. Dissolve 0.4 g of the substance under examination in 100 ml of *methanol*.

Reference solution. Dissolve with the aid of heat 0.016 g of *4-acetamidobenzalazine IPRS* in 150 ml of *methanol*, cool and dilute to 200 ml with *methanol* and further dilute 5 ml of the solution to 50 ml with *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air, spray with *2 M nitric acid* and within 2 minutes examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.1 g in 60 ml of *methanol* by heating at 60° in a water-bath, add slowly 20 ml of hot *methanolic silver nitrate solution*, maintain the solution at 60° until the precipitate coagulates and leaves a clear supernatant liquid. Cool, filter through a sintered-glass crucible (porosity No. 4), wash the residue with *methanol* until the washings are free from silver nitrate and dry to constant weight at 105°.

1 g of residue is equivalent to 0.4606 g of $C_{10}H_{12}N_4OS$.

Storage. Store protected from light and moisture.

Thiacetazone and Isoniazid Tablets

Thiacetazone and Isoniazid Tablets contain one part by weight of Thiacetazone and two parts by weight of Isoniazid.

Thiacetazone and Isoniazid Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amounts of thiacetazone, $C_{10}H_{12}N_4OS$, and isoniazid, $C_6H_7N_3O_2$.

Category. Antitubercular.

Usual strengths. Thiacetazone, 37.5 mg and Isoniazid, 75 mg; Thiacetazone, 75 mg and Isoniazid, 150 mg; Thiacetazone, 150 mg and Isoniazid, 300 mg.

Identification

A. Extract a quantity of the powdered tablets containing about 30 mg of Thiacetazone with 70 ml of *ethanol (95 per cent)* with the aid of heat for 15 minutes with occasional shaking. Cool, dilute to 100 ml with *ethanol (95 per cent)* and filter. Dilute 1 ml to 100 ml with *ethanol (95 per cent)*. The solution complies with the following test.

When examined in the range 230 nm to 360 nm (2.4.7), the solution shows an absorption maximum at about 328 nm, absorbance at about 328 nm is about 0.55.

B. Shake a quantity of the powdered tablets containing 0.1 g of isoniazid with 50 ml of water and filter. To 5 ml of filtrate, add 5 ml of *ethanol (95 per cent)*, 0.1 g of *borax* and 5 ml of a 5 per cent w/v solution of *1-chloro-2,4-dinitrobenzene* in *ethanol (95 per cent)*, evaporate to dryness on a water-bath and continue heating for a further 10 minutes. To the residue add 10 ml of *methanol* and mix; a reddish purple colour is produced.

Tests

Disintegration (2.5.1). Not more than 30 minutes.

Other tests. Comply with the tests stated under Tablets.

Assay. *For thiacetazone* — Weigh and powder 20 tablets. Disperse a quantity of the powder containing 30 mg of Thiacetazone in 70 ml of *ethanol (95 per cent)* and heat on a water-bath for 15 minutes with intermittent shaking. Cool, add sufficient *ethanol (95 per cent)* to produce 100.0 ml and filter. To 1.0 ml of the filtrate add sufficient *ethanol (95 per cent)* to produce 100.0 ml and measure the absorbance of the resulting solution at the maximum at about 328 nm (2.4.7), using *ethanol (95 per cent)* as the blank.

Calculate the content of $C_{10}H_{12}N_4OS$ from the absorbance obtained by repeating the operation using *thiacetazone IPRS* in place of the tablets under examination.

For isoniazid — Weigh a quantity of the powdered tablets containing 0.2 g of Isoniazid, dissolve as completely as possible in 100 ml of *water* and filter. Wash the residue with *water*, combine the filtrate and washings and dilute to 250.0 ml with *water*. To 50.0 ml of the resulting solution add 50 ml of water, 20 ml of *hydrochloric acid* and 0.2 g of *potassium bromide*. Titrate with *0.0167 M potassium bromate*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.0167 M potassium bromate* is equivalent to 0.003429 g of $C_6H_7N_3O_2$.

Storage. Store protected from light and moisture.

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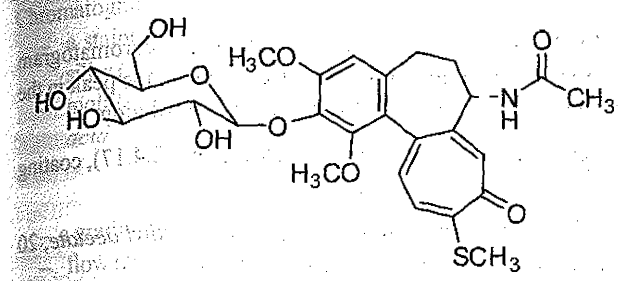
Thiocolchicoside



$C_{27}H_{33}NO_{10}S$ Mol. Wt. 563.5

Thiocolchicoside is 2-demethoxy-2-glucosidoxythiocolchicine.

Thiocolchicoside contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{27}H_{33}NO_{10}S$, calculated on the dried basis.

Description. A yellow crystalline powder.

Category. Muscle relaxant.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *thiocolchicoside IPRS* or with the reference spectrum of thiocolchicoside.

B. When examined in the range of 220 nm to 440 nm (2.4.7), a 0.0015 per cent w/v solution in *water* shows absorption maxima at the same wavelength as obtained with the reference solution.

Tests

pH (2.4.24). 6.0 to 7.5, determined in a 0.5 per cent w/v solution in *carbon-dioxide free water*.

Specific optical rotation (2.4.22). – 580° to – 550° at 23°, determined on 0.5 per cent w/v solution in *water*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 50 mg of the substance under examination in 50.0 ml of *methanol*.

Reference solution (a). A 0.05 per cent w/v solution of *colchicoside IPRS* in *methanol*.

Reference solution (b). A 0.05 per cent w/v solution of *thiocolchicoside IPRS* in *methanol*.

Reference solution (c). Dilute 1.0 ml each of reference solution (a) and (b) to 100.0 ml with *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with silica (5 µm),
- mobile phase: A. *n- heptane* containing *2 per cent acetic acid*,
  B. *chloroform*,
  C. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 360 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)	Mobile phase C (per cent v/v)
0	86	10	4
50	0	65	35
51	86	10	4
60	86	10	4

Inject reference solution (c). The test is not valid unless the theoretical plates is not less than 20000, the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent for the peak due to thiocolchicoside.

The relative retention time with reference to thiocolchicoside for colchicine is about 0.55, for N- deacetyl N- formyl thiocolchicoside is about 1.05 and for colchicoside is about 1.10.

Inject reference solution (c) and test solution. In the chromatogram obtained with the test solution, the area of each peak corresponding to N-deacetyl N-formyl thiocolchicoside and colchicoside is not more than the area of thiocolchicoside and colchicoside peak, respectively in the chromatogram obtained with reference solution (c) (0.5 per cent) and the sum of the areas of all other secondary peaks is not more than the area of peak due to thiocolchicoside in the chromatogram obtained with reference solution (c) (0.5 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 4.0 per cent, determined on 1.0 g by drying in oven at 105° for 3 hours.

Microbial contamination (2.2.9). Total microbial count not more than 1000 CFU per g, total yeast and mould count not more than 100 CFU per g, 1 g is free from *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacteriaceae* and 10 g is free from *Salmonella* and *Shigella*.

Thiocolchicoside intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

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Bacterial endotoxins (2.2.3). Not more than 87.5 Endotoxin Unit per mg.

Thiocolchicoside intended for use in the manufacture of parenteral preparations without a further appropriate sterilisation procedure complies with the following additional requirement.

Sterility (2.2.11). Complies with the test for sterility.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of substance under examination in 100.0 ml of *water*. Dilute 10.0 ml of the solution to 100.0 ml with *water*.

Reference solution. A 0.1 per cent w/v solution of *thiocolchicoside IPRS* in *water*. Dilute 10.0 ml of the solution to 100.0 ml with *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: A. *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- spectrophotometer set at 370 nm,
- injection volume: 20 µl.

Time (in min.)	Flow (ml/min.)	Mobile phase A (per cent /v/v)	Mobile phase B (per cent v/v)
0	1.0	90	10
10	1.0	90	10
11	1.0	75	25
12	1.8	75	25
15	1.8	75	25
17	1.8	70	30
18	1.8	70	30
20	1.8	90	10
22	1.0	90	10

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate content of $C_{27}H_{33}NO_{10}S$.

Storage: Store protected from light and at a temperature not exceeding 30°.

Thiocolchicoside Capsules

Thiocolchicoside Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of thiocolchicoside, $C_{27}H_{33}NO_{10}S$.

Usual strengths. 4 mg; 8 mg.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *ethyl acetate*, 20 volumes of *ethanol* and 10 volumes of *water*.

Test solution. Dissolve 50 mg of the substance under examination in 5.0 ml of *methanol*.

Reference solution. A 1.0 per cent w/v solution of *thiocolchicoside IPRS* in *methanol*.

Apply to the plate 10 µl of each solution. After development, dry the plate in a current of air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *phosphate buffer pH 7.5*,
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.044 per cent w/v solution of *thiocolchicoside IPRS*. Dilute 1.0 ml of the solution to 100.0 ml with the dissolution medium.

0.001 per cent w/v solution of *thiocolchicoside IPRS* in *water*.

Use the chromatographic condition as described under Assay.

Calculate the content of $C_{27}H_{33}NO_{10}S$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{27}H_{33}NO_{10}S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse the content of the capsules containing about 50 mg of Thiocolchicoside in 50.0 ml of *methanol*.

Reference solution (a). A 0.05 per cent w/v solution of *colchicoside IPRS* in *methanol*.

Reference solution (b). A 0.05 per cent w/v solution of *thiocolchicoside IPRS* in *methanol*.

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Reference solution (c). Dilute 1.0 ml each of reference solution (a) and (b) to 100.0 ml with *methanol.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with silica (5 µm),
- mobile phase: A. *n- heptane* containing 2 per cent *acetic acid,*
  B. *chloroform,*
  C. *methanol,*
- a gradient programme using the conditions given below,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 360 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)	Mobile phase C (per cent v/v)
0	86	10	4
50	0	65	35
51	86	10	4
60	86	10	4

The relative retention time with reference to thiocolchicoside for colchicine is about 0.55, for N-deacetyl N-formyl thiocolchicoside is about 1.05 and for colchicoside is 1.10.

Inject reference solution (c). The test is not valid unless the column efficiency is not less than 20000 theoretical plates, the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent for the peak due to thiocolchicoside.

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution the area of each peak corresponding to N-deacetyl N-formyl thiocolchicoside and colchicoside is not more than the area of corresponding peak in the chromatogram obtained with reference solution (c) (0.5 per cent) and the sum of the areas of all other secondary peaks is not more than the area of peak due to thiocolchicoside in the chromatogram obtained with reference solution (c) (0.5 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the content of 20 capsules containing about 10 mg of Thocolchicoside with 100.0 ml of *water.*

Reference solution. A 0.01 per cent w/v solution of *thiocolchicoside IPRS* in *water.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: A. *water,*
  B. *acetonitrile,*
- a gradient programme using the conditions given below,
- spectrophotometer set at 370 nm,
- injection volume: 20 µl.

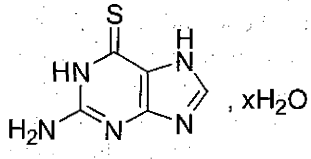
Time (in min.)	Flow (ml/min.)	Mobile phase A (per cent /v/v)	Mobile phase B (per cent v/v)
0	1.0	90	10
10	1.0	90	10
11	1.0	75	25
12	1.8	75	25
15	1.8	75	25
17	1.8	70	30
18	1.8	70	30
20	1.8	90	10
22	1.0	90	10

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{33}NO_{10}S$ in the capsules.

Thioguanine



$C_5H_5N_5S$ (anhydrous) Mol.Wt. 167.2

$C_5H_5N_5S.\ ½\ H_2O$ Mol.Wt. 176.2

Thioguanine is 6H -Purine-6-thione, 2-amino-1,7-dihydro; 2-Aminopurine-6(1H)-thione hemihydrate.

Thioguanine is anhydrous or contains one-half molecule of water of hydration. It contains not less than 96.0 per cent and not more than 100.5 per cent of $C_5H_5N_5S$, calculated on the dried basis.

CAUTION — Thioguanine is potentially cytotoxic; Great care should be taken in handling the powder and preparing the solutions.

Category. Cytotoxic.

Description. A pale yellow, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *thioguanine IPRS* or with the reference spectrum of thioguanine.

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B. Transfer about 100 mg of of the substance under examination, previously dried, to a 100.0 ml volumetric flask. Dissolve in a mixture of 15 ml of *water* and 1.5 ml of *1 M sodium hydroxide*, dilute with *water* to volume, and mix. Transfer 1.0 ml of the solution to a 200.0-ml volumetric flask and add 1 per cent v/v solution of *hydrochloric acid* to volume, and mix. The UV absorption spectrum (2.4.7) of the solution so obtained, exhibits maxima and minima at the same wavelengths as that obtained with *thioguanine IPRS* treated in the same manner.

Tests

Sulphur compounds. Dissolve 50 mg of thioguanine in 5.0 ml of *1 M sodium hydroxide*, the resulting solution obtained is clear.

Inorganic phosphate. Not more than 0.03 per cent as phosphate, determined by the following method.

Ammonium molybdate solution. Dissolve 8.3 g of *ammonium molybdate* in 40.0 ml of *water*, add 33.0 ml of *dilute sulphuric acid* and dilute with *water* to 100.0 ml.

NOTE— This solution is stable for about 2 weeks.

Weigh 50.0 mg of the substance under examination to a large test tube, add 1.0 ml of *dilute sulphuric acid* and heat in a water bath for 5 minutes. Cautiously add *nitric acid*, dropwise, continue heating until the mixture becomes colourless, and then heat for further one minute. Cool, dilute with *water* to 10 ml, and transfer the solution to a 25.0 ml volumetric flask with the aid of a few ml of *water*. To the flask add 0.75 ml of ammonium molybdate solution and 1.0 ml of *aminonaphthol-sulphonic acid*, dilute with *water* to volume, mix and measure the absorbance of the resulting solution at the maximum at about 620 nm (2.4.7), using a blank solution prepared in the same manner but omitting the substance under examination. The absorbance is not greater than that produced by repeating the operation using a solution prepared in the same manner using 1.5 ml of *phosphate standard solution (10 ppm PO_4)*.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution (a). *0.04* per cent solution of the substance under examination in *0.1 M sodium hydroxide*.

Test solution (b). Dilute 1.0 volume of test solution (a) to 10.0 volumes with mobile phase.

Reference solution (a). A 0.004 per cent w/v solution of *guanine IPRS* in *0.01 M sodium hydroxide*.

Reference solution (b). Dilute 1.0 volume of reference solution (a) to 100.0 volumes with mobile phase.

Reference solution (c). Transfer 1.0 ml of reference solution (a) into a 100.0 ml volumetric flask, and dilute with test solution (b) to volume.

The relative retention times are about 0.6 for guanine and 1.0 for thioguanine respectively.

Inject reference solution (b) and (c). The test is not valid unless the resolution between the peaks due to guanine and thioguanine is not less than 3.0, in the chromatogram obtained with reference solution (c). In the chromatogram obtained with reference solution (b), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject reference solution (b) and test solution (b). In the chromatogram obtained with test solution (b) the area of any peak due to guanine is not more than 2.5 times the area of principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent).

Selenium. Not more than 30 ppm, determine by the oxygen flask method (2.3.34),

Test Solution. Burn 200 mg of the substance under examination using a 1000 ml combustion flask and using 25.0 ml of *dilute nitric acid* as the absorbing liquid, proceed as described under *oxygen flask combustion* (2.3.34). After completion of the combustion, add a few ml of *water* in the cup, loose the stopper, and rinse the stopper, and the sides of the flask with about 10.0 ml of *water*. Transfer the solution with the aid of about 20.0 ml of *water* to a 150 ml beaker, and heat gently to the boiling temperature. Boil for 10 minutes, and allow the solution to cool to room temperature.

Reference solution (a). Dissolve 40 mg of selenium in 100.0 ml of 50 per cent v/v solution of *nitric acid* in a 1000 ml volumetric flask, heat on a steam-bath, if necessary and make up the volume with *water*. Dilute 5.0 ml of the solution to 200.0 ml with *water*.

Reference solution (b). Dissolve 100 mg of *2,3-diamino-napthalene* and 500 mg of *hydroxylamine hydrochloride* in 100.0 ml of *0.1 M hydrochloric acid*.

Reference solution (c). Dilute 6.0 ml of reference solution (a) with 25.0 ml *dilute nitric acid* and 25.0 ml of *water*.

Treat the reference solution (c), the test solution, and the reagent blank consisting of 25.0 ml of *dilute nitric acid* and 25.0 ml of *water*, concomitantly, as follows. Add *ammonium hydroxide solution (0.5 per cent, v/v)* to adjusted to pH 2.0. Dilute with *water* to 60.0 ml and transfer to a low-actinic separator with the aid of 10.0 ml of *water*, adding the 10 ml of rinsings to the separator. Add 200 mg of *hydroxylamine hydrochloride*, swirl to dissolve, immediately add 5.0 ml of reference solution (b), insert the stopper, and swirl to mix. Allow the solution to stand at room temperature for 100 minutes. Add 5.0 ml of *cyclohexane*, shake vigorously for 2 minutes, and allow the layers to separate. Discard the aqueous layer, and centrifuge the cyclohexane extract to remove any dispersed *water*. Determine the absorbances of the

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cyclohexane extracts of the test solution and the reference solution (c) in a 1-cm cell at the wavelength of maximum absorbance at about 380 nm, with a suitable spectrophotometer, using the cyclohexane extract as the blank, and compare the absorbances, the absorbance of the test solution is not more than that of the reference solution (c)

Nitrogen (2.3.30). 40.2 to 43.1 per cent, determined on 100 mg using Method E.

1 ml of *0.05 M sulphuric acid* is equivalent to 0.001401 g of N.

Loss on drying (2.4.19). Not more than 6.0 per cent, determined on 1.0 g by drying in an oven at 105° for 5 hours.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with following modifications.

Solvent mixture. A mixture of 1 volume of *orthophosphoric acid* and 99 volumes of *water*.

Test solution (a). 0.04 per cent w/v solution of the substance under examination in *0.01 M sodium hydroxide*.

Test solution (b). Dilute 1.0 volume of test solution (a) to 10.0 volumes with solvent mixture.

Reference solution (a). A 0.04 per cent solution of *thioguanine IPRS* in *0.01 M sodium hydroxide*.

Reference solution (b). Dilute 1.0 volume of reference solution (a) to 10.0 volumes with solvent mixture.

Chromatographic system
- a stainless steel column 5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A buffer solution of *0.05 M monobasic sodium phosphate* adjusted to pH 3.0 with *orthophosphoric acid*.
- flow rate: 2 ml per minute,
- spectrophotometer set at 248 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (b) and test solution (b).

Calculate the content of $C_5H_5N_5S$.

Storage. Store protected from moisture.

Labelling. Label it to indicate its state of hydration.

Thioguanine Tablets

Thioguanine Tablets contain not less than 93.0 per cent and not more than 107.0 per cent of the stated amount of thioguanine, $C_5H_5N_5S$,.

CAUTION — Thioguanine is potentially cytotoxic; Great care should be taken in handling the powder and preparing the solutions.

Usual strength. 40 mg.

Identification

A. Shake a portion of the powdered tablets containing 0.5 g of thioguanine with 10 ml of *1 M sodium hydroxide*, and filter. Acidify the filtrate with *hydrochloric acid*, filter, and dissolve the precipitate in 25 per cent v/v of *ammonia water*. Evaporate to dryness, and dry the residue at 105° at a pressure not exceeding 0.1 psi for 5 hour. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *thioguanine IPRS* or with the reference spectrum of thioguanine.

B. In the Assay, the principal peaks in the chromatogram obtained with test solution corresponds to the peak in the chromatogram obtained with reference solution (b).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter, discarding about 10.0 ml of the filtrate.

Test solution. Transfer 2 ml of the filtrate and 2 ml of *1M hydrochloric acid* to a 20-ml volumetric flask, and dilute with *water* to volume.

Reference solution. A solution of *thioguanine IPRS* in *0.1M hydrochloric acid* suitably diluted with the same solvent to obtain a solution having same concentration as that of the test solution.

Measure the absorbance of the test solution at about 348 nm (2.4.7), using *0.1 M hydrochloric acid* as the blank. Calculate the content of $C_5H_5N_5S$ from the absorbance obtained from the reference solution.

Q. Not less than 75 per cent of the stated amount of $C_5H_5N_5S$.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Weigh and powder 20 tablets, weigh a quantity of the powder containing 40 mg of Thioguanine with 10.0 ml of *0.01 M sodium hydroxide* and sonicate for 5 minutes, add sufficient mobile phase to produce 100.0 ml, mix and filter.

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Reference solution (a) A 0.04 per cent w/v solution each of *thioguanine IPRS* and *guanine IPRS* in 10 per cent v/v solution of *orthophosphoric acid*. Sonicate to dissolve.

Reference solution (b) Dilute 1.0 ml of reference solution (a) to 10.0 ml with mobile phase.

Reference solution (c). A 0.0008 per cent w/v solution of *thioguanine IPRS* and 0.016 per cent w/v solution of *guanine IPRS* in *0.01 M sodium hydroxide*.

Reference solution (d) Dilute 1.0 ml of reference solution (c) to 10.0 ml with mobile phase.

Inject reference solution (b). The test is not valid unless the resolution between thioguanine and guanine peaks is not less than 3.0.

Inject reference solution (d) and the test solution. In the chromatogram obtained with test solution the area of any peak due to guanine is not more than the area of principal peak in the chromatogram obtained with reference solution (d) (4.0 per cent), and the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 22.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (4.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets, weigh a quantity of the powder containing 40 mg of Thioguanine with 100.0 ml of *0.01 M sodium hydroxide solution* and shake for 15 minutes. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). 0.04 per cent w/v of *thioguanine IPRS* in *0.01 M sodium hydroxide*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml in mobile phase.

Chromatographic system

- a stainless steel column 5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a buffer solution prepared by dissolving 6.0 g of *anhydrous monobasic sodium phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 248 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the tailing factor is not more than 2.0 and relative standard deviation for replicate injections is not more than 2.0 per cent.

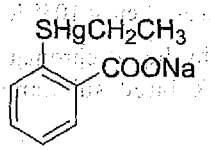
Inject reference solution (b) and the test solution.

Calculate the content of $C_5H_5N_5S$ in the tablets.

Storage. Store protected from moisture, at a temperature between 15° to 25°.

Thiomersal

Thimerosal



$C_9H_9HgNaO_2S$ Mol. Wt. 404.8

Thiomersal is the sodium salt of [(2-carboxyphenyl)thio] ethylmercury.

Thiomersal contains not less than 97.0 per cent and not more than 101.0 per cent of $C_9H_9HgNaO_2S$, calculated on the dried basis.

Category. Antiseptic; pharmaceutical aid (antimicrobial preservative).

Description. A light cream, crystalline powder.

Identification

A. Dissolve 0.1 g in 10 ml of *water* and add 2 ml of *silver nitrate solution*; a pale yellow precipitate is produced.

B. Dissolve 0.5 g in 10 ml of *water* and add 2 ml of *2 M hydrochloric acid*; a white precipitate is produced which, after washing with *water* and drying over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa, melts at about 110° (2.4.21).

Tests

pH (2.4.24). 6.0 to 8.0, determined in a 1.0 per cent w/v solution.

Inorganic mercury compounds. Not more than 0.7 per cent, determined by the following method.

NOTE — Protect the solutions from light.

Solution A. A 33.2 per cent w/v solution of *potassium iodide*.

Test solution. Dissolve 25 mg of the substance under examination in *water* and dilute to 25.0 ml with *water*.

Reference solution. A 0.0095 per cent w/v solution of *mercuric chloride* in *water*.

Label five 10-ml volumetric flasks A, B, C, D and E. Place 5 ml of the test solution in flasks A, B, C and D. To each of flasks C

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and D add 0.5 ml of the reference solution. Dilute the contents of flasks A and C to 10.0 ml with *water* (blank preparation A and C). Dilute the contents of flasks B and D to 10 ml with a freshly prepared solution A (test preparation B and reference preparation D). Place 5 ml of solution A in flask E. Dilute to 10 ml with *water* (blank preparation E). Measure the absorbance of each solution (A_a, A_b, A_c, A_d, A_e) at 323 nm (2.4.7), using *water* as the blank.

Calculate the content of inorganic mercury compounds, expressed as Hg, in the substance under examination from the expression

$$\frac{(A_b - A_a - A_e) \times m_R \times 0.1847}{(A_d - A_c - A_b + A_a) \times m_T}$$

Where, m_R = mass of mercuric chloride in the reference solution in milligrams,

m_T = mass of the substance under examination in milligrams.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying over *phosphorus pentoxide* at a pressure not exceeding 0.7 kPa for 24 hours.

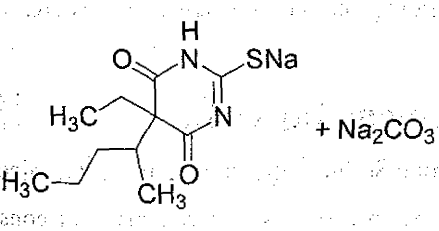
Assay. Weigh 0.5 g, transfer to a 100-ml long-necked flask, add 5 ml of *sulphuric acid* and heat gently until charring occurs; continue to heat and add *hydrogen peroxide solution (100 vol)* dropwise, until the mixture is colourless. Dilute with *water*, evaporate until slight fuming occurs, dilute to 10 ml with *water*, cool and titrate with *0.1 M ammonium thiocyanate* using *ferric ammonium sulphate solution* as indicator.

1 ml of *0.1 M ammonium thiocyanate* is equivalent to 0.02024 g of $C_9H_9HgNaO_2S$.

Storage. Store protected from light and moisture.

Thiopentone Sodium

Thiopental Sodium



$C_{11}H_{17}N_2NaO_2S$ Mol. Wt. 264.3

Thiopentone Sodium is a mixture of sodium (*RS*)-5-ethyl-5-(1-methylbutyl)-2-thiobarbiturate and anhydrous sodium carbonate.

Thiopentone Sodium contains not less than 84.0 per cent and not more than 87.0 per cent of $C_{11}H_{18}N_2O_2S$ and not less than 10.2 per cent and not more than 11.2 per cent of Na, both calculated on the dried basis.

Category. General anaesthetic.

Description. A yellowish white powder; hygroscopic.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests B and D may be omitted if tests A, C and E are carried out.

A. Acidify 10 ml of a 10 per cent w/v solution in *carbon dioxide-free water* with *2 M hydrochloric acid*; the solution effervesces. Shake the solution with 20 ml of *ether*, separate the ether layer, wash with 10 ml of *water* and dry over *anhydrous sodium sulphate*. Filter, evaporate the filtrate to dryness and dry the residue at 105°.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *thiopentone IPRS* or with the reference spectrum of thiopentone.

B. Complies with the test for identification of barbiturates (2.3.2), but using the following solutions.

Test solution. A 0.1 per cent w/v solution of the substance under examination in *water*.

Reference solution. Dissolve 85 mg of *thiopentone IPRS* in 10 ml of *2 M sodium hydroxide* and dilute to 100 ml with *water*.

C. Acidify 5 ml of a 5 per cent w/v solution with *dilute acetic acid* and filter. Wash the precipitate with *water*, recrystallise from *water* and dry at 70°; the crystals melt at about 160° (2.4.21).

D. Dissolve 1 mg of the crystals obtained in test A in 1 ml of *0.1 M sodium hydroxide*. Add about 1 mg of *sodium nitroprusside* and, after 15 minutes, 1 ml of *dilute hydrochloric acid*; a reddish violet colour is produced.

E. It gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution in *carbon dioxide-free water* (solution A) is clear (2.4.1), and not more intensely coloured than reference solution GYS3 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14)

Test solution. Dissolve 20.0 mg of the substance under examination in the mobile phase and dilute to 20.0 ml with the mobile phase.

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Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 10.0 ml with the mobile phase

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with end-capped octadecylsilane bonded to porous silica (5 µm)
- mobile phase: a mixture of 65 volumes of 0.1 per cent w/v solution of *phosphoric acid* and 35 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 10 µl.

Allow the chromatography to proceed for twice the retention time of thiopental.

Name	Relative retention time	Correction factor
Thiopental impurity A[1]	0.3	—
Thiopental impurity B[2]	0.4	1.5
Thiopental impurity C[3]	0.9	—
Thiopental (retention time about: 20 minutes)	1.0	—
Thiopental impurity D[4]	1.3	—

[1]5-[(1*RS*)-1-methylbutyl]-2-thioxo-2,3-dihydropyrimidine-4,6(1*H*,5*H*)-dione,

[2]5-ethyl-5-[(1*RS*)-1-methylbutyl]pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione,

[3]5-ethyl-5-(1-ethylpropyl)-2-thioxo-2,3-dihydropyrimidine-4,6(1*H*,5*H*)-dione,

[4]mixture of (2*RS*,3*RS*)-2-(carbamothioylcarbamoyl)-2-ethyl-3-methylhexanoic acid and (2*RS*,3*SR*)-2-(carbamothioylcarbamoyl)-2-ethyl-3-methylhexanoic acid.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with test solution the area of any peak corresponding to thiopental impurity C is not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (3.0 per cent), the area of any peak corresponding to thiopental impurity B is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (1.0 per cent), the area of any peak corresponding to thiopental impurity D is not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (0.3 per cent), the area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (0.1 per cent), the sum of the areas of any secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (5.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (0.05 per cent).

Chlorides (2.3.12). To 10 ml of solution A add 30 ml of *water* and 10 ml of *2 M nitric acid*. Shake successively with three quantities, each of 25 ml, of *ether*, discard the ether layers and heat the aqueous solution on water-bath to remove any residual ether. 30 ml of the aqueous layer complies with the limit test for chlorides (330 ppm).

Loss on drying (2.4.19). Not more than 2.5 per cent, determined on 0.5 g by drying in an oven at 100° at a pressure of 1.5 to 2.5 kPa for 4 hours.

Assay. *For thiopentone* — Weigh 0.15 g, dissolve in 5 ml of water, add 2 ml of *1 M sulphuric acid* and extract with four quantities, each of 10 ml, of *chloroform*. Filter the combined chloroform extracts, evaporate the filtrate to dryness on a water-bath and dissolve the residue in 30 ml of *dimethylformamide*, previously neutralised with *0.1 M lithium methoxide*. Titrate immediately with *0.1 M lithium methoxide*, using 1 drop of a 0.2 per cent w/v solution of *thymol blue* in *methanol* as indicator, until a blue colour is obtained. Protect the solution from absorption of carbon dioxide during the titration.

1 ml of *0.1 M lithium methoxide* is equivalent to 0.02423 g of $C_{11}H_{18}N_2O_2S$.

For sodium — Weigh 0.4 g, dissolve in 30 ml of *water*, add 1 drop of *methyl red solution* and titrate with *0.05 M sulphuric acid* until the yellow colour changes to red. Boil gently for 2 minutes, cool and, if necessary, continue the titration with *0.05 M sulphuric acid* until the red colour is restored.

1 ml of *0.05 M sulphuric acid* is equivalent to 0.002299 g of Na.

Thiopentone sodium intended for use in the manufacture of parenteral preparations without a further appropriate sterilization procedure complies with the following additional requirement.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store protected from light and moisture.

Thiopentone Injection

Thiopentone Sodium Injection; Thiopental Injection

Thiopentone Injection is a sterile material consisting of Thiopentone Sodium with or without auxiliary agents. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

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The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Thiopentone Injection contains thiopentone, $C_{11}H_{18}N_2O_2S$ that is not less than 77.0 per cent and not more than 92.0 per cent and sodium, Na that is not less than 9.4 per cent and not more than 11.8 per cent of the stated amount of thiopentone sodium.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Usual strengths. 250 mg; 500 mg; 1 g; 5 g.

Description. A yellowish white powder; hygroscopic.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests B and D may be omitted if tests A, C and E are carried out.

A. Acidify 10 ml of a 10 per cent w/v solution in *carbon dioxide-free water* with *2 M hydrochloric acid*; the solution effervesces. Shake the solution with 20 ml of *ether*, separate the ether layer, wash with 10 ml of *water* and dry over *anhydrous sodium sulphate*. Filter, evaporate the filtrate to dryness and dry the residue at 105°.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *thiopentone IPRS* or with the reference spectrum of thiopentone.

B. Complies with the test for identification of barbiturates (2.3.2), but using the following solutions.

Test solution. 0.1 per cent w/v solution of the substance under examination in *water*.

Reference solution. Dissolve 85 mg of *thiopentone IPRS* in 10 ml of *2 M sodium hydroxide* and dilute to 100 ml with *water*.

C. Acidify 5 ml of a 5 per cent w/v solution with *dilute acetic acid* and filter. Wash the precipitate with *water*, recrystallise from *water* and dry at 70°; the crystals melt at about 160° (2.4.21).

D. Dissolve 1 mg of the crystals obtained in test A in 1 ml of *0.1 M sodium hydroxide*. Add about 1 mg of *sodium nitroprusside* and, after 15 minutes, 1 ml of *dilute hydrochloric acid*; a reddish violet colour is produced.

E. It gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution in *carbon dioxide-free water* (solution A) is clear (2.4.1), and not more intensely coloured than reference solution GYS3 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve a quantity of the contents of a sealed container containing 0.1 g of thiopental sodium in 80 ml of the mobile phase, add sufficient mobile phase to produce 100.0 ml filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 5.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 2.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS 3).
- mobile phase: a mixture of 65 volumes of *0.02 M orthophosphoric acid* and 35 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 10 µl.

Allow the chromatography to proceed for twice the retention time of thiopental.

Name	Relative retention time	Correction factor
Thiopental impurity A[1]	0.3	—
Thiopental impurity B[2]	0.4	1.6
Thiopental impurity C[3]	0.9	—
Thiopental (retention time: about 19 minutes)	1.0	—
Thiopental impurity D[4]	1.3	—

[1]5-[(1*RS*)-1-methylbutyl]-2-thioxo-2,3-dihydropyrimidine-4,6(1*H*,5*H*)-dione,

[2]5-ethyl-5-[(1*RS*)-1-methylbutyl]pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione,

[3]5-ethyl-5-(1-ethylpropyl)-2-thioxo-2,3-dihydropyrimidine-4,6(1*H*,5*H*)-dione,

[4]mixture of (2*RS*,3*RS*)-2-(carbamothioylcarbamoyl)-2-ethyl-3-methylhexanoic acid and (2*RS*,3*SR*)-2-(carbamothioylcarbamoyl)-2-ethyl-3-methylhexanoic acid.

Inject reference solution (b). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

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Inject reference solution (a), (b) , (c) and the test solution. In the chromatogram obtained with test solution the area of any peak corresponding to thiopental impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent), the area of any peak corresponding to thiopental impurity B is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent), the area of any peak corresponding to thiopental impurity C is not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent), the area of any peak corresponding to thiopental impurity D is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent), the sum of the areas of any secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (5.0 per cent). Disregard any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Loss on drying (2.4.19). Not more than 2.5 per cent, determined on 0.5 g by drying in an oven at 100° at a pressure of 1.5 to 2.5 kPa for 4 hours.

Bacterial endotoxins (2.2.3). Not more than 1.0 Endotoxin Unit per mg of thiopentone sodium.

Assay. *For thiopentone* — Mixed the contents of 10 containers and Weigh 0.15 g, of the contents, dissolve in 5 ml of *water*, add 2 ml of *1 M sulphuric acid* and extract with four quantities, each of 10 ml, of *chloroform*. Filter the combined chloroform extracts, evaporate the filtrate to dryness on a water-bath and dissolve the residue in 30 ml of *dimethylformamide*, previously neutralised with *0.1 M lithium methoxide*. Titrate immediately with *0.1 M lithium methoxide*, using 1 drop of a 0.2 per cent w/v solution of *thymol blue* in *methanol* as indicator, until a blue colour is obtained. Protect the solution from absorption of carbon dioxide during the titration.

1 ml of *0.1 M lithium methoxide* is equivalent to 0.02423 g of $C_{11}H_{18}N_2O_2S$.

For sodium — Mixed the contents of 10 containers and Weigh 0.4 g of the contents, dissolve in 30 ml of *water*, add 1 drop of *methyl red solution* and titrate with *0.05 M sulphuric acid* until the yellow colour changes to red. Boil gently for 2 minutes, cool and, if necessary, continue the titration with *0.05 M sulphuric acid* until the red colour is restored

1 ml of *0.05 M sulphuric acid* is equivalent to 0.002299 g of Na.

Storage. Store in single dose containers.

Labelling. The label states the amount of active ingredient in terms of Thiopentone Sodium.

Thiotepa



$C_6H_{12}N_3PS$ Mol. Wt. 189.2

Thiotepa is tris(1-aziridinyl)phosphine sulphide.

Thiotepa contains not less than 97.0 per cent and not more than 102.0 per cent of $C_6H_{12}N_3PS$, calculated on the anhydrous basis.

Category. Anticancer.

Description. Fine, white, crystalline flakes.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *thiotepa IPRS* or with the reference spectrum of thiotepa.

B. Determine on 20 mg by the oxygen-flask method (2.3.34), using 5 ml of *1.25 M sodium hydroxide* as the absorbing liquid. When the process is complete, dilute to 25 ml with *water* (solution A). To 5 ml of solution A add 0.1 ml of *hydrogen peroxide solution (100 vol)* and 1 ml of *1 M hydrochloric acid*, mix and add 0.05 ml of *barium chloride solution*; a turbidity is produced.

C. To 2 ml of solution A add 40 ml of *water* and 4 ml of *ammonium molybdate-sulphuric acid solution*, mix, add 0.1 g of *L-ascorbic acid* and boil for 1 minute; a blue colour is produced.

Tests

Appearance of solution. A 2.0 per cent w/v solution is clear (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.35 g of the substance under examination 100.0 ml of the *water*.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *water*. Further dilute 1.0 ml to 10.0 ml with *water*.

Reference solution (b). Dissolve 10 mg of the substance under examination in 2 ml of *methanol* in a ground glass-stoppered tube, add 50 µl of a 0.1 per cent v/v solution of *orthophosphoric acid*, stopper the tube and heat in a water

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bath at 65° for 50 seconds (generation of methoxythiotepa). Allow the solution to cool and add 1 ml of *methanol*.

Reference solution (c). Dissolve 15 mg of the substance under examination in 10 ml of *water*, add 1 g of *sodium chloride*, boil in a water bath for 10 minutes and cool (generation of chloro- adduct).

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm) (Such as Nucleosil C18),
- mobile phase: a mixture of 15 volumes of *acetonitrile* and 85 volumes of *0.1 M phosphate buffer pH 7.0*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject reference solution (b) and (c). The test is not valid unless the resolution between the two principal peaks is not less than 3. The relative retention time with reference to thiotepa for methoxythiotepa is about 1.3 and for chloro-adduct (thiotepa impurity A) is about 3.75.

Inject reference solution (a) and the test solution. Run the chromatogram 4 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to the chloro-adduct is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent). The area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent).

Water (2.3.43). Not more than 2.0 per cent, determined on 1.2 g.

Assay. Weigh 0.2 g into a stoppered flask, add 50 ml of a 20 per cent w/v solution of *sodium thiosulphate* and titrate immediately with *0.1 M hydrochloric acid*, using *methyl orange solution* as indicator, until a faint red colour persists for 10 seconds. Stopper the flask, allow to stand for 30 minutes and titrate with *0.1 M sodium hydroxide* using *phenolphthalein solution* as indicator. Subtract the volume of *0.1 M sodium hydroxide* used from the volume of *0.1 M hydrochloric acid* used. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of *hydrochloric acid* required.

1 ml of *0.1 M hydrochloric acid* is equivalent to 0.006307 g of $C_6H_{12}N_3PS$.

Storage. Store protected from light and moisture, at a temperature between 2° to 8°. At higher temperatures it tends to polymerise with loss of activity.

Thiotepa Injection

Thiotepa Injection is a sterile material consisting of Thiotepa with or without auxiliary agents. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Thiotepa Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of thiotepa, $C_6H_{12}N_3PS$.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Usual strength. 15 mg.

Description. A white powder.

Identification

A. Determine on 20 mg by the oxygen-flask method (2.3.34), using 5 ml of *1.25 M sodium hydroxide* as the absorbing liquid. When the process is complete, dilute to 25 ml with *water* (solution A). To 5 ml of solution A add 0.1 ml of *hydrogen peroxide solution (100 vol)* and 1 ml of *1 M hydrochloric acid*, mix and add 0.05 ml of *barium chloride solution*; a turbidity is produced.

B. To 2 ml of solution A add 40 ml of *water* and 4 ml of *ammonium molybdate-sulphuric acid solution*, mix, add 0.1 g of *L-ascorbic acid* and boil for 1 minute; a blue colour is produced.

Tests

Appearance of solution. Dissolve a quantity containing 15 mg of Thiotepa in 4 ml of *water*; the solution is clear (2.4.1).

pH (2.4.24). 5.5 to 7.5, determined in a 1.0 per cent w/v solution in *carbon dioxide-free water*.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Use freshly prepared solutions.

Test solution. Dissolve a quantity of the contents of the sealed container containing 15 mg of Thiotepa in 4 ml of *water*, filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *water*. Further dilute 1.0 ml to 10.0 ml with *water*.

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Reference solution (b). Dissolve 10 mg of *thiotepa IPRS* in 2 ml of *methanol* in a ground-glass-stoppered tube, add 50 µl of a 0.1 per cent v/v solution of *orthophosphoric acid*, stopper the tube and heat in a water-bath at 65° for 50 seconds (generates methoxy-thiotepa). Allow the solution to cool and add 1 ml of *methanol*.

Reference solution (c). Dissolve 15 mg of *thiotepa RS* in 10 ml of *water*, add 1 g of *sodium chloride*, boil in a water-bath for 10 minutes and cool (generates chloro-adduct impurity).

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm) (Such as Nucleosil C18),
- mobile phase: a mixture of 15 volumes of *acetonitrile* and 85 volumes of *0.1 M mixed phosphate buffer pH 7.0*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

The chromatogram obtained with reference solution (b) shows a peak corresponding to methoxy-thiotepa with a retention time relative to thiotepa of about 1.3 and the chromatogram obtained with reference solution (c) shows a peak corresponding to the chloro- adduct impurity with a retention time relative to thiotepa of about 3.1.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 3.0.

Inject reference solution (a) and the test solution. Run the chromatogram 4 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to the chloro-adduct impurity is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent), the area of any other secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). The area of not more than two such peaks is more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than four times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent).

Bacterial endotoxins (2.2.3). Not more than 6.25 Endotoxin Units per mg of Thiotepa.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a suitable quantity of the injection containing 0.15 g of Thiotepa in 100 ml of *water*.

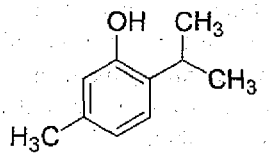
Reference solution. A 0.15 per cent w/v solution of *thiotepa IPRS* in *water*.

Chromatographic system
- a stainless steel column 20 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 70 volumes of *water* and 30 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Calculate the content of $C_6H_{12}N_3PS$ in the injection.

Storage. Store protected from light and moisture, at a temperature between 2° to 8°. If solid matter separates from the constituted injection, the solution should not be used.

Thymol



$C_{10}H_{14}O$ Mol. Wt. 150.2

Thymol is 2-isopropyl-5-methylphenol.

Thymol contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{10}H_{14}O$.

Category. Pharmaceutical aid (antimicrobial preservative; antiseptic).

Description. Colourless crystals.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *thymol IPRS* or with the reference spectrum of thymol.

B. Dissolve 0.2 g with heating in 2 ml of *2 M sodium hydroxide*, add 0.2 ml of *chloroform* and heat on a water-bath; a violet colour develops.

C. Dissolve about 2 mg in 1 ml of *anhydrous acetic acid*, add 0.15 ml of *sulphuric acid* and 0.05 ml of *nitric acid*; a bluish green colour develops.

D. Melting range (2.4.21). 48° to 52°.

Tests

Appearance of solution. Dissolve 1.0 g in 10 ml of *2 M sodium hydroxide*. The solution is not more opalescent than opalescence standard OS4 (2.4.1), and not more intensely coloured than reference solution RS6 (2.4.1).

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Acidity. To 1.0 g in a glass-stoppered flask add 20 ml of *water*, boil until dissolution is complete, cool, stopper the flask and shake vigorously for 1 minute. Add a few crystals of the substance under examination to induce crystallisation, shake vigorously for 1 minute and filter. To 5 ml of the filtrate add 0.05 ml of *methyl red solution* and 0.05 ml of *0.01 M sodium hydroxide*; the solution is yellow.

Related substances. Determine by gas chromatography (2.4.13).

Test solution. Dissolve 0.1 g in sufficient *ethanol (95 per cent)* to produce 10 ml.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *ethanol (95 per cent)*.

Reference solution (b). Dilute 1 ml of reference solution (a) to 10 ml with *ethanol (95 per cent)*.

Reference solution (c). Dilute 5 ml of reference solution (b) to 10 ml with *ethanol (95 per cent)*.

Chromatographic system
- a glass column 4 m × 2 mm, packed with diatomaceous support (125 to 180 mesh) impregnated with a mixture suitable for the separation of free fatty acids (Such as FFAP),
- temperature:
  column 80° for 2 minutes, increase @ 8° to 240° stand for 15 minutes,
  inlet port at 250° and detector at 300°,
- flow rate: 30 ml per minute of the carrier gas.

Inject 1 µl of reference solution (a), (c) and the test solution.

In the chromatogram obtained with the test solution he sum of the areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a). Ignore any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (c).

Residue on evaporation. Evaporate 2.0 g on a water-bath and heat at 105° for 1 hour. The residue weighs not more than 1.0 mg (0.05 per cent).

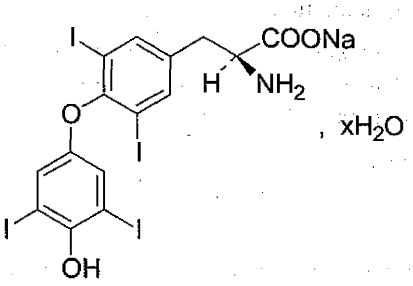
Assay. Weigh 0.1 g, transfer to an iodine flask and dissolve in 25 ml of *1 M sodium hydroxide*. Add 20 ml of hot *dilute hydrochloric acid* and immediately titrate with *0.05 M bromine* to within 1 to 2 ml of the calculated end-point. Warm the solution to about 75°, add 0.1 ml of *methyl orange solution* and shake vigorously. If the solution is red, continue the titration, dropwise and with shaking until the red colour is discharged. Repeat the alternate addition of *0.05 M bromine* and *methyl orange solution* until the red colour is discharged after the addition of the methyl orange solution.

1 ml of *0.05 M bromine* is equivalent to 0.003755 g of $C_{10}H_{14}O$.

Storage. Store protected from light and moisture.

Thyroxine Sodium

Levothyroxine Sodium; *L*-Thyroxine Sodium



$C_{15}H_{10}I_4NNaO_4,xH_2O$ Mol. Wt. 798.9 (anhydrous)

Thyroxine Sodium is sodium *O*⁴-(4-hydroxy-3,5-diiodophenyl)-3,5-diiodo-*L*-tyrosinate and contains a variable quantity of water of crystallisation.

Thyroxine Sodium contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{15}H_{10}I_4NNaO_4$, calculated on the anhydrous basis.

Category. Thyroid hormone.

Description. A white or slightly brownish yellow powder, slightly coloured, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *levothyroxine sodium IPRS* or with the reference spectrum of levothyroxine sodium.

B. To 200 mg, add 2 ml of *1 M sulphuric acid*. Heat on a water-bath followed by heating carefully over a naked flame, increasing the temperature to about 600°. Continue ignition until most of the black particles have disappeared. Dissolve the residue in 2 ml of *water*, the solution gives reaction (A) of sodium salts (2.3.1).

Tests

Appearance of solution. Dissolve 0.5 g in 23 ml of a gently boiling mixture of 4 volumes of *ethanol (95 per cent)* and 1 volume of *1 M hydrochloric acid*. Cool and dilute to 25 ml with the same mixture of solvents (solution A). The freshly prepared solution is not more intensely coloured than reference solution BYS3 (2.4.1).

Specific optical rotation (2.4.22). + 16.0° to + 20.0°, determined in solution A.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 30 volumes of mobile phase A and 60 volumes of *ethanol (95 per cent)*.

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Test solution. Dissolve 25 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture. Dilute 10.0 ml of the solution to 25.0 ml with the solvent mixture.

Reference solution (a). A solution containing 0.0002 per cent w/v each of *levothyroxine sodium IPRS* and *liothyronine sodium IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm × 4.0 mm, packed with endcapped octadecylsilane bonded to porous silica (3 µm),
- mobile phase: A. 0.1 per cent w/v solution of *orthophosphoric acid* in *water*,
  B. 0.1 per cent w/v solution of *orthophosphoric acid* in *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 25 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
10	70	30
40	20	80
50	20	80
55	70	30

Name	Relative retention time
Liothyronine[1]	0.5
levothyroxine (retention time about: 11 minutes)	1.0
levothyroxine impurity F[2]	2.0
levothyroxine impurity G[3]	2.4

[1](2S)-2-amino-3-[4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl] propanoic acid (liothyronine),

[2](2S)-2-amino-3-[4-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenoxy]-3,5-diiodophenyl]propanoic acid,

[3]Unknown structure.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to levothyroxine and liothyronine is not less than 5.0.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to liothyronine is not more than the area of the corresponding peak of in the chromatogram obtained with reference solution (a) (1.0 per cent), the area of any peak corresponding to levothyroxine impurity F is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any peak corresponding to levothyroxine impurity G is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent), the area of any other secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of the all the impurities is not more than 2.0 per cent. Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.3.43). 6.0 per cent to 12.0 per cent, determined on 0.1 g.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *0.1 M sodium hydroxide* and *methanol*.

Test solution. Dissolve 10 mg of the substance under examination in 40 ml of *0.1 M sodium hydroxide*. Shake for about 30 minutes, add 40 ml of *methanol*, cool, mix well and dilute to 100 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the same solvent.

Reference solution. A 0.001 per cent w/v solution of *levothyroxine sodium IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with nitrile groups chemically bonded to porous silica (5 to 10 µm),
- mobile phase: a mixture of 65 volumes of *water*, 1 volume of *orthophosphoric acid* and 35 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{10}I_4NNaO_4$.

Storage. Store protected from light and moisture.

Thyroxine Tablets

Thyroxine Sodium Tablets; Levothyroxine Tablets; Levothyroxine Sodium Tablets; L-Thyroxine Sodium Tablets

Thyroxine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of anhydrous thyroxine sodium, $C_{15}H_{10}I_4NNaO_4$.

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Usual strengths. 12.5 µg; 25 µg; 50 µg; 75 µg; 100 µg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

NOTE — All containers that are in contact with solutions containing levothyroxine sodium are to be made of glass. Modified peak vessels can be used to eliminate coning.

Apparatus No. 2 (Paddle),

Medium. 500 ml of 0.2 per cent w/v solution of *sodium lauryl sulphate* in *0.01 M hydrochloric acid,*

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.01 per cent w/v solution of *levothyroxine sodium IPRS* in *methanol.* Dilute suitably with the dissolution medium to obtain a solution of about the similar concentration as the test solution.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with nitrile groups chemically bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of *acetonitrile,* 65 volumes of *water* and 0.05 volume of *orthophosphoric acid,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 100 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 4.0 per cent.

Inject the reference solution and the test solution.

Q. Not less than 70 per cent of the stated amount of $C_{15}H_{10}I_4NNaO_4$ in the medium.

Liothyronine Sodium. Determine by liquid chromatography (24.14).

Solvent mixture. 60 volumes of *methanol,* 40 volumes of *water* and 0.05 volume of *orthophosphoric acid.*

Solution A. Equal volumes of *0.02 M sodium hydroxide* and *methanol.*

Test solution. Disperse a quantity of powdered tablets containing 100 µg of Levothyroxine sodium in 10.0 ml of the mobile phase, add 2 glass beads and mix on a vortex mixer for 3 minutes. Centrifuge and use the supernatant.

Reference solution (a). A 0.04 per cent w/v solution of *levothyroxine sodium IPRS* in solution A.

Reference solution (b). A 0.04 per cent w/v solution of *liothyronine sodium IPRS* in solution A. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 5.0 ml of reference solution (a) and 10.0 ml of reference solution (b) to 200.0 ml with the mobile phase.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 20.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with nitrile groups chemically bonded to porous silica (5 µm),
- mobile phase: 40 volumes of *acetonitrile,* 60 volumes of *water* and 0.05 volume of *orthophosphoric acid,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 100 µl.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to levothyroxine and liothyronine is not less than 5.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent for levothyroxine peak.

Inject reference solution (d) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to liothyronine is not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (2.0 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay, using the following solutions.

Test solution. To one tablet, add 10 ml of solvent mixture and shake for about 30 minutes, dilute with solvent mixture to produce a solution containing 0.000125 per cent per cent w/v of Thyroxine Sodium.

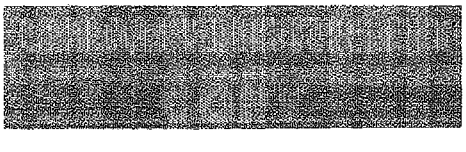
Reference solution. A 0.0125 per cent per cent w/v solution of *levothyroxine sodium IPRS* in a mixture of equal volumes of *0.1 M sodium hydroxide* and *methanol.* Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Calculate the content of $C_{15}H_{10}I_4NNaO_4$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. A mixture of 600 volumes of *water,* 400 volumes of *acetonitrile* and 0.5 volume of *ortho phosphoric acid.*



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Test solution. Weigh and powder 20 or more tablets. Disperse a quantity of the powder containing about 1 mg of Thyroxine Sodium, in 40 ml of the solvent mixture with the aid of ultrasound and dilute to 100.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *levothyroxine sodium IPRS* in a mixture of equal volumes of *0.1 M sodium hydroxide* and *methanol*. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with a stationary phase consisting of porous silica particles (5 to 10 µm) to which nitrile groups are chemically bonded,
- mobile phase: a mixture of 65 volumes of *water*, 1 volume of *orthophosphoric acid* and 35 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

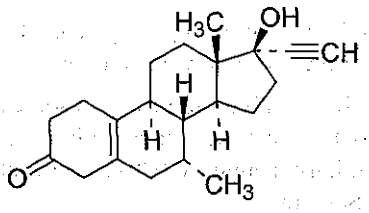
Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{10}I_4NNaO_4$ in the tablets.

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of the equivalent amount of anhydrous thyroxine sodium.

Tibolone



$C_{21}H_{28}O_2$ Mol. Wt. 312.5

Tibolone is 17-hydroxy-7α-methyl-19-nor-17α-pregn-5(10)-en-20-yn-3-one.

Tibolone contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{21}H_{28}O_2$, calculated on the dried basis.

Category. Steroid with estrogenic and progestogenic properties.

Description. A white or almost white, crystalline powder or crystals. It shows polymorphism (2.5.11).

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tibolone IPRS* or with the reference spectrum of tibolone.

Tests

Specific optical rotation (2.4.22). + 100° to + 106°, determined on 1.0 per cent w/v solution in *ethanol*.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 25 volumes of *water* and 75 volumes of *acetonitrile*.

Test solution. Dissolve 40 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with endcapped octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 8 volumes of *methanol*, 40 volumes of *acetonitrile* and 52 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 5 µl.

Name	Relative retention time	Correction factor
Tibolone impurity A[1]	0.22	1.7
Tibolone impurity B[2]	0.24	1.5
Tibolone impurity C[3]	0.58	2.1
Tibolone (retention time: about 14 minutes)	1.0	—
Tibolone impurity D[4]	1.12	—
Tibolone impurity E[5]	2.24	—

[1]10,17-dihydroxy-7α-methyl-19-nor-10ξ,17α-pregn-4-en-20-yn-3-one,
[2]10-hydroperoxy-17-hydroxy-7α -methyl-19-nor-10ξ,17α -pregn-4-en- 20-yn-3-one,
[3]17-hydroxy-7α -methyl-19-nor-10ξ,17α -pregn-4-en-20-yn-3-one,
[4]17-hydroxy-7β-methyl-19-nor-17α -pregn-5(10)-en-20-yn-3-one,
[5]3,3-dimethoxy-7α -methyl-19-nor-17α -pregn-5(10)-en-20-yn-17-ol.

Inject the reference solution and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak due to tibolone impurity A and E is not more than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent). The area of any peak due to tibolone impurity B is not more than 0.4 times

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the area of the principal peak in the chromatogram obtained with the reference solution (0.4 per cent). The area of any peak due to tibolone impurity C is not more than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent). The area of any peak due to tibolone impurity D is not more than 0.2 times the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent), the area of any other secondary peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 0.25 g in 60 ml of *tetrahydrofuran*. Add 25 ml of a 10 per cent w/v solution of *silver nitrate*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25).

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.03125 g of $C_{21}H_{28}O_2$.

Storage. Store at a temperature between 2° to 8°.

Tibolone Tablets

Tibolone Tablets contain not less than 90.0 per cent and not more than 105.0 per cent of the stated amount of tibolone, $C_{21}H_{28}O_2$.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Usual strength. 2.5 mg.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 500 ml of 0.25 per cent w/v solution of *sodium lauryl sulphate*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with a 0.25 per cent w/v solution of *sodium lauryl sulphate* to obtain a solution containing 0.0005 per cent w/v of Tibolone.

Reference solution. A 0.0005 per cent w/v solution of *tibolone IPRS* in a mixture of 1 volume of *methanol* and 99 volumes of 0.25 per cent w/v solution of *sodium lauryl sulphate*.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- column temperature: 40°,
- mobile phase: a mixture of 23 volumes of *water* and 77 volumes of *methanol*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 200 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{28}O_2$, in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{21}H_{28}O_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing 25 mg of Tibolone in 40 ml of *ethanol* with the aid of ultrasound and dilute to 50.0 ml with *ethanol*, centrifuge and use the supernatant.

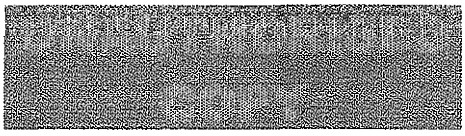
Reference solution (a). A 0.05 per cent w/v solution of *tibolone impurity standard IPRS (containing impurity B, impurity C and 1 per cent w/w of impurity A)* in *ethanol*.

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with *ethanol*.

Chromatographic system

- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (4 µm) (Such as Nova-pak C18),
- column temperature: 40°,
- mobile phase: a mixture of 28 volumes of *tetrahydrofuran* and 72 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 10 µl.

Inject reference solution (a) and (b). The test is not valid unless the chromatogram obtained with reference solution (a) closely resembles the chromatogram supplied with tibolone impurity standard RS and the resolution between the peaks due to tibolone impurity A and tibolone impurity B is not less than 2.0 and the signal-to-noise ratio of the peak due to tibolone impurity A is not less than 10 in the chromatogram obtained with reference solution (b).



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Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to tibolone impurity A (10,17-dihydroxy-7α-methyl-19-nor-10ξ,17α-pregn-4-en-20-yn-3-one) is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (1.0 per cent). The area of any peak corresponding to tibolone impurity B (10-hydroperoxy-17-hydroxy-7α-methyl-19-nor-10ξ,17α-pregn-4-en-20-yn-3-one) is not more than the area of the peak due to tibolone impurity A in the chromatogram obtained with reference solution (a) (1.0 per cent). The area of any peak corresponding to tibolone impurity C (17-hydroxy-7α-methyl-19-nor-10ξ,17α-pregn-4-en-20-yn-3-one), multiplied with correction factor 0.79 is not more than 5 times the area of the peak due to tibolone impurity A in the chromatogram obtained with reference solution (a) (5.0 per cent), the area of any other secondary peak is not more than 0.5 times the area of the peak due to tibolone impurity A in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than 6.5 times the area of the peak due to tibolone impurity A (6.5 per cent). Ignore any peak with an area less than the area of the peak due to tibolone impurity A in the chromatogram obtained with reference solution (b) (0.1 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14).

Test solution. Disperse 1 powdered tablet in *ethanol* with the aid of ultrasound for 10 minutes to obtain a solution containing 0.01 per cent w/v of Tibolone and centrifuge.

Reference solution (a). A 0.01 per cent w/v solution of *tibolone IPRS* in *ethanol.*

Reference solution (b). A 0.05 per cent w/v solution of *tibolone impurity standard IPRS* in *ethanol.*

Use the chromatographic system as described under Related substances with the following modifications

- spectrophotometer set at 210 nm,
- injection volume: 50 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to tibolone impurity A and tibolone impurity B is not less than 2.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{21}H_{28}O_2$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing 25 mg of Tibolone in 40 ml of *ethanol* with the aid of ultrasound and dilute to 50.0 ml with *ethanol* and centrifuge.

Reference solution (a). A 0.05 per cent w/v solution of *tibolone IPRS* in *ethanol.*

Reference solution (b). A 0.05 per cent w/v solution of *tibolone impurity standard IPRS* in *ethanol.*

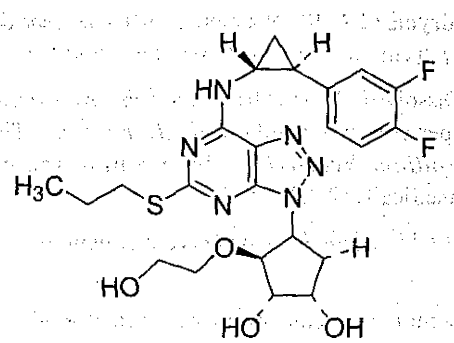
Use chromatographic system as described under Related substances using detection wavelength: 210 nm.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to tibolone impurity A and tibolone impurity B is not less than 2.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{21}H_{28}O_2$ in the tablets.

Ticagrelor



$C_{23}H_{28}F_2N_6O_4S$ Mol. Wt. 522.6

Ticagrelor is 1,2-Cyclopentanediol, 3-[7-[[(1R,2S)-2-(3,4-difluorophenyl) cyclopropyl] amino]-5-(propylthio)-3H-1,2,3-triazolo[4,5-d] pyrimidin-3-yl]-5-(2-hydroxyethoxy)-(1*S*, 2*S*, 3*R*, 5*S*).

Ticagrelor contains not less than 97.5 per cent and not more than 102. 0 per cent of $C_{23}H_{28}F_2N_6O_4S$, calculated on anhydrous basis.

Category. Anti-platelet.

Description. A white or almost white to pale pink powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ticagrelor IPRS* or with the reference spectrum of ticagrelor.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Impurity E. Determine by liquid chromatography (2.4.14).

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Buffer solution. Dissolve 156 g of *sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*.

Test solution. Dissolve 150 mg of the substance under examination in 10 ml of equal volumes of *acetonitrile* and *water* and dilute to 50.0 ml with *water*. Centrifuge at 3500 rpm for 15 minutes or until a clear solution is obtained. Use the clear solution.

Reference solution. A 0.000045 per cent w/v solution of *ticagrelor impurity E IPRS ((R)-mandelate salt)* in *acetonitrile*. Dilute 1.0 ml of the solution to 10.0 ml with *water*.

NOTE — 1 mg of ticagrelor impurity E ((1R,2S)-2-(3,4-Difluorophenyl)cyclopropan-1-amine) corresponds to 1.9 mg of impurity E as mandelic acid salt).

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (1.8 µm) (Such as Zorbax SB C-18)
- column temperature: 55°,
- mobile phase: A. a mixture of 89 volumes of *water*, 10 volumes of *acetonitrile* and 1 volume of the buffer solution,

  B. a mixture of 29 volumes of *water*, 70 volumes of *acetonitrile* 1 volume of the buffer solution,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 222 nm,
- injection volume: 100 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	94	6
8	94	6
9	0	100
20	0	100
20.1	94	6
25	94	6

The relative retention time with reference to ticagrelor (retention time- about 11.5 minutes) for ticagrelor impurity E is about 0.6.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 10.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to impurity E is not more than the area of the principal peak in the chromatogram obtained with the reference solution (8 ppm).

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Carry out the test protected from light.

Buffer solution. Dissolve 156 g of *sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*.

Solvent mixture. 35 volumes of *acetonitrile* and 65 volumes of *water*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution (a). A 0.005 per cent w/v solution of *ticagrelor RS* in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution (b). A solution containing 0.05 per cent w/v of *ticagrelor RS* and 0.0001 per cent w/v of *ticagrelor impurity B RS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm × 3.0 mm packed with phenyl group bonded to porous silica (3 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 89 volumes of *water*, 10 volumes of *acetonitrile* and 1 volume of the buffer solution,

  B. a mixture of 29 volumes of *water*, 70 volumes of *acetonitrile* and 1 volume of the buffer solution,
- a gradient programme using the conditions given below,
- flow rate: 0.65 ml per minute,
- spectrophotometer set at 242 nm,
- injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
2	80	20
42	25	75
47	25	75
47.1	80	20
55	80	20

Name	Relative retention time	Correction factor
Ticagrelor impurity A[1]	0.15	0.5
Ticagrelor	1.0	—
Ticagrelor impurity B[2]	1.06	—
Ticagrelor impurity C[3]	1.23	—
Ticagrelor impurity D[4]	1.49	—

[1](*1S,2S,3R,5S*)-3-{7-amino-5-(propylthio)-3*H*-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl}-5-(2-hydroxyethoxy)cyclopentane-1,2-diol,

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[2](1*S*,2*S*,3*R*,5*S*)-3-({3-[(1*R*,2*S*)-2-(3,4-Difluorophenyl)cyclopropyl]-5-(propylthio)-3*H*-[1,2,3]triazolo[4,5-d]pyrimidin-7-yl}amino)-5-(2-hydroxyethoxy) cyclopentane-1,2-diol,

[3]2-{[(1*S*,2*S*,3*S*,4*R*)-4-(7-{[(1*R*,2*S*)-2-(3,4-Difluorophenyl) cyclopropyl]amino}-5-(propylthio)-3*H*-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl)-2,3-dihydroxycyclopentyl]oxy}ethyl acetate,

[4]2-{[(3*aR*,4*S*,6*R*,6*aS*)-6-(7-{[(1*R*,2*S*)-2-(3,4-difluorophenyl) cyclopropyl]amino}-5-(propylthio)-3*H*-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl)-2,2-dimethyltetrahydro-4H-cyclopenta[d][1,3]dioxol-4-yl]oxy}ethanol.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to ticagrelor and ticagrelor impurity B is not less than 4.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to ticagrelor impurity A and impurity B, each of, is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any peak corresponding to ticagrelor impurity C is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the area of any peak corresponding to ticagrelor impurity D is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.6 per cent, using platinum crucible.

Water (2.3.43). Not more than 0.5 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14),

NOTE — Carry out the test protected from light.

Solvent mixture. 35 volumes of *acetonitrile* and 65 volumes of *water*.

Buffer solution. Dissolve 156 g of *sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution. A 0.05 per cent w/v solution of *ticagrelor IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 3.0 mm packed with phenyl group bonded to porous silica (3 µm),
- column temperature: 40°,
- mobile phase: a mixture of 56 volumes of *water*, 43 volumes of *acetonitrile* and 1 volume of the buffer solution,
- flow rate: 0.65 ml per minute,
- spectrophotometer set at 242 nm,
- injection volume: 5 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{28}F_2N_6O_4S$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Ticagrelor Tablets

Ticagrelor Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of ticagrelor, $C_{23}H_{28}F_2N_6O_4S$.

Usual strength. 90 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of 0.2 per cent v/v of *polysorbate 80* in *water*,
Speed and time. 75 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with dissolution medium if necessary, at the maximum at about 300 nm (2.4.7). Calculate the content of $C_{23}H_{28}F_2N_6O_4S$ in the medium from the absorbance obtained from a solution of known concentration of *ticagrelor IPRS* in the same medium.

Q. Not less than 70 per cent of the stated amount of $C_{23}H_{28}F_2N_6O_4S$.

Related substances. Determine by liquid chromatography (2.4.14) as described under Assay with the following modifications.

NOTE — protect the solution from light.

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Reference solution (a). A solution containing 0.05 per cent w/v of *ticagrelor IPRS* and 0.0001 per cent w/v of *ticagrelor related compound B IPRS* in the solvent mixture.

Reference solution (b). A 0.0001 per cent w/v solution of *ticagrelor IPRS* in the solvent mixture.

Reference solution (c). Dilute 5.0 ml of reference solution (b) to 10.0 ml with the solvent mixture.

Chromatographic system
- mobile phase: A. 1 volume of the buffer solution, 89 volumes of *water* and 10 volumes of *acetonitrile*, B. 1 volume of the buffer solution, 29 volumes of *water* and 70 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
7	35	65
15	35	65
16	0	100
23	0	100
28	90	10

Name	Relative retention time
Ticagrelor related compound A[1*]	0.44
Ticagrelor	1.0
Ticagrelor related compound B[2*]	1.07
Ticagrelor related compound C[3*]	1.45
Ticagrelor related compound D[4*]	1.68

*Process impurity include for identification only and not included in the calculation of total degradation products,

[1](1*S*,2*S*,3*R*,5*S*)-3-{7-amino-5-(propylthio)-3*H*-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl}-5-(2-hydroxyethoxy)cyclopentane-1,2-diol,

[2](1*S*,2*S*,3*R*,5*S*)-3-({3-[1*R*,2*S*)-2-(3,4-Difluorophenyl)cyclopropyl]-5-(propylthio)-3*H*-[1,2,3]triazolo[4,5-d]pyrimidin-7-yl}amino)-5-(2-hydroxyethoxy) cyclopentane-1,2-diol,

[3]2-{[(1*S*,2*S*,3*S*,4*R*)-4-(7-{[(1*R*,2*S*)-2-(3,4-Difluorophenyl) cyclopropyl]amino}-5-(propylthio)-3*H*-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl)-2,3-dihydroxycyclopentyl]oxy}ethyl acetate,

[4]2-{[(3*aR*,4*S*,6*R*,6*aS*)-6-(7-{[(1*R*,2*S*)-2-(3,4-difluorophenyl) cyclopropyl]amino}-5-(propylthio)-3*H*-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl)-2,2-dimethyltetrahydro-4*H*-cyclopenta[d][1,3]dioxol-4-yl]oxy}ethanol.

Inject reference solution (a), (b) and (c). The test is not valid unless the resolution between the peaks due to ticagrelor and ticagrelor related compound B is not less than 1.5 in the chromatogram obtained with reference solution (a), the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (b) and the signal to noise ratio for the principal peak is not less than 10.0 in the chromatogram obtained with reference solution (c). Ignore any peak with an area 0.5 time the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 35 volumes of *acetonitrile* and 65 volumes of *water*.

Buffer solution. 1 M sodium phosphate, adjusted to pH 3.0 with *orthophosphoric acid.*

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of Ticagrelor with 60 ml of the solvent mixture, with the aid of ultrasound with intermittent shaking and dilute to 100.0 ml with the solvent mixture.

Reference solution. A 0.05 per cent w/v solution of *ticagrelor IPRS* in solvent mixture.

Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (1.8 µm),
- column temperature: 55°,
- mobile phase: a mixture of 52 volumes of *acetonitrile* and 1 volume of the buffer solution and 47 volumes of *water*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 242 nm,
- injection volume: 5 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{28}F_2N_6O_4S$ in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.



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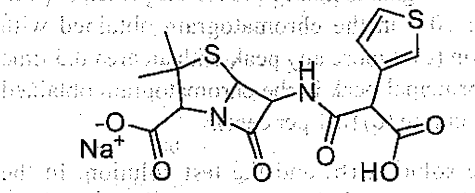
Ticarcillin Monosodium

Ticarcillin Monosodium Monohydrate



$C_{15}H_{15}N_2NaO_6S_2, H_2O$ Mol. Wt. 424.4

Ticarcillin Monosodium is 4-Thia-1-azabicyclo[3.2.0]heptane-2-carboxylicacid, 6-[(carboxy-3-thienylacetyl)amino]-3,3-dimethyl-7-oxo, monosodium salt, [2S-[2α, 5α, 6β (S*)]]-, monohydrate.

Ticarcillin Monosodium Monohydrate contains not less than 890 µg per mg of ticarcillin $C_{15}H_{16}N_2O_6S_2$, calculated on the anhydrous basis.

Category. Beta-Lactam Antibacterial.

Description. A white to pale yellow powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ticarcillin monosodium monohydrate IPRS* or with the reference spectrum of ticarcillin monosodium.

B. In the Assay, the principal peaks in the chromatogram obtained with the test solution correspond to the peak in the chromatogram obtained with the reference solution.

C. Specific optical rotation (see Test).

D. A 5 per cent w/v solution gives reaction (A) of sodium (2.3.1).

Tests

***N, N*-Dimethylaniline** (2.3.21). Not more than 20 ppm.

pH (2.4.24). 2.5 to 4.0, determined in a 1.0 per cent w/v solution of ticarcillin.

Specific optical rotation (2.4.22). + 181.0° to + 197.0°, determined in a 1.0 per cent w/v solution prepared by dissolving 6.9 g of *monobasic sodium phosphate* in 800 ml of *water* adjusted to pH 6.4 with *10 M sodium hydroxide* and dilute to 1000 ml with *water.*

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 20 ml of mobile phase A and dilute to 50.0 ml with mobile phase A.

Reference solution. A 0.002 per cent w/v solution of *ticarcillin monosodium monohydrate IPRS* in mobile phase A.

Chromatographic system

- a stainless steel column 25 cm × 4 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution prepared by dissolving 1.3 g of *dibasic ammonium phosphate* in *water,* adjusted to pH 7.0 with *orthophosphoric acid* and dilute to 1000 ml with *water*,
  B. a mixture of 50 volumes of *methanol* and 50 volumes of mobile phase A,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
30	30	70
40	30	70
45	100	0

Name	Relative retention time
Ticarcillin	0.95, 1.0
Ticarcillin related compound A[1]	2.1

[1]Decarboxy ticarcillin; (2,5-3,3-dimethyl-7-oxo-6[[(thiophen-3yl) acetyl]-4-thia-azabicyclo[3,2,0]heptane-2-carboxylic acid.

Inject the reference solution. The test is not valid unless the resolution between two ticarcillin peaks is not less than 2.0 and the relative standard deviation for replicate injections is not more than 5 per cent for the sum of ticarcillin peaks.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to ticarcillin related compound A is not more than twice the area of the sum of principal peaks in the chromatogram obtained with the reference solution (4.0 per cent) and the area of any other secondary peak is not more than 1.25 times the area of the sum of principal peaks in the chromatogram obtained with the reference solution (2.5 per cent).

Water (2.3.43). 4.0 per cent to 6.0 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in 70 ml of the mobile phase, with the aid of ultrasound and dilute to 200.0 ml with the mobile phase.

Reference solution. A 0.01 per cent w/v solution of *ticarcillin mono sodium monohydrate IPRS* in the mobile phase.

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Chromatographic system
- a stainless steel column 25 cm × 4 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 80 volumes of a buffer solution prepared by dissolving 1.3 gm of *dibasic ammonium phosphate* in 800 ml of *water*, adjusted to pH 7.0 with *orthophosphoric acid* and dilute to 1000 ml with the *water* and 20 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the resolution between two ticarcillin peaks is not less than 1.5 and the relative standard deviation for replicate injections is not more than 1.0 per cent for the sum of ticarcillin peaks.

Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{16}N_2O_6S_2$.

Ticarcillin Monosodium Monohydrate intended for use in the manufacture of Parenteral Preparations without a further procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.05 Endotoxin unit per mg of ticarcillin.

Ticarcillin Monosodium Monohydrate intended for use in the manufacture of Parenteral Preparations without a further sterilisation procedure complies with the following additional requirement.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store protected from moisture, at a temperature between 2° to 8°.

Labelling. The label states whether it is intended for use in the manufacture of parenteral preparations.

Ticarcillin and Clavulanic Acid Injection

Clavulanic Acid and Ticarcillin Injection

Ticarcillin and Clavulanic Acid Injection is a sterile iso-osmotic solution of Ticarcillin Monosodium and Clavulanate Potassium in Water for Injections. It contains one or more suitable buffering agents and a tonicity adjusting agent.

Ticarcillin and Clavulanic Acid Injection contains not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of ticarcillin, $C_{15}H_{16}N_2O_6S_2$ and not less than 85.0 per cent and not more than 120.0 per cent of the stated amount of clavulanic acid, $C_8H_9NO_5$.

Ticarcillin and Clavulanic Acid for Injection

Ticarcillin and Clavulanic acid for Injection is a sterile, dry mixture of Ticarcillin Monosodium and Clavulanate Potassium.

The constituted solution complies with the requirements for clarity of solution and particulate matter stated under Parenteral Preparations (Injections).

Usual Strength. Ticarcillin 3 g and Clavullanic acid 100 mg.

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 5.5 to 7.5.

Bacterial endotoxins (2.2.3). Not more than 0.07 Endotoxin Unit per mg of ticarcillin.

Sterility (2.2.11). Complies with the test for sterility.

Water (2.3.43). Not more than 4.2 per cent.

Other tests. Comply with the tests stated under Parenteral Preparations (Powders for Injections).

Assay. Determine by liquid chromatography (2.4.14).

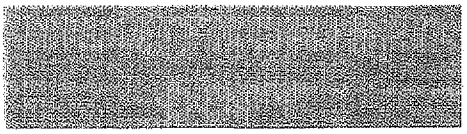
Sodium phosphate buffer pH 4.3. Dissolve 13.8 g of *monobasic sodium phosphate* in 900 ml of *water*, adjusted to pH 4.3 with *orthophosphoric acid* or *10 M sodium hydroxide*, dilute to 1000 ml with *water*.

Sodium phosphate buffer pH 6.4. Dissolve 6.9 g of *monobasic sodium phosphate* in 900 ml of *water*, adjusted to pH 6.4 with *10 M sodium hydroxide*, dilute 1000 ml with *water*.

Clavulanate lithium stock solution. A 0.06 per cent w/v solution of *clavulanate lithium IPRS* in *sodium phosphate buffer pH 6.4*.

Test solution. Dissolve a quantity of powder in sufficient *water* and dilute with *sodium phosphate buffer pH 6.4* to obtain a 0.09 per cent w/v solution of Ticarcillin.

Reference solution. Transfer about 100 mg of *ticarcillin monosodium monohydrate IPRS* to a 100-ml volumetric flask and add 150/J ml of *clavulanate lithium stock solution* (J is the ratio of the stated amount of ticarcillin to the stated amount of clavulanic acid in the powder) and dilute to 100.0 ml with *sodium phosphate buffer pH 6.4*.



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Chromatographic system
- a stainless steel column 30 cm x 4 mm, packed with octadecylsilane chemically bonded to porous silica (5 µm),
- mobile phase: a mixture of 95 volumes of *sodium phosphate buffer pH 4.3* and 5 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

The relative retention time with reference to ticarcillin for clavulanic acid is about 0.2.

Inject the reference solution. The test is not valid unless the column efficiency of the principal peaks is not less than 1000 theoretical plates; the tailing factor for the principal peaks are not more than 2.0; the resolution between the peaks due to ticarcillin and clavulanic acid is not less than 5.0; and the relative standard deviation for replicate injections is not more than 2.0 per cent.

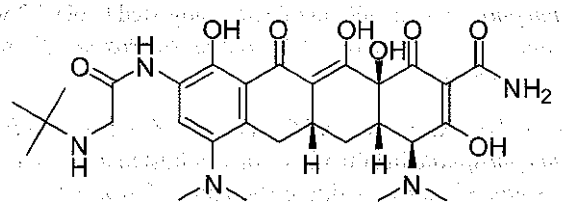
Inject the reference solution and the test solution.

Calculate the content of $C_{15}H_{16}N_2O_6S_2$ and $C_8H_9NO_5$ in the injection.

Storage. Store in single dose or multiple dose containers.

Labelling. The label states (1) that the injection is to be thawed just prior to use; (2) conditions for proper storage of the resultant solution; (3) directs that the solution is not to be refrozen.

Tigecycline



$C_{29}H_{39}N_5O_8$ Mol. Wt. 585.7

Tigecycline is 2-Naphthacenecarboxamide,4,7-bis (dimethylamino)-9-[[[(1,1-dimethylethyl)amino]acetyl] amino]-1,4,4a,5,5a,6,11,12a-octahydro-3,10,12,12a-tetrahydroxy-1,11-dioxo-(4*S*,4a*S*,5a*R*,12a*S*).

Tigecycline contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{29}H_{39}N_5O_8$, calculated on the anhydrous basis.

Category. Antibacterial.

Description. An orange powder. It shows polymorphism (2.5.11).

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tigecycline IPRS* or with the reference spectrum of tigecycline.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

NOTE — Handle standard and sample under moisture controlled condition, to prevent degradation.

pH (2.4.24). 7.7 to 8.2, determined in a 1.0 per cent w/v solution.

Specific optical rotation (2.4.22). −240.0° to −215.0°, determined in a 0.1 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 4.35 g of *dibasic potassium phosphate* and 0.5 g of *sodium bisulphite* in 900 ml of *water*, adjusted to pH 8.0 with *1 M potassium hydroxide* and dilute to 1000 ml with *water*.

NOTE — Protect the solutions from light. Store these solutions at 10° immediately after preparation.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution (a). A 0.0005 per cent w/v solution of *tigecycline IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.05 per cent w/v of *tigecycline IPRS* and 0.00024 per cent w/v of *tigecycline impurity B IPRS* in the solvent mixture.

Reference solution (c). Dilute 5.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (3µm) (Such as Phenomenex Luna C18),
- sample temperature: 10°,
- mobile phase: A. a mixture of 95 volumes of a buffer solution prepared by dissolving 4.58 g of *dibasic potassium phosphate* and 0.98 g of *disodium edetate* in 1000 ml of *water*, adjusted to pH 6.4 with *orthophosphoric acid* and 5 volumes of *acetonitrile*,

  B. a mixture of 50 volumes of a buffer solution prepared by dissolving 8.7 g of *dibasic potassium phosphate* and 1.96 g of *disodium edetate* in 1000 ml of *water*, adjusted to pH 6.4 with *orthophosphoric acid* and 50 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,

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– flow rate: 1 ml per minute,
– spectrophotometer set at 248 nm,
– injection volume: 25 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
40	57	43
55	0	100
58	0	100
59	85	15
66	85	15

Name	Relative retention time
Specified unidentified impurity	0.12
Specified unidentified impurity	0.23
Tigecycline open ring[1,2]	0.36
Specified unidentified impurity	0.50
Tigecycline 12-oxo-11-hydroxy[3]	0.55
Tigecycline impurity B[4]	0.64
Tigecycline epimer[5]	0.74
Specified unidentified impurity	0.76
Tigecycline	1.0
Tigecycline quinine analog[6]	1.3
Specified unidentified impurity	1.5
Minocycline	1.6
Tigecycline tricyclic analog[7]	1.7
Specified unidentified impurity	1.9

[1]To be reported if there is a peak with maximum absorbance at either 390 or 640 nm. The impurity is quantitated at 248 nm. The system may resolve two peaks. The limit is for the sum of the two peaks,

[2](3*S*,4*R*)-7-[2-(*tert*-Butylamino)acetamido]-3-[4-carbamoyl-2-(dimethylamino)-3,5,6-trihydroxybenzyl]-5-(dimethylimino)-1,4,8-trihydroxy-3,4,5,8-tetrahydronaphthalene-2-carboxylate,

[3](4*S*,4a*S*,12a*S*)-9-[2-(*tert*-Butylamino)acetamido]-4,7-bis(dimethylamino)- 3,10,11,12a-tetrahydroxy-1,12-dioxo-1,4,4a,5,12,12a-hexahydro-2-naphthacenecarboxamide,

[4](4*S*,4a*S*,5a*R*,12a*S*)-9-Amino-4,7-bis(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide hydrochloride,

[5](4*R*,4a*S*,5a*R*,12a*S*)-9-[2-(*tert*-Butylamino)acetamido]-4,7-bis(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,10,12,12a-tetrahydroxy-1,11-dioxo-2-naphthacenecarboxamide,

[6](*S*)-4-({6-[2-(*tert*-Butylamino)acetamido]-8-(dimethylamino)-5-hydroxy-4-oxo-1,2,3,4-tetrahydronaphthalen-2-yl}methyl)-2,5-dihydroxy-3,6-dioxocyclohexa-1,4-dienecarboxamide,

[7](1*S*,4a*R*,4b*R*,10a*R*,11a*S*)-7-[2-(*tert*-Butylamino)acetamido]-9-(dimethylamino)-1,4,4a,6-tetrahydroxy-2,5,12-trioxo-1,2,4a,5,10,10a,11,11a-octahydro-1,4b-methanobenzo[*b*]fluorene-3-carboxamide.

Inject reference solution (a), (b) and (c).The test is not valid unless the resolution between the peaks due to tigecycline epimer and tigecycline impurity B is not less than 1.5, in the chromatogram obtained with reference solution (b), the tailing factor is between 0.7 to 1.5 and the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (a) and the signal- to- noise ratio is not less than 10 in the chromatogram obtained with reference solution (c).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to tigecycline open ring is not more than 0.02 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent). The area of any peak corresponding to tigecycline 12-oxo-11-hydroxy, tigecycline quinine analog, tigecycline tricyclic analog and at relative retention time 0.12, 0.23, 0.5, 0.76, 1.5, 1.9, each of, is not morethan 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent). The area of any peak corresponding to tigecycline impurity B and minocycline, each of, is not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent). The area of any peak corresponding to tigecycline epimer is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent), the area of any other secondary peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 2.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.2 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 2.5 per cent, determined on 0.5 g in 10 ml of *dimethyl sulfoxide*.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 4.35 g of *dibasic potassium phosphate* and 0.5 g of *sodium bisulphite* in 900 ml of *water*, adjusted to pH 8.0 with *1 M potassium hydroxide* and dilute to 1000 ml with *water*.

NOTE — Protect the solutions from light. Store these solutions at 10° immediately after preparation.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution (a). A 0.01 per cent w/v solution of *tigecycline IPRS* in the solvent mixture.

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Reference solution (b). Dissolve 20 mg of *tigecycline IPRS* in 10 ml of *water*. Add 1 or 2 drops *trifluoro acetic acid* and heat the solution at about 65° for 15 minutes and cool. Dilute 5.0 ml of the solution to 100.0 ml with *water*.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- sample temperature: 10°,
- mobile phase: a mixture of 86 volumes of a buffer solution prepared by dissolving 4.35 g of *dibasic potassium phosphate* and 0.93 g of *disodium edetate* in 1000 ml of *water*, adjusted to pH 6.2 with *orthophosphoric acid* and 14 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 248 nm,
- injection volume: 20 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to tigecycline and tigecycline epimer is not less than 3.0 in the chromatogram obtained with reference solution (b), the tailing factor is between 0.7 to 1.5 and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{29}H_{39}N_5O_8$.

Tigecycline intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 1.0 Endotoxin Unit per mg of tigecycline.

Storage. Store protected from light and moisture, under nitrogen, at a temperature 2° to 8°.

Labelling. When it is intended for use in preparing injectable dosage forms, the label states that it is sterile or must be subjected to further processing during the preparation of injectable dosage forms.

Tigecycline Injection

Tigecycline Injection is a sterile material consisting of Tigecycline with or without excipients. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections or other suitable solvent, immediately before use.

The constituted solution complies with the requirements for the Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Tigecycline Injection contains not less than 96.0 per cent and not more than 116.0 per cent of the stated amount of tigecycline, $C_{29}H_{39}N_5O_8$.

Usual strength. 50 mg per vial.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injections) and with the following requirements.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

NOTE — Handle standard and sample under moisture controlled condition, to prevent degradation.

pH (2.4.24). 4.5 to 5.5, determined in the reconstituted solution.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 4.35 g of *dibasic potassium phosphate* and 0.5 g of *sodium bisulphite* in 900 ml of *water*, adjusted to pH 8.0 with *1 M potassium hydroxide* and dilute to 1000 ml with *water*.

NOTE — Protect the solutions from light using low-actinic glass ware. Store these solutions at 10° immediately after preparation.

Test solution. Mix the content of 5 vials. Disperse a quantity containing 50 mg of Tigecycline in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution (a). A 0.0005 per cent w/v solution of *tigecycline IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.05 per cent w/v of *tigecycline IPRS* and 0.00024 per cent w/v of *tigecycline impurity B IPRS* in the solvent mixture.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 20.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- sample temperature: 10°,
- mobile phase: A. a mixture of 95 volumes of a buffer solution prepared by dissolving 4.58 g of *dibasic potassium phosphate* and 0.98 g of *disodium edetate* in 1000 ml of *water*, adjusted to pH 6.4 with *orthophosphoric acid* and 5 volumes of *acetonitrile*,

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B. a mixture of 50 volumes of buffer solution prepared by dissolving 8.7 g of *dibasic potassium phosphate* and 1.96 g of *disodium edetate* in 1000 ml of *water*, adjusted to pH 6.4 with *orthophosphoric acid* and 50 volumes of *acetonitrile*,

– a gradient programme using the conditions given below,
– flow rate: 1 ml per minute,
– spectrophotometer set at 248 nm,
– injection volume: 25 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
40	57	43
55	0	100
58	0	100
59	85	15
66	85	15

Name	Relative retention time
Tigecycline open ring[1,2]	0.36
Tigecycline 12-oxo-11-hydroxy[3]	0.55
Tigecycline impurity B[4]	0.64
Tigecycline epimer[5]	0.74
Tigecycline	1.0
Tigecycline quinine analog[6]	1.3
Minocycline	1.6
Tigecycline tricyclic analog[7]	1.7

[1]To be reported if there is a peak with maximum absorbance at either 390 or 640 nm. The impurity is quantitated at 248 nm. The system may resolve two peaks. The limit is for the sum of the two peaks,

[2](3*S*,4*R*)-7-[2-(*tert*-Butylamino)acetamido]-3-[4-carbamoyl-2-(dimethylamino)-3,5,6-trihydroxybenzyl]-5-(dimethylimino)-1,4,8-trihydroxy- 3,4,5,8-tetrahydronaphthalene-2-carboxylate,

[3](4*S*,4a*S*,12a*S*)-9-[2-(*tert*-Butylamino)acetamido]-4,7-bis(dimethylamino)- 3,10,11,12a-tetrahydroxy-1,12-dioxo-1,4,4a,5,12,12a-hexahydro-2-naphthacenecarboxamide,

[4](4*S*,4a*S*,5a*R*,12a*S*)-9-Amino-4,7-bis(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide hydrochloride,

[5](4*R*,4a*S*,5a*R*,12a*S*)-9-[2-(*tert*-Butylamino)acetamido]-4,7-bis(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,10,12,12a-tetrahydroxy-1,11- dioxo-2-naphthacenecarboxamide,

[6](*S*)-4-({6-[2-(*tert*-Butylamino)acetamido]-8-(dimethylamino)-5-hydroxy-4-oxo-1,2,3,4-tetrahydronaphthalen-2-yl}methyl)-2,5-dihydroxy- 3,6-dioxocyclohexa-1,4-dienecarboxamide,

[7](1*S*,4a*R*,4b*R*,10a*R*,11a*S*)-7-[2-(*tert*-Butylamino)acetamido]-9-(dimethylamino)-1,4,4a,6-tetrahydroxy-2,5,12-trioxo-1,2,4a,5,10,10a,11,11a- octahydro-1,4b-methanobenzo[*b*]fluorene-3-carboxamide.

Inject reference solution (a), (b) and (c). The test is not valid unless the resolution between the peaks due to tigecycline impurity B and tigecycline epimer is not less than 1.5, in the chromatogram obtained with reference solution (b), the tailing factor is between 0.7 to 1.5 and the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (a) and signal-to- noise ratio is not less than 10 in the chromatogram obtained with reference solution (c).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to tigecycline open ring is not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent). The area of any peak corresponding to tigecycline 12-oxo-11-hydroxy and tigecycline tricyclic analog,each of, is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). The area of any peak corresponding to tigecycline quinine analog is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). The area of any peak corresponding to tigecycline impurity B is not more than 0.7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent). The area of any peak corresponding to tigecycline epimer is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent), the area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (6.0 per cent).

Sterility (2.2.11). Complies with the test for sterility.

Bacterial endotoxins (2.2.3). Not more than 1.75 Endotoxin Unit per mg of tigecycline.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 4.35 g of *dibasic potassium phosphate* and 0.5 g of *sodium bisulphite* in 900 ml of *water*, adjusted to pH 8.0 with *1 M potassium hydroxide* and dilute to 1000 ml with *water*.

NOTE — Protect the solutions from light using low-actinic glass ware. Store these solutions at 10° immediately after preparation.

Test solution. Mix the content of 5 vials. Disperse a quantity containing 50 mg of Tigecycline in the solvent mixture and dilute to 50.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

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Reference solution (a). A 0.01 per cent w/v solution of *tigecycline IPRS* in the solvent mixture.

Reference solution (b). Dissolve 10 mg of *tigecycline IPRS* in 10 ml of *water*. Add 1 or 2 drops *trifluoroacetic acid* and heat the solution at about 65° for 15 minutes and cool. Dilute 5.0 ml of the solution to 50.0 ml with *water*.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- sample temperature: 10°,
- mobile phase: a mixture of 86 volumes of a buffer solution prepared bydissolving 4.35 g of *dibasic potassium phosphate* and 0.93 g of *disodium edetate* in 1000 ml of *water* adjusted to a pH of 6.2 with *orthophosphoric acid* and 14 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 248 nm,
- injection volume: 20 µl.

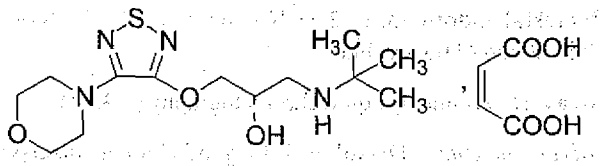
Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to tigecycline and tigecycline epimer is not less than 3.0,in the chromatogram obtained with reference solution (b), the tailing factor is between 0.7 to 1.5 and the relative standard deviation for replicate injections is not more than 1.1 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{29}H_{39}N_5O_8$ in the injection.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Timolol Maleate



$C_{13}H_{24}N_4O_3S,C_4H_4O_4$ Mol. Wt. 432.5

Timolol Maleate is (*S*)-1-*tert*-butylamino-3-(4-morpholino-1,2,5-thiadiazol-3-yloxy)propan-2-ol hydrogen maleate.

Timolol Maleate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{13}H_{24}N_4O_3S,C_4H_4O_4$, calculated on the dried basis.

Category. Beta-adrenoceptor antagonist (Antiglaucoma).

Description. A white or almost white, crystalline powder or colourless crystals.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *timolol maleate IPRS* or with the reference spectrum of timolol maleate.

B. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a).

C. Triturate 0.1 g with a mixture of 1 ml of *2 M sodium hydroxide* and 3 ml of *water* and shake with three quantities, each of 5 ml, of *ether*. To 0.1 ml of the aqueous layer add a solution of 10 mg of *resorcinol* in 3 ml of *sulphuric acid* and heat on a water-bath for 15 minutes; no violet-red colour is produced. Neutralise the remainder of the aqueous layer with *1 M sulphuric acid*, add 1 ml of *bromine water*, heat on a water-bath for 15 minutes, then heat to boiling and cool. To 0.2 ml of the solution add a solution of 10 mg of *resorcinol* in 3 ml of *sulphuric acid* and heat on a water-bath for 15 minutes; a violet-red colour is produced. Add 0.2 ml of a 10 per cent w/v solution of *potassium bromide* and heat on a water-bath for 5 minutes; the colour changes to violet-blue.

Tests

Appearance of solution. A 2.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution BS8 (2.4.1).

pH (2.4.24). 3.8 to 4.3, determined in a 2.0 per cent w/v solution.

Specific optical rotation (2.4.22). −6.2° to −5.7°, determined in a 10.0 per cent w/v solution in *1 M hydrochloric acid*.

Enantiomeric purity. Determine by liquid chromatography (2.4.14).

NOTE—Protect the solutions from light.

Solvent mixture. 10 volumes of *dichloromethane* and 30 volumes of *2-propanol*.

Test solution. Dissolve 30 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). A 0.3 per cent w/v solution of *timolol maleate IPRS* in the solvent mixture.

Reference solution (b). A 0.003 per cent w/v solution of *timolol impurity A IPRS ((R)-timolol IPRS)* in the solvent mixture.

Reference solution (c). Dilute 1 ml of reference solution (a) to 100 ml with the solvent mixture. Mix 1 ml of the solution with 1 ml of reference solution (b).

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Reference solution (d). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with silica gel OD for chiral separations (5 µm),
- mobile phase: a mixture of 2 volumes of *diethylamine*, 40 volumes of *2-propanol* and 960 volumes of *hexane*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 297 nm,
- injection volume: 5 µl.

Timolol impurity A is eluted first. The retention times of the principal peaks due to (*S*)-enantiomer in the chromatograms obtained with the test solution and reference solution (a) are identical.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to timolol impurity A and the (*S*)-enantiomer is not less than 4.0.

Inject reference solution (d) and the test solution. The area of any peak due to timolol impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (1.0 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in mobile phase A and dilute to 20 ml with mobile phase A.

Reference solution (a). A 0.05 per cent w/v solution of *timolol maleate IPRS* in the mobile phase A.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with mobile phase A.

Chromatographic system
- a stainless steel column 12.5 cm x 4 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of equal volumes of *methanol* and a 0.43 per cent w/v solution of *sodium octanesulphonate* previously adjusted to pH 3.0 with *glacial acetic acid*,

  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 2 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	97.5	2.5
10	97.5	2.5
11	70	30
14.5	70	30
17	97.5	2.5

Name	Relative retention time	Correction factor
Maleic acid	0.1	—
Timolol impurity D[1]	0.3	0.6
Timolol impurity E[2]	0.4	—
Timolol impurity F[3]	0.6	—
Timolol impurity B[4]	0.7	—
Timolol (Retention time: about 5 minutes)	1.0	—
Timolol impurity C[5]	2.8	—

[1]4-(morpholin-4-yl)-1,2,5-thiadiazol-3-ol,

[2](2*Z*)-4-[(1*S*)-1-[[(1,1-dimethylethyl)amino]methyl]-2-[[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy]ethoxy]-4-oxobut-2-enoic acid,

[3]4-(4-chloro-1,2,5-thiadiazol-3-yl)morpholine,

[4](2*RS*)-3-[(1,1-dimethylethyl)amino]-2-[[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy]propan-1-ol,

[5](2*RS*)-*N*-(1,1-dimethylethyl)-2,3-bis[[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy]propan-1-amine.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to timolol impurities B, C, D, E, F is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent) and ignore any peak due to maleic acid.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.35 g in 60 ml of *anhydrous glacial acetic acid.* Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.04325 g of $C_{13}H_{24}N_4O_3S, C_4H_4O_4$.

Storage. Store protected from light and moisture.

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Timolol Eye Drops

Timolol Maleate Eye Drops

Timolol Eye Drops are a sterile solution of Timolol Maleate in Purified Water. They may contain suitable antimicrobial preservatives, buffering agents, stabilisers and suitable substances to increase the viscosity of the solution.

Timolol Eye Drops contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of timolol, $C_{13}H_{24}N_4O_3S$.

Usual strengths. The equivalent of 0.25 per cent w/v and 0.5 per cent w/v of timolol.

Identification

A. Add a volume containing 50 mg of timolol to an equal volume of *carbonate buffer pH 9.7* and extract with two quantities, each of 40 ml, of *dichloromethane*. Reserve the aqueous layer for test B. Filter the combined dichloromethane extracts with *anhydrous sodium sulphate* and evaporate to dryness. Dry the residue at 60° at a pressure of 2 kPa for 15 minutes.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *timolol maleate IPRS*, treated in the same manner or with the reference spectrum of timolol.

B. Filter the aqueous layer reserved in test A and evaporate to about 1 ml. Add 1 ml of *bromine solution*, heat in a water-bath for 10 minutes, boil, cool and add 0.1 ml of the solution to a solution of 10 mg *resorcinol* in 3 ml of *sulphuric acid*; a bluish black colour is produced on heating in a water-bath for 15 minutes.

Tests

pH (2.4.24). 6.5 to 7.5.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Use the undiluted eye drops.

Reference solution (a). Dilute 1 volume of the eye drops to 250 volumes with the mobile phase.

Reference solution (b). Dilute 1 volume of the eye drops to 500 volumes with the mobile phase.

Reference solution (c). A 0.3 per cent w/v solution of *maleic acid* in the mobile phase.

Chromatographic system

- a stainless steel column 20 cm × 4 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 57.5 volumes of *methanol* and 42.5 volumes of *0.02 M sodium octanesulphonate*, adjusted to pH 3.0 with *glacial acetic acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 20 µl.

Inject reference solution (c), for identification of peak due to maleic acid.

Inject reference solution (a), (b) and the test solution. Run the chromatogram of the test solution for 4 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any secondary peak, other than the peak corresponding to maleic acid, is not greater than the area of the peak in the chromatogram obtained with reference solution (a) (0.4 per cent) and not more than two such peaks have an area greater than that of the peak obtained with reference solution (b) (0.2 per cent).

Other tests. Comply with the tests stated under Eye Drops.

Assay. To a volume containing about 25 mg of timolol add *water* to produce 50.0 ml and mix. To 5.0 ml add 15 ml of *carbonate buffer pH 9.7* and extract with three quantities, each of 20 ml, and one quantity of 10 ml of *toluene*. Wash each extract successively with the same 10-ml volume of *carbonate buffer pH 9.7*. Combine the toluene extracts and extract with four quantities, each of 20 ml, of *0.05 M sulphuric acid*. Combine the extracts, dilute to 100.0 ml, filter and measure the absorbance of the filtrate at the maximum at about 295 nm (2.4.7), using as the blank a solution prepared by treating 5.0 ml of *water* in the same manner beginning at the words "add 15 ml of *carbonate buffer pH 9.7*...".

Calculate the content of $C_{13}H_{24}N_4O_3S$ taking 279 as the specific absorbance at 295 nm.

Storage. Store protected from light and moisture.

Labelling. The label states (1) the strength in terms of the equivalent amount of timolol; (2) the names and concentration of any added antimicrobial preservatives.

Timolol Tablets

Timolol Maleate Tablets

Timolol Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of timolol maleate, $C_{13}H_{24}N_4O_3S,C_4H_4O_4$.

Usual strength. 10 mg.

Identification

A. To a quantity of the powdered tablets containing 70 mg of Timolol Maleate add 20 ml of *carbonate buffer pH 9.7* and extract with two quantities, each of 40 ml, of *dichloromethane*. Reserve the aqueous layer for test B. Dry the extracts with

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anhydrous sodium sulphate, evaporate to dryness using a rotary evaporator, dry the residue at 60° at a pressure of 2 kPa for 15 minutes.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *timolol maleate IPRS*, treated in the same manner or with the reference spectrum of timolol.

B. Filter the aqueous layer reserved in test A and evaporate to about 1 ml. Add 1 ml of *bromine water*, heat on a water-bath for 15 minutes, then heat to boiling and cool. Add 0.1 ml of the solution to a solution of 10 mg of *resorcinol* in 3 ml of *sulphuric acid* and heat on a water-bath for 15 minutes; a bluish black colour is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 500 ml of *0.1 M hydrochloric acid*,
Speed and time. 100 rpm and 20 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a suitable quantity of *timolol maleate IPRS* in the dissolution medium to obtain a solution having the same concentration as that to the test solution.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{13}H_{24}N_4O_3S,C_4H_4O_4$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{13}H_{24}N_4O_3S,C_4H_4O_4$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets containing 0.1 g of Timolol Maleate with 10 ml of the mobile phase for 10 minutes and filter.

Reference solution (a). A 0.004 per cent w/v of *timolol maleate IPRS* with the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 20 cm × 4 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 57.5 volumes of *methanol* and 42.5 volumes of *0.02 M sodium octanesulphonate*, adjusted to pH 3.0 with *glacial acetic acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 20 µl.

Inject reference solution (a), (b) and the test solution. Run the chromatogram of the test solution for 4 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any secondary peak, other than the peak corresponding to maleic acid, is not more than the area of the peak in the chromatogram obtained with reference solution (a) (0.4 per cent) and not more than two such peaks have an area more than that of the peak obtained with reference solution (b) (0.2 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay with the following modification.

Test solution. Disperse one tablet in 60 ml of the solvent mixture, with the aid of ultrasound and dilute to obtain a solution having the similar concentration of reference solution (a) with the solvent mixture.

Calculate the content of $C_{13}H_{24}N_4O_3S,C_4H_4O_4$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

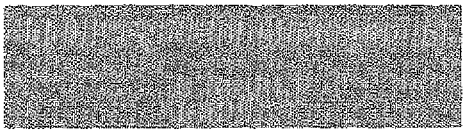
Solvent mixture. A mixture of 80 volumes of a buffer solution prepared by dissolving 11.04 g of *monobasic sodium phosphate* in 1000 ml of *water*, adjusted to pH 2.8 with *orthophosphoric acid* and 20 volumes of *methanol*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 10 mg of Timolol Maleate in the solvent mixture, with the aid of ultrasound and dilute to 100.0 ml with the solvent mixture.

Reference solution (a). A 0.01 per cent w/v solution of *timolol maleate IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.01 per cent w/v of *timolol maleate IPRS* and 0.001 per cent w/v *timolol related compound D IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 10 cm x 2.1 mm, packed with octadecylsilane bonded to porous silica (2.6 µm),
- mobile phase: A. a 0.05 per cent v/v solution of *trifluoroacetic acid* in *water*,
  B. a 0.05 per cent v/v solution of *trifluoroacetic acid* in *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 0.4 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 10 µl.



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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	84	16
2.4	84	16
8.0	20	80
8.1	84	16
11	84	16

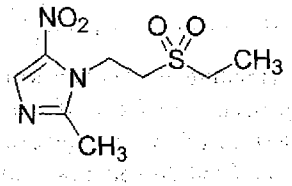
Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to timolol maleate and timolol related compound D is not less than 2.5 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 2.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{13}H_{24}N_4O_3S,C_4H_4O_4$ in the tablets.

Storage. Store protected from moisture.

Tinidazole



$C_8H_{13}N_3O_4S$ Mol. Wt. 247.3

Tinidazole is 1-[2-(ethylsulphonyl)ethyl]-2-methyl-5-nitroimidazole.

Tinidazole contains not less than 98.0 per cent and not more than 100.5 per cent of $C_8H_{13}N_3O_4S$, calculated on the dried basis.

Category. Antiprotozoal.

Description. Pale yellow crystals or a crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tinidazole IPRS* or with the reference spectrum of tinidazole.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *methanol* shows an absorption maximum at about 310 nm; absorbance at about 310 nm, about 0.35.

C. To about 5 mg, add 5 ml of *0.1 M hydrochloric acid*, 50 mg of *zinc powder*, 4 ml of *hydrochloric acid* and allow to stand for 30 minutes. Add 4 ml of a 1 per cent w/v solution of *vanillin*, heat on a boiling water-bath for 20 minutes, allow to cool to room temperature and dilute to 20 ml with *water*; a greenish yellow colour is produced.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Protect the solutions from light.

Test solution. Dissolve 10 mg of the substance under examination in 10.0 ml of *methanol* and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg each of *tinidazole impurity A IPRS (2-methyl-5-nitro-1H-imidazole IPRS)* and *tinidazole impurity B IPRS (1-[2-(ethylsulphonyl)ethyl]-2-methyl-4-nitro-1H-imidazoleRS)* in 10 ml of *methanol* and dilute to 100.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 3 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 10 volumes of *acetonitrile*, 20 volumes of *methanol* and 70 volumes of *water*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 320 nm,
- injection volume: 20 µl.

The relative retention time with reference to tinidazole for tinidazole impurity A is about 0.6 and for tinidazole impurity B is about 0.7.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to tinidazole impurity A and B is not less than 2.0.

Inject reference solution (a), (c) and the test solution. Run the chromatogram 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak due to tinidazole impurity A and B is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 4 times the

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area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 2.0 per cent, determined on 1.0 g by drying in an oven at 105° for 4 hours.

Assay. Dissolve 0.5 g in 30 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, using 0.15 ml of *nile blue A solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02473 g of $C_8H_{13}N_3O_4S$.

Storage. Store protected from light and moisture.

Tinidazole Tablets

Tinidazole Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of tinidazole, $C_8H_{13}N_3O_4S$.

Usual strengths. 150 mg; 300 mg; 500 mg.

Identification

A. When examined in the range 230 nm to 360 nm (2.4.7), the final solution obtained in the Assay shows an absorption maximum at about 310 nm.

Extract a quantity of the powdered tablets containing 0.1 g of Tinidazole with 10 ml of *methanol*, filter and evaporate the filtrate to dryness. The residue complies with the following tests.

B. To about 5 mg add 5 ml of *0.1 M hydrochloric acid*, 50 mg of *zinc powder*, 4 ml of *hydrochloric acid* and allow to stand for 30 minutes. Add 4 ml of a 1 per cent w/v solution of *vanillin*, heat on a boiling water-bath for 20 minutes, allow to cool to room temperature and dilute to 20 ml with *water*; a greenish yellow colour is produced.

C. Melting range (2.4.21). 125° to 128°.

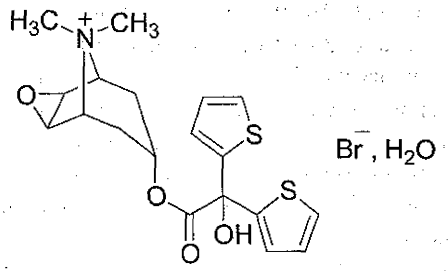
Tests

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.15 g of Tinidazole, add 20 ml of *methanol*, shake well and add sufficient *methanol* to produce 100.0 ml. Mix well and filter. Dilute 10.0 ml of the solution to 100.0 ml with *methanol* and further dilute 10.0 ml of the solution to 100.0 ml with *methanol*. Measure the absorbance of the resulting solution at the maximum at about 310 nm (2.4.7). Calculate the content of $C_8H_{13}N_3O_4S$ taking 356 as the specific absorbance at 310 nm.

Storage. Store protected from light and moisture.

Tiotropium Bromide Monohydrate



$C_{19}H_{22}BrNO_4S_2, H_2O$ Mol. Wt. 490.4

Tiotropium Bromide Monohydrate is (1*R*,2*R*,4*S*,5*S*,7*s*)-7-[[Hydroxy[di(thiophen-2-yl)]acetyl]oxy]-9,9-dimethyl-3-oxa-9-azatricyclo[3.3.1.$0^{2,4}$]nonan-9-ium bromide monohydrate.

Tiotropium Bromide Monohydrate contains not less than 98.5 per cent and not more than 101.5 per cent of tiotropium bromide, $C_{19}H_{22}NO_4S_2Br$, calculated on the anhydrous basis.

Category. Bronchodilator.

Description. A white to off-whitepowder or crystals.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tiotropium bromide monohydrate IPRS* or with the reference spectrum of tiotropium bromide monohydrate.

B. It gives reaction (a) of bromide (2.3.1).

Tests

Appearance of solution. A 1.0 per cent w/v solution is clear (2.4.1) and not more intensely coloured than reference solution YS6 (2.4.1).

Impurities G and H. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

NOTE — Prepare the solutions immediately before use.

Solvent mixture. Dilute 1 ml of *3M hydrochloric acid* to 100 ml with *methanol*.

Mobile phase. A mixture of 10 volumes of *water*, 15 volumes of *anhydrous formic acid*, 35 volumes of *acetonitrile* and 50 volumes of *methylene chloride*.

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Test solution. Dissolve 0.4 g of the substances under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). Dissolve the contents of a vial of *tiotropium impurity mixture IPRS* (containing impurities G and H) in 1.0 ml of the solvent mixture.

Reference solution (b). Mix 0.1 ml of the test solution with 0.1 ml of reference solution (a).

Apply to the plate 10 µl of the test solution and reference solution (a), and 20 µl of reference solution (b). After development, dry the plate in air and expose to iodine vapors till spots appear (about 15 minutes), remove the plate and examine immediately. In the chromatogram obtained with the test solution, any secondary spot corresponding to, each of, tiotropium impurity G and H is not more intense than the corresponding spot of tiotropium impurity G and H in the chromatogram obtained with reference solution (a) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots, of each, tiotropium impurity G and H and tiotropium with R_f values of about 0.33, 0.38 and 0.64, respectively.

Related substances.Determine by liquid chromatography (2.4.14).

NOTE — Carry out the test protected from light.

Test solution. Dissolve 50 mg of the substance under examination in mobile phase B and dilute to 25.0 ml with mobile phase B.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase B. Dilute 1.0 ml of the solution to 10.0 ml with mobile phase B.

Reference solution (b). Dissolve 4 mg of *tiotropium for system suitability IPRS* (containing impurity C) in 2.0 ml of mobile phase B.

Chromatographic system

- a stainless steel column 15 cm x 3.0 mm packed with propylsilane bonded to porous silica (3.5 µm),
- column temperature; 50°,
- mobile phase A: dissolve 1.0 g of *sodium methanesulphonate* and 5.0 g of *potassium dihydrogen phosphate* in 980 ml of *water* adjusted to pH 3.0 with *dilute phosphoric acid* and dilute to 1000 ml with *water*,
  B: a mixture of 10 volumes of *methanol*, 40 volumes of *acetonitrile* and 50 volumes of mobile phase A,
- a gradient programme using the conditions given below,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
3	90	10
17	80	20
28	25	75
30	25	75
35	90	10

The relative retention time with reference to tiotropium (retention time: about 15 minutes) for tiotropium impurity C is about 1.2.

Inject reference solution (b). The test is not valid unless the resolution between the peak due to tiotropium and tiotropium impurity C is not less than 2.4.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of anysecondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). 2.5 per cent to 4.0 per cent, determined on 0.3 g.

Assay. Determine by liquid chromatography (2.4.14).

*Test solution.*Dissolve 20 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.002 per cent w/v solution of *tiotropium bromide monohydrate IPRS* equivalent to tiotropium bromide in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 55 volumes of a buffer solution prepared by dissolving 3.85 g of *ammonium acetate* in 1000 ml of *water*, adjusted to pH 5.5 with *dilute acetic acid* and 45 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 235 nm,
- injection volume: 20 µl.

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Inject the reference solution. The test is not valid unless the column efficiency in not less than 3000 theoretical plates, tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{22}NO_4S_2,Br$.

Storage. Store protected from light and moisture.

Tiotropium Bromide Powder for Inhalation

Tiotropium Powder for Inhalation consists of Tiotropium Bromide Monohydrate in microfine powder either alone or admixed with Lactose in a pre-metered unit for use in a suitable powder inhaler.

Tiotropium Powder for Inhalation contains not less than 90.0 per cent and not more than 125.0 per cent of the stated amount of tiotropium, $C_{19}H_{22}NO_4S_2$ per unit dose.

Usual strengths. 9 µg; 18 µg

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Other tests. Comply with the tests stated under Inhalation Preparations (Powders for Inhalation).

Follow the procedure described under Assay with suitable dilution of the reference solution wherever the amount of active substance is to be determined in any test.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 90 volumes of 0.05 per cent v/v *orthophosphoric acid* and 10 volumes of *acetonitrile*.

Test solution. Dissolve a quantity of the mixed contents of 20 capsules in sufficient of the solvent mixture to get a solution containing 1.8 µg per ml of Tiotropium.

Reference solution. A solution of *tiotropium bromide IPRS* equivalent to tiotropium 1.8 µg per ml in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 80 volumes of a buffer solution prepared by dissolving 2 ml *triethylamine* in 1000 ml of *water* and adjusted to pH 2.5 with *orthophosphoric acid*, and 20 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 237 nm,
- inject 200 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 4500 theoretical plates, the tailing factor is not more than 1.7 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{22}NO_4S_2$ per unit.

Storage. Store protected from moisture, at temperature not exceeding 30°.

Labelling. The label states the quantity of tiotropium per pre-metered unit.

Titanium Dioxide

TiO_2 Mol. Wt. 79.9

Titanium Dioxide contains not less than 98.0 per cent and not more than 100.5 per cent of TiO_2.

Category. Pharmaceutical aid and topical protectant.

Description. A white or almost white, infusible powder.

Identification

A. When strongly heated it becomes pale yellow; the colour is discharged on cooling.

B. To 0.5 g, add 5 g of *anhydrous sodium sulphate* and 10 ml of *water* and mix. Add 10 ml of *sulphuric acid* and boil gently until clear; cool, add slowly 30 ml of a 25 per cent v/v solution of *sulphuric acid* and dilute with *water* to 100 ml (solution A). To 5 ml of solution A add 0.1 ml of *strong hydrogen peroxide solution*; an orange-red colour is produced.

C. To 5 ml of solution A add 0.5 g of *zinc*, in granules; after 45 minutes a violet-blue colour is produced.

Tests

Appearance of solution. Solution A is not more opalescent than opalescence standard OS2 (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. Shake 5.0 g with 50 ml of *carbon dioxide-free water* for 5 minutes and centrifuge until a clear solution is obtained. To 10 ml of the solution add 0.1 ml of *bromothymol blue solution*. Not more than 1.0 ml of either *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the solution.

Water-soluble substances. Not more than 0.5 per cent, determined by the following method. Boil 10.0 g for 5 minutes

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with 150 ml of *water* containing 0.5 g of *ammonium sulphate*. Cool, dilute to 200 ml with *water* and filter until a clear solution is obtained. Evaporate 100 ml of the filtrate to dryness ignite and weigh.

Arsenic (2.3.10). To 0.2 g in a 100 ml Kjeldahl flask, add 2 g of *anhydrous sodium sulphate*, 7 ml of *sulphuric acid* and 5 ml of *nitric acid*. Heat gently until a clear solution is obtained, cool, add 10 ml of *water*, cool again and add 5 g of *hydrazine reducing mixture* and 10 ml of *hydrochloric acid*. Immediately attach an air condenser and distil into 15 ml of cooled *water* until a total volume of 30 ml is obtained. Rinse the condenser with 5 ml of *water* and dilute the combined distillate and rinsings to 40 ml with *water*. 20 ml of the resulting solution complies with the limit test for arsenic. Use a mixture of 0.5 ml of *arsenic standard solution (1 ppm As)* and 24.5 ml of *water* to prepare the standard (5 ppm).

Barium. Shake 20.0 g with 30 ml of *hydrochloric acid*, add 100 ml of *distilled water* and boil. Filter while hot through a hardened filter paper until a clear filtrate is obtained. Wash the filter with 60 ml of *distilled water* and dilute the combined filtrate and washings to 200 ml with *distilled water*. To 10 ml of the solution add 1 ml of *1 M sulphuric acid*. After 30 minutes any opalescence is not more intense than that of a mixture of 10 ml of the test solution and 1 ml of *distilled water*.

Heavy metals (2.3.13). Dilute 10 ml of the solution prepared in the test for Barium to 20 ml with *water*. 12 ml of the solution complies with the limit test for heavy metals, Method D (20 ppm), using 10 ml of *lead standard solution (1 ppm pb)*.

Iron. To 8 ml of solution A, add 4 ml of *water*, mix and add 0.05 ml of *bromine water*, allow to stand for 5 minutes, remove the excess of bromine with a current of air and add 3 ml of *potassium thiocyanate solution*. Any colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using a mixture of 4 ml of *iron standard solution (2 ppm Fe)* and 8 ml of a 20 per cent w/v solution of *sulphuric acid* (200 ppm).

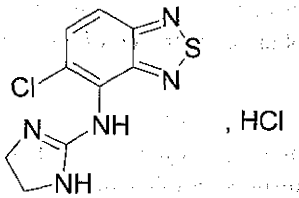
Assay. Weigh 0.5 g, transfer to a 300 ml Kjeldahl flask, add 5 g of *anhydrous sodium sulphate* and 10 ml of *water*, mix and add 10 ml of *sulphuric acid*. Boil gently until clear, cool, add slowly 40 ml of cooled *sulphuric acid* (25 per cent), cool again and dilute with *water* to 100.0 ml (solution B). To 300 g of *zinc*, in granules, add 300 ml of a 2 per cent w/v solution of *mercuric nitrate* and 2 ml of *nitric acid*, shake for 10 minutes and wash with *water*. Pack the zinc amalgam into a glass tube (400 mm x 20 mm) fitted with a tap and a filter plate. Pass through the column, at a rate of about 3 ml per minute, 100 ml of *1 M sulphuric acid* followed by 100 ml of *water*, ensuring that the amalgam is covered with liquid throughout. Pass slowly through the column, at a rate of about 3 ml per minute, 200 ml of *0.5 M sulphuric acid* followed by 100 ml of *water*. Collect the combined eluates in a 500-ml conical flask containing 50 ml of a 15 per cent w/v solution of *ferric ammonium sulphate* in *sulphuric acid (25 per cent)* and titrate immediately with *0.1 M ceric ammonium nitrate* using *ferroin solution* as indicator until a greenish colour is obtained (n_1 ml). Pass slowly through the column 100 ml of *0.5 M sulphuric acid* followed by 20.0 ml of solution B, wash with 100 ml of *0.5 M sulphuric acid* followed by 100 ml of water. Collect the combined eluates in a 500-ml conical flask containing 50 ml of a 15 per cent w/v solution of *ferric ammonium sulphate* in *sulphuric acid (25 per cent)*, rinse the lower end of the column with *water* and titrate immediately with *0.1 M ceric ammonium nitrate* using *ferroin solution* as indicator until a greenish colour is obtained (n_2 ml).

Calculate the percentage content of TiO_2 from the expression $3.99(n_2 - n_1)/w$

Where, *w* is the weight, in g, of the substance under examination used in the preparation of solution B.

Storage. Store protected from moisture. Avoid contact with aluminium.

Tizanidine Hydrochloride



$C_9H_8ClN_5S, HCl$ Mol. Wt. 290.2

Tizanidine Hydrochloride is 5-chloro-*N*-(2-imidazolin-2-yl)-2,1,3-benzothiadiazol-4-yl amine.

Tizanidine Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_9H_8ClN_5S.HCl$, calculated on the dried basis.

Category. α-adrenergic agonist.

Description. A white to yellowish white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tizanidine hydrochloride IPRS*.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

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C. It gives reaction (A) for chlorides (2.3.1).

Tests

pH (2.4.24). 3.5 to 5.3, determined on 5.0 per cent w/v solution in *water*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of the mobile phase.

Reference solution (a). A 0.1 per cent w/v solution of *tizanidine hydrochloride IPRS* in the mobile phase.

Reference solution (b). Dilute 1 ml of reference solution (a) to 100 ml with mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: a mixture of 80 volumes of buffer solution prepared by dissolving 3.5 g of *sodium-1-pentane sulphonate* in 1000 ml of *water*, adjusted to pH 3.0 with 12 per cent *orthophosphoric acid solution* or *1 M sodium hydroxide* and 20 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the tailing factor is not more than 2.0 and the column efficiency is not less than 5000 theoretical plates.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than the area of the peak in the chromatogram obtained with reference solution (b) (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method A (20 ppm).

Total Chloride. 11.9 per cent to 12.5 per cent.

Weigh 0.5 g and dissolve in 50 ml of *water*. Titrate with *0.1 M silver nitrate*. Determine the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of chloride.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1 g by drying in an oven at 105° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in 50.0 ml of the mobile phase. Dilute 10.0 ml of the solution to 50.0 ml with the same solvent.

Reference solution. A 0.01 per cent w/v solution of *tizanidine hydrochloride IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 15 cm × 4.6 mm packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of a buffer solution prepared by dissolving 6.8 g of *monobasic potassium phosphate* in 1000 ml of *water* adjusted to pH 7.5 with *5.3 M potassium hydroxide*, and 50 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_9H_8ClN_5S.HCl$.

Storage. Store protected from light.

Tizanidine Tablets

Tizanidine Hydrochloride Tablets

Tizanidine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of tizanidine, $C_9H_8ClN_5S$.

Usual strengths. 2 mg; 4 mg; 6 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium: 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, suitably diluted with dissolution

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medium if necessary, at the maximum at about 320 nm (2.4.7). Calculate the content of $C_9H_8ClN_5S$ in the medium from the absorbance obtained from a solution of known concentration of *tizanidine Hydrochloride IPRS* in the same medium.

Q. Not less than 70 per cent of the stated amount of $C_9H_8ClN_5S$.

Uniformity of content. Complies with the tests stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay.

Test solution. Crush one tablet and disperse in 50 ml *phosphate buffer pH 6.6*, dilute to 100.0 ml with *acetonitrile* and filter.

Reference solution. Dissolve about 10 mg of *tizanidine hydrochloride IPRS*, in 25 ml of *phosphate buffer pH 6.6* and dilute to 50.0 ml with *acetonitrile*. Dilute a suitable volume of the solution in a mixture of equal volumes of *phosphate buffer pH 6.6* and *acetonitrile* to obtain a concentration similar to the test solution.

Calculate the content of $C_9H_8ClN_5S$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powdered tablet containing about 20 mg of Tizanidine, disperse in 50 ml *phosphate buffer pH 6.6* and dilute to 100.0 ml with *acetonitrile* and filter.

Reference solution. Weigh 10 mg of *tizanidine hydrochloride IPRS*, dissolve in 25 ml *phosphate buffer pH 6.6* and dilute to 50.0 ml with *acetonitrile*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 80 volumes of *phosphate buffer pH 6.6* and 20 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 320 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

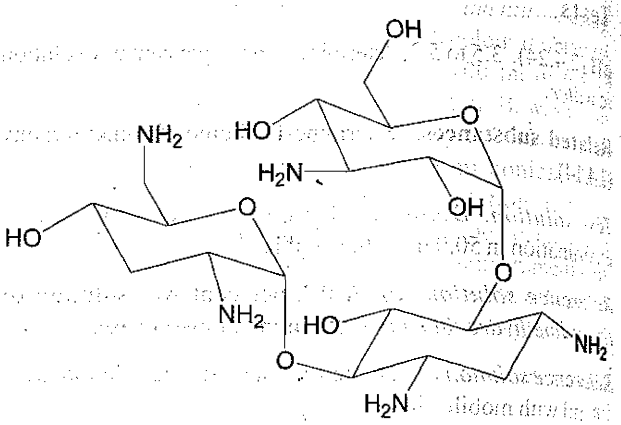
Inject the reference solution and the test solution.

Calculate the content of $C_9H_8ClN_5S$.

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of the equivalent amount of Tizanidine.

Tobramycin



$C_{18}H_{37}N_5O_9$ Mol. Wt. 467.3

Tobramycin is 6-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-2-deoxy-4-*O*-(2,6-diamino-2,3,6-trideoxy-α-D-*ribo*-hexopyranosyl)-D-streptamine, an antimicrobial substance produced by *Streptomyces tenebrarius* or by any other means.

Tobramycin has potency not less than 900 mcg of $C_{18}H_{37}N_5O_9$ per mg, calculated on anhydrous basis.

Category. Antibacterial.

Description. A white or almost white powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H*.

Mobile phase. A mixture of 60 volumes of *methanol*, 40 volumes of *strong ammonia solution* and 20 volumes of *chloroform*.

Test solution. Dissolve 0.4 g of the substance under examination in 100 ml of *water*.

Reference solution (a). A 0.4 per cent w/v solution of *tobramycin IPRS* in *water*.

Reference solution (b). A solution containing 0.4 per cent w/v each of *kanamycin sulphate IPRS*, *neomycin sulphate IPRS* and *tobramycin IPRS* in *water*.

Apply to the plate 5 µl of each solution. After development, dry the plate in warm air, spray with a mixture of equal volumes of a 46 per cent w/v solution of *sulphuric acid* and a 0.2 per cent w/v solution of *1,3-naphthalenediol* in *ethanol (95 per cent)* and heat at 105° for 10 minutes. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated principal spots.

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B. Dissolve about 5 mg in 5 ml of *water*, add 5 ml of a 0.1 per cent w/v solution of *ninhydrin* in *ethanol (95 per cent)* and heat in a water-bath for 3 minutes; a violet-blue colour develops.

Tests

pH (2.4.24). 9.0 to 11.0, determined in a 10.0 per cent w/v solution.

Specific optical rotation (2.4.22). + 138° to + 148°, determined in a 4.0 per cent w/v solution.

2-Methyl-1-propanol. Not more than 1.0 per cent w/w, determined by gas chromatography (2.4.13).

Test solution (a). A 10 per cent w/v solution of the substance under examination in *water*.

Test solution (b). A solution containing 10 per cent w/v of the substance under examination and 0.2 per cent v/v of *2-propanol* (internal standard).

Reference solution. A solution containing 0.1 per cent w/v of *2-methyl-1-propanol* and 0.2 per cent v/v of the internal standard.

Chromatographic system
- a glass column 1.5 m x 4 mm, packed with porous polymer beads (80 to 100 mesh) (Such as Porapak Q),
- temperature: column 165°,

Calculate the percentage w/w of 2-methyl-1-propanol.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H*.

Mobile phase. A mixture of equal volumes of *strong ammonia solution*, *2-butanone* and *ethanol (95 per cent)*.

Test solution. Dissolve 0.8 g of the substance under examination in 100 ml of *0.02 M ammonia*.

Reference solution. A 0.008 per cent w/v solution of the substance under examination in *0.02 M ammonia*.

Apply to the plate 5 µl of each solution. After development, dry the plate in warm air, heat at 110° for 10 minutes and spray the hot plate with a solution prepared immediately before use by diluting *sodium hypochlorite solution (3 per cent Cl)* with *water* to contain 0.5 per cent w/v of available chlorine. Dry in a current of cold air until a sprayed area of the plate below the line of application gives at most a very faint blue colour with a drop of *potassium iodide and starch solution*; avoid prolonged exposure to cold air. Spray the plate with *potassium iodide and starch solution*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Sulphated ash (2.3.18). Not more than 0.3 per cent.

Water (2.3.43). Not more than 8.0 per cent, determined on 0.3 g.

Assay. Determine by liquid chromatography (2.4.14).

2, 4-Dinitrofluorobenzene reagent. A 1.0 per cent w/v solution of *2,4-dinitrofluorobenzene* in *absolute alcohol*. This solution may be used for 5 days if refrigerated when not in use.

Tris (hydroxymethyl) aminomethane reagent. A 1.5 per cent w/v solution of *tris (hydroxymethyl) aminomethane* in *water*. This solution may be used for 1 month if refrigerated when not in use. To 40 ml of the solution add *dimethyl sulphoxide* with mixing, dilute with *dimethyl sulphoxide* to 200 ml, and mix. Use this reagent within 4 hours (*NOTE — If kept immersed in an ice –water bath below 10°, the reagent may be used for up to 8 hours*).

Test solution (a). Dissolve 55 mg of the substance under examination in 1 ml of *0.5M sulphuric acid* and sufficient *water* and dilute to 50.0 ml with *water*. Dilute 10.0 ml of the solution to 50.0 ml with *water*.

Test solution (b). To 4.0 ml of test solution (a), add 10 ml of *2,4 dinitrofluorobenzene reagent* and 10 ml of *tris(hydroxymethyl)aminomethane reagent*, shake, and insert the stopper. Place the flask in a constant temperature bath at 60 ± 2°, and heat for 50 ± 5 minutes. Remove the flasks from the bath, and allow to stand for 10 minutes. Add *acetonitrile* to about 2 ml below the 50 ml mark, allow to cool to room temperature, then dilute with *acetonitrile* to volume, and mix.

Reference solution (a). Dissolve 55 mg of *tobramycin IPRS* in 1 ml of *0.5M sulphuric acid* and enough *water* and dilute to 50.0 ml with *water*. Dilute 10.0 ml of the solution to 50.0 ml with *water*.

Reference solution (b). To 4.0 ml of reference solution (a), add 10 ml of *2,4 dinitrofluorobenzene reagent* and 10 ml of *tris(hydroxymethyl)aminomethane reagent*, shake, and insert the stopper. Place the flask in a constant temperature bath at 60 ± 2 °, and heat for 50 ± 5 minutes. Remove the flasks from the bath, and allow to stand for 10 minutes. Add *acetonitrile* to about 2 ml below the 50 ml mark, allow to cool to room temperature, then dilute with *acetonitrile* to volume, and mix.

Reference solution (c). A 0.024 per cent w/v solution of *p-naptholbenzein* in *acetonitrile*. Dilute 2.0 ml of the solution with reference solution (b) to 10.0 ml and use promptly.

Blank solution. To 4.0 ml of *water*, add 10 ml of *2,4 dinitrofluorobenzene reagent* and 10 ml of *tris(hydroxymethyl)aminomethane reagent*, shake, and insert the stopper. Place the flask in a constant temperature bath at 60 ± 2°, and heat for 50 ± 5 minutes. Remove the flasks from the bath, and allow to stand for 10 minutes. Add acetonitrile to about 2 ml below the 50 ml mark, allow to cool to room temperature, then dilute with *acetonitrile* to volume, and mix.

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NOTE — Heat all solutions at the same temperature and for the same duration of time as indicated. Move all flasks to and from the 60 ± 2° constant temperature bath at the same time.

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (1.5 to 10 µm) (Such as Waters Bondapack),
- mobile phase : dissolve 2.0 g of *tris(hydroxymethyl) aminomethane* in about 800 ml of *water*. To this solution, add 20 ml of *0.5 M sulphuric acid*, dilute with *acetonitrile* to obtain 2000 ml of solution,
- flow rate : 1.2 ml per minute,
- spectrophotometer set at 365 nm,
- injection volume : 20 µl.

The relative retention time with respect to tobramycin for *p*-naphtholbenzein is about 0.6.

Inject the blank solution and record the responses to identify the solvent and reagent peaks.

Inject reference solution (b) and (c). The test is not valid unless resolution between the peaks due to p-naphtholbenzein and tobramycin is not less than 4.0 in the chromatogram obtained with reference solution (c) and relative standard deviation for replicate injection is not more than 2.0 per cent in reference solution (b).

Inject reference solution (b) and test solution (b).

Calculate the content of $C_{18}H_{37}N_5O_9$.

Tobramycin intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 2.0 Endotoxin Units per mg of tobramycin.

Tobramycin intended for use in the manufacture of parenteral preparations or eye drops without a further appropriate sterilisation procedure complies with the following additional requirements.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store protected from moisture. If the material is sterile, the container should be sterile, tamper-evident and sealed so as to exclude micro-organisms.

Labelling. The label states (1) the number of Units per mg; (2) where applicable, that it is sterile; (3) where applicable, that it is free from bacterial endotoxins and depressor substances.

Tobramycin Injection

Tobramycin Sulphate Injection

Tobramycin Injection is a sterile solution of Tobramycin Sulphate in Water for Injections or of Tobramycin in Water for Injections containing sufficient Sulphuric Acid to adjusted to pH 3.5 to 6.0.

Tobramycin Injection contains not less than 90.0 per cent and not more than 120.0 per cent of the stated amount of tobramycin, $C_{18}H_{37}N_5O_9$.

Usual strengths. 10 mg per ml; 40 mg per ml.

Description. A colourless solution.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H*.

Mobile phase. A mixture of 60 volumes of *methanol*, 40 volumes of *strong ammonia solution* and 20 volumes of *chloroform*.

Test solution. Dilute a suitable volume of the injection with *water* to produce a solution containing 0.4 per cent w/v solution of tobramycin.

Reference solution (a). A 0.4 per cent w/v solution of *tobramycin IPRS* in *water*.

Reference solution (b). A solution containing 0.4 per cent w/v each of *kanamycin sulphate IPRS*, *neomycin sulphate IPRS* and *tobramycin IPRS* in *water*.

Apply to the plate 5 µl of each solution. After development, dry the plate in warm air, spray with a mixture of equal volumes of a 46 per cent w/v solution of *sulphuric acid* and a 0.2 per cent w/v solution of *1,3-naphthalenediol* in *ethanol (95 per cent)* and heat at 105° for 10 minutes. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated principal spots.

B. It gives the reactions of sulphates (2.3.1).

Tests

pH (2.4.24). 3.5 to 6.0.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel H*.

Mobile phase. A mixture of equal volumes of *strong ammonia solution*, *2-butanone* and *ethanol (95 per cent)*.

Test solution. Dilute a suitable volume of the injection with *0.01 M ammonia* to obtain a solution containing 40 mg of

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Tobramycin in 4 ml. Shake with 10 ml of *ether* and use the aqueous layer.

Reference solution. A 0.02 per cent w/v solution of the substance under examination in *0.02 M ammonia.*

Apply to the plate 5 µl of each solution. After development, dry the plate in warm air, heat at 110° for 10 minutes and spray the hot plate with a solution prepared immediately before use by diluting *sodium hypochlorite solution (3 per cent Cl)* with *water* to contain 0.5 per cent w/v of available chlorine. Dry in a current of cold air until a sprayed area of the plate below the line of application gives at most a very faint blue colour with a drop of *potassium iodide and starch solution*; avoid prolonged exposure to cold air. Spray the plate with *potassium iodide and starch solution*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Bacterial endotoxins (2.2.3). Not more than 2.0 Endotoxin Units per mg of tobramycin.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by the microbiological assay of antibiotics, Method B (2.2.10).

Calculate the content of tobramycin in the injection, taking each 1000 Units found to be equivalent to 1 mg of tobramycin.

Tobramycin Inhalation Solution

Tobramycin Inhalation Solution is a sterile, non pyrogenic, preservative free solution of Tobramycin or Tobramycin Sulphate in Water for Injection containing *sodium chloride*.

Tobramycin Inhalation Solution contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of tobramycin $C_{18}H_{37}N_5O_9$.

Usual strength. 300 mg per 5 ml.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution(b).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution A 0.0192 per cent of the substance under examination in *water.*

Reference solution(a). A 0.11 per cent w/v solution of *tobramycin IPRS* in *water*, and adjusted to pH 6.0 with *0.5 M sulphuric acid,* and dilute with *water.*

Reference solution(b). A 0.022 per cent of tobramycin from the reference solution (a) in *water.*

Reference solution (c). Heat a portion of reference solution (a) in a suitable sealed glass container at 100° for 8-9 hours.Cool to room temperature, and dilute with *water* to obtain a 0.022 per cent w/v solution of tobramycin.

Reference solution(d). A 0.11 per cent w/v solution of *tobramycin IPRS* prepared by adding *0.5M sulphuric acid,* using 2.0 per cent of the final volume, and enough water to dissolve the tobramycin, and dilute with *water* to volume.

Reference solution(e). 0.00011 per cent of tobramycin from the reference solution (d) in *water.*

Blank.Water.

Derivatization procedure. Heat all solutions at the same temperature and for the same duration as indicated. Move all flasks to 60° constant water-bath at the same time. To separate 50 ml flasks, transfer 15.0 ml of reference solution(b), 15.0 ml of reference solution (c), 15.0 ml of reference solution (e), 15.0 ml of test solution, and 15.0 ml of blank. To each flask add 10 ml of *2,4-dinitrofluorobenzene reagent* and 10 ml of *tris(hydroxymethyl)aminomethane reagent*, shake, and insert the stopper. Place the flasks in a constant temperature bath at 60°, and heat for 50 minutes. Remove the flasks from the bath, and allow to stand for 10 minutes, Add 2.0 ml of *acetonitrile* allow to cool to room temperature, and dilute with *acetonitrile*, to 50.0 ml. Allow the solutions to stand for 16 hours. The solutions thus obtained are derivatized reference solution (b), derivatized reference solution (c), reference solution (e), test solution, and blank solution.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with phenyl group chemically bonded to porous silica (5 µm),
- mobile phase: A.a mixture of 5 volumes of *acetonitrile*, 95 volumes of *water* and 0.08 volumes of *ortho-phosphoric acid,*
  B.a mixture of 75 volumes of *acetonitrile,* 25 volumes of *water* and 0.08 volumes of *ortho-phosphoric acid,*
- a gradient programme using the conditions given below,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 365 nm,
- injection volume: 45 µl.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	79	21
14	66	34
25	30	70
35	30	70
40	20	80
50	5	95
50.1	79	21
55	79	21

Name	Relative retention time
Specified unidentified impurity	0.36
Deoxystreptamine kanosaminide	0.66
Nebramine	0.94
Kanamycin B	0.96
Tobramycin	1.0

Inject derivatised reference solution (c).The test is not valid unless the capacity factor is not less than 15.5 for tobramycin, and the resolution is not less than 1.0 between the nebramine and kanamycin peaks.

Compare the derivatised reference solution (b) and (c) chromatograms to identify degradation peaks. Deoxystreptamine kanosaminide and nebramine will increase in response in derivatized reference solution (c)

Inject the derivatized reference solution (b), (e) and blank. The relative standard deviation for replicate injections is not more than 2.0 per cent with reference solution (e). Disregard any peak corresponding to those from the derivatised blank solution and substract the quantities of any such peaks found at the relative retention time of 0.36,0.66,0.94 from those found in the derivatized test solution.For unknown peak determination,disregards any peak found in the chromatogram of the derivatised test solution that correspond to those in the chromatogram of derivatised reference solution (b).

Inject reference solution (b) (e), the test solution and blank.The area of impurity due to specified unidentified impurity should not be more than 0.4 times the area of principal peak in the chromatogram obtained with reference solution (e) (0.25 per cent),The area of impurity due to deoxystreptamine kanosaminide should not be more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.3 per cent),the area of impurity due to nebramine should not be more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.4 per cent),the area of any secondary unspecified impurity is not more than 0.17 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.1 per cent),the sum of areas of unspecified impurity is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.2 per cent), the area of total impurity is not more than 1.74 times the area of the principal peak in the chromatogram obtained with reference solution (e) (1.0 per cent).

Content of sodium chloride. Sodium chloride contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of sodium chloride.

Solution A. 2.0 g of gelatin and add 50.0 ml of *nitric acid* in 1000 ml of *water*.

Transfer 25 ml of tobramycin inhalation solution in a flask add 80 ml of *water* and 10 ml of solution A. titrate potentiometrically with *0.1 M silver nitrate* using silver electrode.

1ml of *0.1M silver nitrate* is equivalent to 0.005844 g of sodium chloride.

Light absorption (2.4.7). Absorbance of the sample at the maximum at about 410 nm in a 1-cm cell is not more than 0.30.

Other tests. Comply with the test stated under Inhalation Prepration and Parentral Preparation(Injections).

pH (2.4.24). 4.5 to 6.5.

Bacterial endotoxin (2.2.3). Not more than 60 Endotoxin Units per ml.

Sterility (2.2.11).Complies with the test for sterility.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. A 0.0192 per cent w/v of tobramycin from the inhalation solution in *water*.

Reference solution(a). A 0.11 per cent w/v solution of *tobramycin IPRS* prepared by adding *0.5M sulphuric acid*, using 2.0 per cent of the final volume, and enough *water* to dissolve the tobramycin, and dilute with *water* to volume.

Reference solution (b). A 0.022 per cent w/v of *tobramycin IPRS* from the reference solution (a) in *water*.

Blank. Water.

Derivatization procedure. Heat all solutions at the same temperature and for the same duration of time as indicated. Move all flasks to 60° constant water-bath at the same time. To separate 50 ml volumetric flasks, transfer 4.0 ml of the reference solution (b), 4.0 ml of the test solution, and 4.0 ml of the *water*. To each flask add 10 ml of *2,4-dinitrofluoro-benzene reagent* and 10 ml of *tris(hydroxymethyl) aminomethane* reagent, shake, and insert the stopper. Place the flasks in a constant temperature bath at 60°, and heat for 50 minutes. Remove the flasks from the bath, and allow to stand for 10 minutes. Add 2.0 ml of *acetonitrile* allow to cool to room temperature, then dilute with *acetonitrile* to 50.0 ml.

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The solutions thus obtained are the derivatized reference solution(b), derivatized test solution and derivatized blank solution, respectively.

Reference solution(c). A 0.024 per cent of *p-naphtholbenzein* in *acetonitrile*: Transfer 2.0 ml of the solution to a 10.0 ml volumetric flask, dilute with derivatized reference solution (b) to volume, and use promptly.

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: Dissolve 2.0 g of *tris(hydroxymethyl) aminomethane* in about 800 ml of *water*. To this solution add 20 ml of *0.5 M sulphuric acid*, and dilute with *acetonitrile* to obtain 2000 ml of solution,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 365 nm,
- injection volume: 20 µl.

Inject derivatized reference solution (b) and derivatized blank solution, and reference solution(c). The relative retention times for p-naphtholbenzein and tobramycin are about 0.6 and 1.0, respectively. Identify the solvent and reagent peaks using the derivatized blank solution. Resolution between p-naphtholbenzein and tobramycin is not less than 4.0 obtained with reference solution (c) and relative standard deviation is not more than 2.0 per cent for derivatized reference solution (b).

Inject the reference solution(b) and the test solution.

Calculate the contents of tobramycin, $C_{18}H_{37}N_5O_9$.

Storage. Store protected from light and keep in a refrigerator.

Tobramycin and Fluorometholone Acetate Ophthalmic Suspension

Tobramycin and Fluorometholone Acetate Ophthalmic Suspension is a sterile aqueous solution of Tobramycin Sulphate and Fluorometholone Acetate in a suitable aqueous vehicle. It may contain suitable buffers, dispersants, tonicity-adjusting agents and preservatives.

Tobramycin and Fluorometholone Acetate Ophthalmic Suspension contains not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of tobramycin $C_{18}H_{37}N_5O_9$ and not less than 90.0 per cent and not more than 115.0 per cent of the labelled amount of fluorometholone acetate $C_{24}H_{31}FO_5$.

Usual strength. Tobramycin 3.0 mg and Fluorometholone Acetate 1.0 mg per ml.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF 254*.

Solvent mixture. A mixture of 100 volumes of *butyl alcohol* and 1 volume of *pyridine*.

Mobile phase. A mixture of 60 volumes of *methanol*, 25 volumes of *chloroform* and 30 volumes of *ammonium hydroxide*.

Test solution. Allow the ophthalmic suspension to settle, and decant 1 ml of the supernatant liquid into a test tube. Add 0.1 g of sodium sulphate and centrifuge. Use the clear supernatant liquid.

Reference solution (a). A 0.6 per cent w/v solution of *tobramycin IPRS* in *water*.

Reference solution (b). A mixture of equal volumes of the test solution and reference solution (a).

Apply to the plate 3 µl of each solution. Allow the mobile phase to rise 8 cm. After development, dry the plate in air, heat the plate at 110° for 15 minutes, spray with 1.0 per cent w/v solution of *ninhydrin* in solvent mixture. The chromatogram obtained with the test solution exhibits a pink fluorescent band due to tobramycin. The Rf value of the principal spot in the chromatogram obtained with the test solution and the chromatogram obtained with reference solution (b), respectively, correspond to those of reference solution (a).

B. In the Assay for fluorometholone acetate, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 6.0 to 7.0.

Other tests. Comply with the tests stated under Eye Drops.

Assay. *For tobramycin* — Determine by liquid chromatography (2.4.14).

Solvent mixture A. A 1.0 per cent w/v solution of *2,4-dinitrofluorobenzene* in *alcohol*.

Solvent mixture B. A 1.5 per cent w/v solution of *tris (hydroxymethyl) aminomethane* in *water*.

Solvent mixture C. A 0.3 per cent w/v solution of *tris (hydroxymethyl) aminomethane* prepared as follows. Transfer 40 ml of solvent mixture B to a 200.0 ml volumetric flask, add *dimethyl sulphoxide* while mixing and dilute with *dimethylsulphoxide* to the volume.

NOTE — Use this reagent within 4 hours. If kept immersed in an ice-water bath below 10°, the reagent may be used up to 8 hours).

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Test solution. Dilute a measured volume of ophthalmic solution to obtain a solution having a concentration of 0.009 per cent w/v of Tobramycin in *water*.

Reference solution (a). Transfer an accurately weighed quantity of 55 mg of *tobramycin IPRS* to a 50.0 ml volumetric flask, add 1ml of *0.5M sulphuric acid* and sufficient *water* to dissolve and dilute to 50.0 ml with *water*.

Reference solution (b). Dilute reference solution (a) with the *water* to obtain a solution containing 0.022 per cent w/v of tobramycin.

Reference solution (c). A 0.024 per cent w/v solution of *p-naphtholbenzein* in *acetonitrile*.

NOTE — Use freshly prepared solution.

Reference solution (d). Dilute 2.0 ml of reference solution (c) to 10.0 ml with the derivatised reference solution (b).

Derivatise the solutions prior to analysis by transferring 4.0 ml of reference solution (b), 10.0 ml of test solution and 4.0 ml of *water* to separate 50 ml volumetric flask. Add 10 ml of solvent mixture A and 10 ml of solvent mixture C to each flask. Shake vigorously for few seconds and heat in a water-bath at 60°± 2° for 50 ± 5 minutes. Cool in cold *water* for 10 minutes and dilute with *acetonitrile*.

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 1.0 g of *tris(hydroxymethyl) aminomethane* in 400 ml of *water*, add 10 ml of *0.5M sulphuric acid*, dilute with *acetonitrile* to 1000 ml and mix. Cool and pass through a filter of 0.2 µm or finer pore size.
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 365 nm,
- injection volume: 20 µl.

The relative retention time with reference to tobramycin for p-naphtholbenzein is about 0.6.

Inject reference solution (d). The test is not valid unless the resolution between the peaks due to p-naphtholbenzein and tobramycin is not less than 4.0.

Inject the derivatised reference solution (b). The test is not valid unless the relative standard deviation for replicate injection is not more than 2.0 per cent.

Inject the derivatised reference solution (b) and the test solution.

Calculate the content of $C_{18}H_{37}N_5O_9$ in Ophthalmic Suspension.

For fluorometholone acetate — Determine by liquid chromatography (2.4.14).

Test solution. Dilute a measured volume of ophthalmic solution to obtain a solution having a concentration of 0.004 per cent w/v of Fluorometholone Acetate in *acetonitrile*. Centrifuge and use the clear supernatant solution.

Reference solution (a). A 0.004 per cent w/v solution of *fluorometholone acetate IPRS* in *acetonitrile*.

Reference solution (b). A 0.004 per cent w/v solution each of *fluorometholone IPRS* and *fluorometholone acetate IPRS* in *acetonitrile*.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of equal volumes of *acetonitrile* and *water*.
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

The relative retention time with reference to fluorometholone acetate for fluorometholone is about 0.7.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to fluorometholone and fluorometholone acetate is not less than 2.0.

Inject reference solution (a). The test is valid unless the column efficiency is not less than 1000 theoretical plates, the tailing factor is not more than 1.35 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

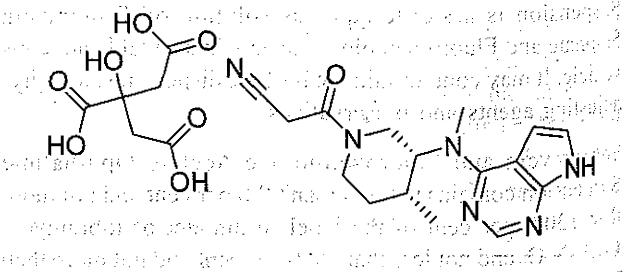
Inject reference solution (a) and the test solution.

Calculate the content of $C_{24}H_{31}FO_5$ in Ophthalmic Suspension.

Storage. Store in tightly closed container.

Labelling. The label states that the contents should be shaken before use.

Tofacitinib Citrate



$C_{22}H_{28}N_6O_8$ Mol. Wt. 504.5

Tofacitinib Citrate is 3-{(3*R*,4*R*)-4-methyl-3-[methyl(7*H*-pyrrolo[2,3-d]pyrimidin-4-yl)amino]piperidin-1-yl}-3-oxo propanenitrile 2-hydroxypropane-1,2,3-tricarboxylic acid.

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Tofacitinib Citrate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{22}H_{28}N_6O_8$, calculated on the anhydrous basis.

Category. Rheumatoid Arthritis.

Description. A white to off-white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tofacitinib citrate IPRS* or with the reference spectrum of tofacitinib citrate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). +26.0° to +32.0°, determined in a 0.25 per cent w/v solution in *water*.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of *water* and 20 volumes of *acetonitrile*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture, with the aid of ultrasound for 5 minutes and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). A solution containing 0.0002 per cent w/v, each of, *DCT IPRS, diastereomer of tofacitinib citrate impurity IPRS* and 0.1 per cent w/v of *tofacitinib citrate IPRS* in the solvent mixture.

Reference solution (b). A 0.00015 per cent w/v solution of *tofacitinib citrate IPRS* in the solvent mixture.

Reference solution (c). Dilute 3.0 ml of reference solution (b) to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as ACE 5 C-18-PFP),
- column temperature: 35°,
- mobile phase: A. a buffer solution prepared by dissolving 2.72 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	79	21
15	79	21
25	35	65
30	35	65
32	79	21
40	79	21

Name	Relative retention time	Correction factor
Citric acid	0.22	—
DCT[1]	0.34	0.67
Tofacitinib	1.0	—
Diastereomer of Tofacitinib Citrate[2]	1.18	—

[1] *N*-methyl-*N*[(3*R*,4*R*)-4-methylpiperidin-3yl]-7*H*-pyrrolo[2,3-d]pyrimidin-4-amine,

[2] 3-[(3*RS*, 4*SR*)-4-Methyl-3- [methyl (7*H*-pyrrolo[2,3-d]pyrimidin-4-yl)amino]piperidin-1-yl]-3-oxopropanenitrile.2-Hydroxy propane-1,2,3-tricarboxylic acid.

Inject reference solution (a), (b) and (c). The test is not valid unless the resolution between the peaks due to tofacitinib and diastereomer of tofacitinib citrate impurity is not less than 3.5 in the chromatogram obtained with reference solution (a), the column efficiency is not less than 9000 theoretical plates, the tailing factor is not more than 1.5, the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (b) and the signal-to-noise ratio is not less than 10 in the chromatogram obtained with reference solution (c).

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to DCT and diastereomer of tofacitinib impurity, each of, is not more than 3.33 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any other secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0. 3 per cent) and the sum of areas of all the secondary peaks is not more than 6.7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore the peak due to citric acid and any peak with an area less than 0.33 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.5 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

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Solvent mixture. Equal volumes of *water* and *acetonitrile*.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution. A 0.005 per cent w/v solution of *tofacitinib citrate IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 75 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen orthophosphate* and 1.75 g of *dipotassium hydrogen orthophosphate* in 1000 ml of *water* and 25 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 1500 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{28}N_6O_8$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Tofacitinib Tablets

Tofacitinib Citrate Tablets

Tofacitinib Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of tofacitinib, $C_{16}H_{20}N_6O$.

Usual strength. 5 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 100 rpm and 15 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *tofacitinib citrate IPRS* in the dissolution medium to obtain a solution of concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 75 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen orthophosphate* and 1.75 g of *dipotassium hydrogen orthophosphate* in 1000 ml of *water*, and 25 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 1500 theoretical plates, the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{20}N_6O$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{16}H_{20}N_6O$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of a buffer solution prepared by dissolving 2.72 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 6.0 with *potassium hydroxide solution* and 20 volumes of *acetonitrile*.

Test solution. Disperse a quantity of powdered tablets containing 30 mg of Tofacitinib in the solvent mixture, with the aid of magnetic stirrer for about 30 minutes and dilute to 100.0 ml with the solvent mixture, filter.

Reference solution (a). A solution containing 0.0001 per cent w/v, each of, *DCT IPRS* and *diastereomer of tofacitinib citrate impurity IPRS* and 0.05 per cent w/v of *tofacitinib citrate IPRS* in the solvent mixture.

Reference solution (b). Dissolve a suitable quantity of *tofacitinib citrate IPRS* in the solvent mixture to obtain a solution containing 0.0003 per cent w/v of tofacitinib.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 20.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as ACE 5 C-18-PFP),

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– column temperature: 35°,
– mobile phase: A. a buffer solution prepared by dissolving 2.72 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*,
B. *acetonitrile*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 210 nm,
– injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	79	21
15	79	21
25	35	65
30	35	65
32	79	21
40	79	21

Name	Relative retention time
Citric acid	0.22
DCT[1]	0.33
Tofacitinib (Retention time: about 11 minutes)	1.0
Diastereomer of tofacitinib citrate[2*]	1.19

*Process impurity include for identification only and not included in the calculation of total degradation products.

[1]*N*-methyl-*N*[(3*R*,4*R*)-4-methylpiperidin-3yl]-7*H*-pyrrolo[2,3-d] pyrimidin-4-amine,

[2]3-[(3*RS*,4*SR*)-4-Methyl-3-[methyl(7*H*-pyrrolo[2,3-d]pyrimidin-4-yl) amino]piperidin-1-yl]-3-oxopropanenitrile.2-Hydroxy propane-1,2,3-tricarboxylic acid.

Inject reference solution (a), (b) and (c). The test is not valid unless the resolution between the peaks due to tofacitinib and diastereomer of tofacitinib citrate impurity is not less than 3.5 in the chromatogram obtained with reference solution (a), the column efficiency is not less than 9000 theoretical plates, the tailing factor is not more than 1.5, the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (b) and the signal-to-noise ratio is not less than 10 in the chromatogram obtained with reference solution (c).

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to DCT is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any other secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent). Ignore the peak due to citric acid and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Disperse one intact tablet in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Inject the reference solution and test solution.

Calculate the content of $C_{16}H_{20}N_6O$ in the tablet.

Water (2.3.43). Not more than 8.0 per cent, determined on 0.5 g.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *water* and *acetonitrile*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 50 mg of Tofacitinib in the solvent mixture, with the aid of magnetic stirrer for about 45 minutes and dilute to 200.0 ml with the solvent mixture, filter. Dilute 2.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution. Dissolve a suitable quantity of *tofacitinib citrate IPRS* in the solvent mixture to obtain a solution containing 0.005 per cent w/v of tofacitinib.

Chromatographic system
– a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
– mobile phase: a mixture of 80 volumes of a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* and 1.75 g of *dipotassium hydrogen phosphate* in 1000 ml of *water*, and 20 volumes of *acetonitrile*,
– flow rate: 1.5 ml per minute,
– spectrophotometer set at 215 nm,
– injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 1500 theoretical plates, the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{20}N_6O$ in the tablets.

1 mg of tofacitinib citrate, $C_{22}H_{28}N_6O_8$ is equivalent to 0.619 mg of tofacitinib, $C_{16}H_{20}N_6O$.

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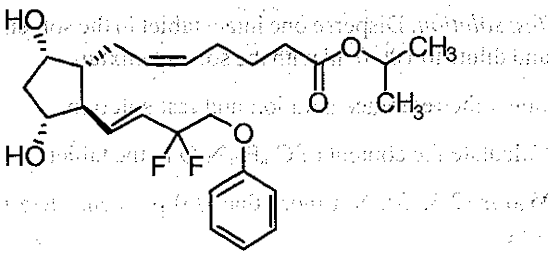
Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of Tofacitinib.

Tofluprost



$C_{22}H_{28}F_2O_5$ Mol Wt. 410.5

Tofluprost is Isopropyl (5*Z*)-7-{(1*R*,2*R*,3*R*,5*S*)-2-[(1*E*)-3,3-difluoro-4-phenoxybut-1-en-1-yl]-3,5-dihydroxycyclopentyl}-5-heptenoate.

Tofluprost contains not less than 96.0 per cent and not more than 102.0 per cent of $C_{22}H_{28}F_2O_5$, calculated on the anhydrous basis.

Category. Antihypertensive.

Description. Yellow to light yellow oil.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 72 volumes of *methanol* and 28 volumes of *water*.

Test solution. Dissolve 10 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- mobile phase: A. *water*,
- B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 219 nm,
- injection volume: 60 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	28	72
4	28	72
8	20	80
13	20	80
14	28	72
22	28	72

Inject the test solution. The area of any secondary peak is not more than 0.5 per cent and the sum of the areas of all the secondary peaks is not more than 1.0 per cent, calculated by area normalization.

Water (2.4.43) . Not more than 2.0 per cent, determined on 10 mg by thermo gravimetric analysis.

Instrument condition

- temperature: 25° to 200° at the rate of 10° per minute,using nitrogen.

Determine weight loss of sample between 25° to 200°.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 72 volumes of *methanol* and 28 volumes of *water*.

Test solution. Dissolve 10 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture. Dilute 10.0 ml of the solution to 20.0 ml with the solvent mixture

Reference solution. A 0.01 per cent w/v solution of *tofluprost IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- mobile phase: A.*water*,
- B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 219 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	28	72
4	28	72
8	20	80
13	20	80
14	28	72
22	28	72

Inject the reference solution. The test is not valid unless, the column efficiency is not less than 1000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

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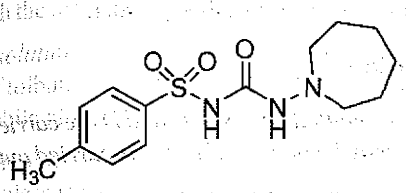
Inject reference solution and the test solution.

Calculate the content of $C_{22}H_{28}F_2O_5$.

Tolazamide



$C_{14}H_{21}N_3O_3S$ Mol. Wt. 311.4

Tolazamide is 1-perhydroazepin-1-yl-3-tolyl-*p*-sulphonylurea.

Tolazamide contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{14}H_{21}N_3O_3S$, calculated on the dried basis.

Category. Hypoglycaemic.

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tolazamide IPRS* or with the reference spectrum of tolazamide.

B. When examined in the range 230 to 350 nm (2.4.7), a 0.04 per cent w/v solution in *ethanol (95 per cent)* exhibits maxima at 256 nm, 263 nm and 275 nm and a shoulder at 268 nm. The absorbances at the maxima are about 0.78, about 0.83 and about 0.62 respectively.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 200 volumes of *chloroform*, 100 volumes of *methanol*, 60 volumes of *cyclohexane* and 23 volumes of *13.5 M ammonia*.

Test solution. Dissolve 1.0 g of the substance under examination in 50 ml of *acetone*.

Reference solution. A 0.01 per cent w/v solution of *toluene-p-sulphonamide* in *acetone*.

Apply to the plate 10 µl of each solution. After removal of the plate, dry in a current of cold air, heat at 110° for 10 minutes, place the hot plate in a tank of chlorine gas prepared by the addition of *hydrochloric acid* to a 5 per cent w/v solution of *potassium permanganate* contained in a beaker placed in the tank and allow to stand for 2 minutes. Dry it in a current of cold air until an area of the plate below the line of application gives at most a very faint blue colour with a 0.5 per cent w/v solution of *potassium iodide* in *starch mucilage*; avoid prolonged exposure to cold air. Spray the plate with a 0.5 per cent w/v solution of *potassium iodide* in *starch mucilage*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 60° at a pressure not exceeding 0.7 kPa.

Assay. Dissolve 0.5 g in 20 ml of *butan-2-one* with the aid of gentle heat. Allow to cool, add 30 ml of *ethanol (95 per cent)* and titrate with *0.1 M sodium hydroxide* using *phenolphthalein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.03114 g of $C_{14}H_{21}N_3O_3S$.

Tolazamide Tablets

Tolazamide Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of tolazamide, $C_{14}H_{21}N_3O_3S$.

Usual strengths. 250 mg; 500 mg.

Identification

Triturate a quantity of the powdered tablets containing about 0.25 g of Tolazamide with 50 ml of *acetone* and filter. Evaporate the filtrate to dryness and dry the residue at 60° at a pressure of 2 kPa for 3 hours. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tolazamide IPRS* or with the reference spectrum of tolazamide.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.05 M Tris (hydroxymethyl)aminomethane pH 7.6*, adjusted to pH 7.6 with *hydrochloric acid*,

Speed and time. 75 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, dilute suitably if necessary with the medium, at the maximum at about 224 nm (2.4.7). Calculate the content of $C_{14}H_{21}N_3O_3S$ in the medium from the absorbance obtained from a solution of known concentration of *tolazamide IPRS* in the dissolution medium.

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Q. Not less than 70 per cent of the stated amount of $C_{14}H_{21}N_3O_3S$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 23 volumes of *13.5 M ammonia*, 60 volumes of *cyclohexane*, 100 volumes of *methanol* and 200 volumes of *chloroform*.

Test solution. Shake a quantity of the powdered tablets containing about 0.2 g of Tolazamide with 10 ml of *acetone* and filter.

Reference solution. A 0.01 per cent w/v solution of *toluene-p-sulphonamide* in *acetone*.

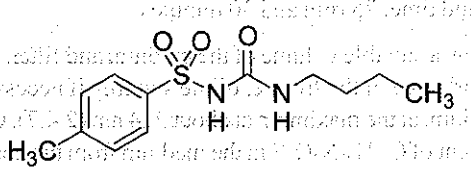
Apply to the plate 10 µl of each solution. After development, dry the plate in a current of cold air, heat at 110° for 10 minutes, place the hot plate in a tank of chlorine gas, prepared by the addition of *hydrochloric acid* to a 5 per cent w/v solution of *potassium permanganate* contained in a beaker placed in the tank and allow to stand for 2 minutes. Dry the plate in a current of cold air until an area of the plate below the line of application gives at most a very faint blue colour with a 0.5 per cent w/v solution of *potassium iodide* in *starch mucilage* avoid prolonged exposure to cold air. Spray the plate with a 0.5 per cent w/v solution of *potassium iodide* in *starch mucilage*. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Shake a quantity of the powdered tablets containing about 0.5 g of Tolazamide with 50 ml of *chloroform*, filter, wash the residue with *chloroform* and evaporate the combined filtrate and washings to dryness. Dissolve the residue in 20 ml of *butan-2-one* with the aid of gentle heat. Allow to cool, add 30 ml of *ethanol (95 per cent)* and titrate the resulting solution with *0.1 M sodium hydroxide*, using *phenolphthalein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.03114 g of $C_{14}H_{21}N_3O_3S$.

Tolbutamide



$C_{12}H_{18}N_2O_3S$ Mol. Wt. 270.4

Tolbutamide is 3-butyl-1-[(4-methylphenyl)sulphonyl]urea.

Tolbutamide contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{12}H_{18}N_2O_3S$, calculated on the dried basis.

Category. Hypoglycaemic.

Description. A white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tolbutamide IPRS* or with the reference spectrum of tolbutamide.

B. Dissolve 25 mg in sufficient *methanol* to produce 100.0 ml.

When examined in the range 245 nm to 360 nm (2.4.7), the resulting solution shows absorption maxima at about 258 nm, 263 nm and 275 nm and a shoulder at about 268 nm. Dilute the solution with *methanol* to produce a 0.001 per cent w/v solution. When examined in the range 220 nm to 235 nm, the resulting solution shows an absorption maximum at about 228 nm; absorbance at about 228 nm, about 0.50.

C. Boil 0.1 g with 8 ml of a 50 per cent v/v solution of *sulphuric acid* under a reflux condenser for 30 minutes. Make the solution strongly alkaline with *sodium hydroxide solution* and steam distil for 30 minutes, receiving the distillate in 20 ml of *0.1 M hydrochloric acid*. To 1 ml of the solution containing the distillate add 0.1 g of *sodium acetate* and 10 ml of *buffer solution pH 9.4*. Cool in an ice-bath for 10 minutes, add 1 ml of *diazotised nitroaniline solution*, set aside for 20 minutes and add dropwise 1 ml of *sodium hydroxide solution*; an orange-red colour is produced.

D. Boil 0.1 g with 8 ml of a 50 per cent v/v solution of *sulphuric acid* under a reflux condenser for 30 minutes. Cool in an ice-bath; a crystalline precipitate of *4-toluenesulphonylamide* is formed, which after recrystallisation from hot *water* and drying at 105° melts at 135° to 140° (2.4.21).

Tests

Appearance of solution. A 2.0 per cent w/v solution in *1 M sodium hydroxide* is clear (2.4.1), and colourless (2.4.1).

pH (2.4.24). 4.5 to 5.5, determined in a solution prepared by dissolving 2.0 g in 50 ml of *carbon dioxide-free water* by heating at 70° for 5 minutes, cooling rapidly and filtering.

Non-sulphonyl urea. Dissolve 0.5 g in 1 ml of *dilute ammonia solution* and 9 ml of *water*; not more than a faint opalescence is produced.

Related substances. Determine by liquid chromatography (2.4.14).

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NOTE—Prepare the solutions immediately before use.

Test solution. Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.001 per cent w/v each of tolbutamide impurity A and tolbutamide impurity B in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of *acetonitrile* and 65 volumes of a 0.14 per cent w/v solution of *potassium dihydrogen phosphate*, adjusted to pH 3.5 with *orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Name	Relative retention time
Tolbutamide impurity B[1]	0.2
Tolbutamide impurity A[2]	0.3
Tolbutamide (Retention time: about 18 minutes)	1.0

[1]toluenesulphonamide,

[2]toluenesulphonylurea.

For identification of impurities, use the chromatogram obtained with reference solution (b) to identify the peak due to tolbutamide impurities A and B.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to tolbutamide impuritites A and B is not less than 2.0.

Inject reference solution (a) and the test solution. Run the chromatogram 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 0.5 g in a mixture of 40 ml of *ethanol (95 per cent)* and 20 ml of *water*. Titrate with *0.1 M sodium hydroxide* using *phenolphthalein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.02704 g of $C_{12}H_{18}N_2O_3S$.

Storage. Store protected from moisture.

Tolbutamide Tablets

Tolbutamide Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of tolbutamide, $C_{12}H_{18}N_2O_3S$.

Usual strength. 500 mg.

Identification

Extract a quantity of the powdered tablets containing 1 g of Tolbutamide with 10 ml of *chloroform*, filter, evaporate the filtrate to dryness, scratching the sides of the vessel, if necessary, to induce crystallisation, and dry the residue at 105° for 30 minutes. The residue complies with the following tests.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tolbutamide IPRS* or with the reference spectrum of tolbutamide.

B. Boil 0.1 g of residue with 8 ml of a 50 per cent v/v solution of *sulphuric acid* under a reflux condenser for 30 minutes. Cool in an ice-bath; a crystalline precipitate of *4-toluenesulphonylamide* is formed, which after recrystallisation from hot *water* and drying at 105° melts at 135° to 140°.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of a solution containing 2.04 per cent w/v of *disodium hydrogen phosphate* and 0.135 per cent w/v of *potassium dihydrogen phosphate*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate suitably diluted if necessary, at the maximum at about 228 nm (2.4.7). Calculate the content of $C_{12}H_{18}N_2O_3S$ in the medium taking 417 as the specific absorbance at 228 nm.

Q. Not less than 70 per cent of the stated amount of $C_{12}H_{18}N_2O_3S$.

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Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 90 volumes of *chloroform*, 8 volumes of *methanol* and 2 volumes of *anhydrous formic acid*.

Test solution. Shake a quantity of the powdered tablets containing 0.5 g of Tolbutamide with 10 ml of *acetone* and filter.

Reference solution (a). A 0.015 per cent w/v solution of *4-toluenesulphonamide* in *acetone*.

Reference solution (b). A mixture of equal volumes of the test solution and reference solution (a).

Apply to the plate 5 µl of each of test solution and reference solution (a) and 10 µl of reference solution (b). After development, dry the plate in a current of warm air and heat at 110° for 10 minutes. While still hot, place the plate in a chromatographic tank with an evaporating dish containing a 5 per cent w/v solution of *potassium permanganate*, add an equal volume of *hydrochloric acid* and close the tank. Leave the plate in the tank for 2 minutes, then place it in a current of cold air until the excess of chlorine is removed and an area of coating below the line of application gives only a very faint blue colour with *potassium iodide and starch solution*; avoid prolonged exposure to cold air. Spray the plate with *potassium iodide and starch solution* and allow to stand for 5 minutes. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a). The chromatogram obtained with reference solution (b) shows two clearly separated spots.

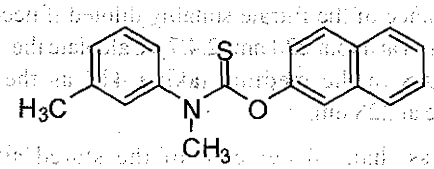
Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.5 g of Tolbutamide, add 50 ml of *ethanol (95 per cent)*, previously neutralised to *phenolphthalein solution*, warm to dissolve, add 25 ml of *water* and titrate with *0.1 M sodium hydroxide* using *phenolphthalein* solution as indicator.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.02704 g of $C_{12}H_{18}N_2O_3S$.

Storage. Store protected from moisture.

Tolnaftate



$C_{19}H_{17}NOS$ Mol.Wt. 307.4

Tolnaftate is *O*-naphthalen-2-yl methyl(3-methylphenyl)-thiocarbamate.

Tolnaftate contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{19}H_{17}NOS$, calculated on the dried basis.

Category. Antifungal.

Description. A white or yellowish white powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tolnaftate IPRS* or with the reference spectrum of tolnaftate.

B. Mix about 1 mg with 0.5 ml of *sulphuric acid*. Add 0.05 ml of *formaldehyde solution*. A greenish-blue colour develops.

C. Melting range (2.4.21). 109° to 112°.

Tests

Appearance of solution. A 5.0 per cent w/v solution in *acetone* is clear (2.4.1) and not more intensely coloured than reference solution YS6 (2.4.1).

Impurity D. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.4 g of the substance under examination in 2 ml of *dichloromethane*. Extract with 3 quantities, each of 3 ml, of *0.01 M hydrochloric acid*. Combine the aqueous phases and dilute to 10.0 ml with *0.01 M hydrochloric acid*.

Reference solution (a). Dissolve 20 mg of tolnaftate impurity D (*N-methyl-m-toluidine*) in 50.0 ml of *dichloromethane*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *dichloromethane*. Take 2.0 ml of the solution and extract with 3 quantities, each of 3 ml, of *0.01 M hydrochloric acid*. Combine the aqueous phases and dilute to 10.0 ml with *0.01 M hydrochloric acid*.

Reference solution (c). Dissolve 10 mg of the substance under examination in 25 ml of *methanol*. Add 2 ml of the solution to 2 ml of reference solution (a) and dilute to 25 ml with *methanol*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 0.1 volume of *trifluoroacetic acid*, 10 volumes of *methanol* and 90 volumes of *water*,
  B. a mixture of 0.1 volume of *trifluoroacetic acid*, 10 volumes of *water* and 90 volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,

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– spectrophotometer set at 254 nm,
– injection volume: 100 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
3	70	30
8	0	100
20	0	100

The relative retention time with reference to tolnaftate (retention time: about 15 minutes) for tolnaftate impurity D is about 0.25.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to tolnaftate impurity D and tolnaftate is not less than 5.0 and the tailing factor for the peak due to tolnaftate impurity D is not more than 1.9.

Inject reference solution (b) and the test solutiuon. In the chromatogram obtained with the test solution, the area of any peak corresponding to tolnaftate impurity D is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (20 ppm).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in 5 ml of *methanol* and dilute to 25.0 ml with *methanol*.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with *methanol*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *methanol*.

Chromatographic system
– a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
– mobile phase: A. a mixture of 0.1 volume of *trifluoroacetic acid*, 30 volumes of *water* and 70 volumes of *methanol*,
B. a mixture of 0.1 volume of *trifluoroacetic acid*, 10 volumes of *water* and 90 volumes of *methanol*,
– a gradient programme using the conditions given below,
– flow rate: 1 ml per minute,
– spectrophotometer set at 254 nm,
– injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
12	100	0
30	0	100
33	0	100
35	100	0

The relative retention time with reference to tolnaftate (retention time is about 18 minutes) for tolnaftate impurity A is about 0.7.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in vacuum at 60° at a pressure not exceeding 0.7 kPa for 3 hours.

Assay. Dissolve 50 mg in 250 ml of *methanol*. Dilute 2 ml of the solution to 50 ml with *methanol*. Measure the absorbance at the maximum at 257 nm (2.4.7).

Calculate the content of $C_{19}H_{17}NOS$ taking 720 as the specific absorbance at 257 nm.

Storage. Store protected from light.

Tolnaftate Cream

Tolnaftate Cream contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of tolnaftate, $C_{19}H_{17}NOS$.

Usual strength. 1 per cent w/w.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. Toluene.

Test solution. Evaporate 10 ml of the Solution A on steam bath to dryness and dissolve the residue in 1.0 ml of *ethanol (95 per cent)*.

Reference solution. A 0.1 per cent w/v solution of *tolnaftate IPRS* in *ethanol (95 per cent)*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the

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chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Other tests. Comply with the tests stated under Creams.

Assay. Transfer a weighed quantity of the cream containing about 10 mg of Tolnaftate to a separator containing 75 ml of *chloroform*. Wash the chloroform solution successively with two 25 ml portions of *0.1 M hydrochloric acid* and 25 ml of *water*. Filter the chloroform layer through a chloroform–washed cotton pledget into a 100-ml volumetric flask. Add *chloroform* to volume and mix (Solution A). Dilute 5.0 ml of the solution with *chloroform* to 50 ml and mix. Measure the absorbance of the resulting solution at the maximum at about 258 nm (2.4.7), using 0.001 per cent w/v solution of *tolnaftate IPRS* in *chloroform*.

Calculate the content of $C_{19}H_{17}NOS$ in the cream.

Storage. Store protected from moisture.

Tolnaftate Gel

Tolnaftate Gel contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of tolnaftate, $C_{19}H_{17}NOS$.

Usual strength. 1 per cent w/w.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica GF254*.

Mobile phase. *Toluene*.

Test solution. Evaporate 10 ml of the Solution A on steam bath to dryness and dissolve the residue in 1.0 ml of *ethanol (95 per cent)*.

Reference solution. A 0.1 per cent w/v solution of *tolnaftate IPRS* in *ethanol (95 per cent)*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Other tests. Comply with the tests stated under Gels.

Assay. Transfer a weighed quantity of the gel containing about 10 mg of Tolnaftate to a separator containing 75 ml of *chloroform*. Wash the chloroform solution successively with two 25 ml portions of *0.1 M hydrochloric acid* and 25 ml of *water*. Dilute to 100 ml with *chloroform* and mix (Solution A). Dilute 5.0 ml of the solution with *chloroform* to 50 ml and mix. Measure the absorbance of the resulting solution at the maximum at about 258 nm (2.4.7), using 0.001 per cent w/v solution of *tolnaftate IPRS* in *chloroform*.

Calculate the content of $C_{19}H_{17}NOS$ in the gel.

Storage. Store protected from moisture.

Tolnaftate Topical Powder

Tolnaftate Topical Powder contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of $C_{19}H_{17}NOS$.

Usual strength. 1 per cent w/w.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. *Toluene*.

Test solution. Evaporate the 5.0 ml portion of the *methanol solution* reserved in the Assay, on a steam bath just to dryness and dissolve the residue in 1.0 ml of *alcohol*.

Reference solution. A 0.1 per cent w/v solution of *tolnaftate IPRS* in *ethanol (95 per cent)*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Assay. Determine by liquid chromatography (2.4.14).

Internal standard solution. A 0.1 per cent w/v solution of *progesterone* in *methanol*.

Test solution. Transfer a weighed quantity of the topical powder containing about 5.0 mg of tolnaftate to a screw-capped, 50 ml centrifuge tube. Add 25 ml of *methanol*, place the cap on the tube and rotate on a rotating device for 10 minutes, and centrifuge at about 2000 rpm for 5 minutes, filter the supernatant and transfer 20 ml of the filtrate to 50-ml volumetric flask, retaining the remaining portion of the filtrate for identification test. Add 5.0 ml of internal standard solution to the flask and dilute to volume with *methanol* and mix.

Reference solution. A 0.02 per cent w/v solution of *tolnaftate IPRS* in *methanol*. To 20 ml of the solution add 5.0 ml of internal standard solution and dilute to 50.0 ml with *methanol*.

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Chromatographic system
- a stainless steel column 30 cm x 4 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 2 volumes of *acetonitrile* and 1 volume of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

The relative retention time of progesterone and tolnaftate are about 0.7 minute and 1.0 minute respectively.

Inject the reference solution. The test is not valid unless the resolution between the tolnaftate and internal standard peak is not less than 3.0 and the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{17}NOS$ in the powder.

Storage. Store protected from moisture.

Tolnaftate Topical Solution

Tolnaftate Topical Solution contains not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of tolnaftate, $C_{19}H_{17}NOS$.

Usual strength. 1 per cent w/v.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. Toluene.

Test solution. Evaporate 25 ml of solution A on steam bath to dryness and dissolve the residue in 1.0 ml of the *ethanol (95 per cent)*.

Reference solution. A 0.1 per cent w/v solution of *tolnaftate IPRS* in *ethanol (95 per cent)*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

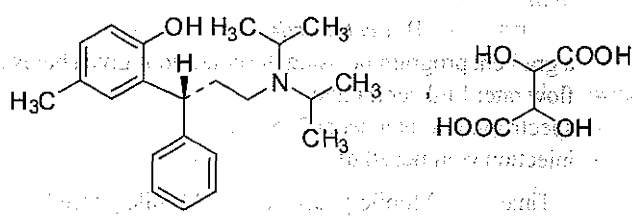
Tests

Assay. Take an measured volume containing about 10 mg of Tolnaftate, add 50 ml of *chloroform* and extract with 50 ml of *0.1 M sodium hydroxide*. Filter the chloroform layer through a chloroform-washed cotton pledget into a 250- ml volumetric flask and extract the aqueous layer with two 45 ml portions of *chloroform*, filtering each into the flask. Add *chloroform* to volume and mix (Solution A). Dilute 25 ml of the solution with *chloroform* to 100 ml and mix. Measure the absorbance of the resulting solution at the maximum at about 258 nm (2.4.7), using 0.001 per cent w/v solution of *tolnaftate IPRS* in *chloroform*.

Calculate the content of $C_{19}H_{17}NOS$ in topical solution.

Storage. Store protected from moisture.

Tolterodine Tartrate



$C_{22}H_{31}NO,C_4H_6O_6$ Mol Wt. 475.6

Tolterodine Tartrate is 2-[(1*R*)-3-[bis(1-methylethyl)amino]-1-phenylpropyl]-4-methylphenol tartrate.

Tolterodine Tartrate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{22}H_{31}NO,C_4H_6O_6$, calculated on the dried basis.

Category. Anticholinergic.

Description. A white to off-white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tolterodine tartrate IPRS* or with the reference spectrum of tolterodine tartrate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). + 27° to + 34°, determined in 1.0 per cent w/v solution in *methanol*.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of mobile phase A and mobile phase B.

Test solution. Dissolve 50 mg of the substance under examination in 10 ml of *water* and dilute to 50.0 ml with the solvent mixture.

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Reference solution (a). Dissolve about 10 mg of *tolterodine tartrate IPRS* in 20 ml of *water* and dilute to 100.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution (b). A 0.03 per cent w/v solution of tartaric acid in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil),
- mobile phase: A. a *0.05 M potassium dihydrogen orthophosphate*, adjusted to pH 3.5 with *orthophosphoric acid*,

B. *acetonitrile*,

- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	65	35
5	65	35
20	50	50
40	30	70
41	65	35
50	65	35

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore the peak due to tartaric acid corresponding to the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 10 volumes of mobile phase A and 10 volumes of mobile phase B.

Test solution. Dissolve 50 mg of the substance under examination in 20 ml of *water*, sonicate and dilute to 100.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. Dissolve about 50 mg of *tolterodine tartrate IPRS* in 20.0 ml of *water*, sonicate and dilute to 100.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil),
- mobile phase: A. a *0.05 M potassium dihydrogen orthophosphate*, adjusted to pH 3.5 with *orthophosphoric acid*,

B. *acetonitrile*,

- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Time (in min)	Mobile Phase A (per cent v/v)	Mobile Phase B (per cent v/v)
0	65	35
5	65	35
20	50	50
21	65	35
25	65	35

Inject reference solution. The test is not valid unless the theoretical plates of the principal peak is not less than 2000 and the tailing factor is not more than 2.0. The relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{22}H_{31}NO.C_4H_6O_6$.

Storage. Store protected from moisture.

Tolterodine Tartrate Tablets

Tolterodine Tablets contain Tolterodine Tartrate.

Tolterodine Tartrate Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amounts of tolterodine tartrate, $C_{26}H_{37}NO_7$.

Usual strengths. 1 mg; 2 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

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Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *gastric juice, artificial* (without enzyme) prepared by dissolving 2 g of *sodium chloride* in 80 ml of *1 M hydrochloric acid* and dilute to 1000 ml with *water*,

Speed and time. 50 rpm and 10 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A solution of *tolterodine tartrate IPRS* prepared by dissolving in *methanol* and diluted with the dissolution medium to get the similar concentration as that of the test solution.

Use chromatographic system as described under Assay using injection volume: 100 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_{26}H_{37}NO_7$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{26}H_{37}NO_7$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of mobile phase A and mobile phase B.

Test solution. Disperse a quantity of powdered tablets containing 10 mg of Tolterodine Tartrate in 1 ml of *methanol*, 5 ml of the solvent mixture with the aid of ultrasound and dilute to 10.0 ml with the solvent mixture, filter.

Reference solution (a). A 0.001 per cent w/v solution of *tolterodine tartrate IPRS* prepared by dissolving in *methanol* and diluted with the solvent mixture.

Reference solution (b). A 0.03 per cent w/v solution of *tartaric acid* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5µm),
- mobile phase: A. dissolve 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml *water*, adjusted to pH 3.5 with *orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	65	35
5	65	35
20	50	50
40	30	70
50	65	35

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent). Ignore the peak due to tartaric acid corresponding to the peak in the chromatogram obtained with reference solution (b).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay using following modification.

Test solution. Disperse 1 intact tablet in the mobile phase with the aid of ultrasound for 20 minutes and dilute to 10.0 ml with the mobile phase, filter. Dilute further with the mobile phase if necessary.

Calculate the content of $C_{26}H_{37}NO_7$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 10 mg of Tolterodine Tartrate in 70 ml of the mobile phase with the aid of ultrasound for 20 minutes and dilute to 100.0 ml with the mobile phase, filter.

Reference solution. A 0.01 per cent w/v solution of *tolterodine tartrate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of buffer solution prepared by mixing 2.2 ml of *orthophosphoric acid* to 1000 ml with *water*, adjusted to pH 3.0 with *triethylamine* and 35 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

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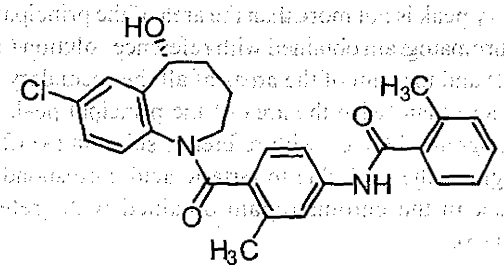
Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{26}H_{37}NO_7$ in the tablets.

Storage. Store at a temperature not exceeding 30°.

Tolvaptan



$C_{26}H_{25}ClN_2O_3$ Mol Wt 448.9

Tolvaptan is *N*-{4-{[(5*R*)-7-chloro-5-hydroxy-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl]carbonyl}-3-methylphenyl}-2-methylbenzamide.

Tolvaptan contains not less than 98.0 per cent and not more than 102.0 per cent, calculated on the anhydrous basis.

Category. Vasopressin Receptor Antagonist.

Description. A white to off-white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tolvaptan IPRS* or with the reference spectrum of tolvaptan.

B. In the assay, the principal peak in the chromatogram obtained with test solution corresponds to the the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 50 volumes of solution A and 50 volumes of *acetonitrile.*

Solution A. A mixture of 95 volumes of buffer prepared by dissolving 1.36 g of *potassium dihydrogen orthophosphate* into 1000 ml of *water*, adjusted to pH 3.0 with *phosphoric acid* and 5 volume of *acetonitrile.*

Test solution. Dissolve 25 mg of the substance under examination in the solvent mixture and dilute to 25.0 ml with the solvent mixture.

Reference solution. A 0.1 per cent w/v solution of *tolvaptan IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 12.5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: a mixture of 57 volumes of solution A and 43 volumes of a mixture of 95 volumes of *acetonitrile* and 5 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates and the tailing factor is not more be than 2.0.

Inject the test solution. The area of any secondary peak is not more than 0.3 per cent and the sum of areas of all the secondary peaks is not more than 1.0 per cent, calculated by area normalization.

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method D (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 0.3 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *tolvaptan IPRS* in the solvent mixture.

Use the chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{26}H_{25}ClN_2O_3$.

Storage. Store protected from moisture.

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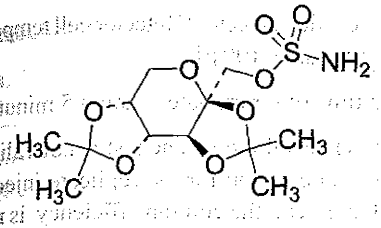
Topiramate



$C_{12}H_{21}NO_8S$ Mol. Wt. 339.4

Topiramate is 2,3:4,5-di-*O*-1-isopropylidene-β-D-fructopyranose sulphamate.

Topiramate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{12}H_{21}NO_8S$, calculated on the anhydrous basis.

Category. Anti-convulsant.

Description. A white to off-white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *topiramate IPRS* or with the reference spectrum of topiramate.

B. Melting range (2.4.21). 120° to 130°.

Tests

Specific optical rotation (2.4.22). – 36.0° to – 28.0°, determined in a 0.4 per cent w/v solution in *methanol* at 20°.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh 0.4 g of the substance under examination and dissolve in 10 ml of a mixture of 50 volumes of *water* and 50 volumes of *acetonitrile*.

Reference solution. A 0.1 per cent w/v solution each of *2,3:4,5-Bis-O-(1-methylethylidene)-beta-D-fructopyranose IPRS* (*topiramate impurity A IPRS*) and *2,3-O-(1-methylethylidene)-beta-D-fructopyranose sulphamate IPRS* (*topiramate impurity B IPRS*) in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 50 volumes of *water* and 50 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- refractive index detector,
- detector cell temperature 50°,
- injection volume: 50 µl.

The retention times of topiramate impurity B and impurity A are about 2.9 and 4.5 minutes and the relative retention times with respect to topiramate are 0.54 and 0.84 respectively.

Inject the reference solution. The test is not valid unless the resolution between the peak due to topiramate impurity A and topiramate impurity B is not less than 3.

Inject the reference solution and the test solution. Run the chromatogram for 40 minutes. For the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: topiramate impurity A = 1.09; topiramate impurity B = 0.94. The area of any secondary peak due to topiramate impurity A and B is not more than 0.15 and the area of any other secondary peak is not more than 0.1 per cent. Ignore any peak due to lactose.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution. A 1.0 per cent w/v solution of *topiramate IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 50°
- mobile phase: a mixture of 50 volumes of *water* and 50 volumes of *acetonitrile*,
- flow rate: 0.6 ml per minute,
- refractive index detector (Detector cell temperature: 50°)
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{12}H_{21}NO_8S$

Storage. Store protected from light, at a temperature not exceeding 30°.

Topiramate Tablets

Topiramate Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of topiramate, $C_{12}H_{21}NO_8S$.

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Usual strengths. 25 mg, 50 mg, 100 mg, 200 mg, 300 mg and 400 mg.

Identification

A. Weigh a quantity of the powdered tablets containing 1 g of Topiramate, disperse in 30 ml of *dichloromethane*, centrifuge at 2000 rpm for 10 minutes and filter. Evaporate the filtrate to dryness. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *topiramate IPRS* or with the reference spectrum of topiramate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium: 500 ml of *water*.
Speed and time: 50 rpm and 45 minutes.
Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14), using the chromatographic conditions described under test for Related substances.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve an weighed quantity of *topiramate IPRS* in the dissolution medium and dilute with the dissolution medium to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Calculate the content of $C_{12}H_{21}NO_8S$ in the medium.

Q: Not less than 70 per cent of the stated amount of $C_{12}H_{21}NO_8S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. To a quantity of the powdered tablets containing 100 mg of Topiramate, add sufficient mobile phase to produce 100 ml, mix and centrifuge. Use the supernatant liquid.

Reference solution. A 0.1 per cent w/v solution of *topiramate IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS 3),
- column temperature: 50°,
- mobile phase: a mixture of 50 volumes of *acetonitrile* and 50 volumes of *water*,
- flow rate: 1 ml per minute,
- refractive index detector (Detector cell temperature 50°),
- injection volume: 100 µl.

The retention time of topiramate is about 5 minutes.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent, the column efficiency is not less than 3000 theoretical plates and the tailing factor is not more than 2.0

Inject the test solution. The content of any individual impurity is not more than 1.0 per cent and the sum of areas of all impurities is not more than 2.0 per cent. Calculated by area normalisation. Ignore any peak due to identified excipients in the chromatogram obtained with the test solution.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14) as described under test for Related substances.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 100 mg of Topiramate, disperse in 100.0 ml with the mobile phase and centrifuge. Use the supernatant liquid.

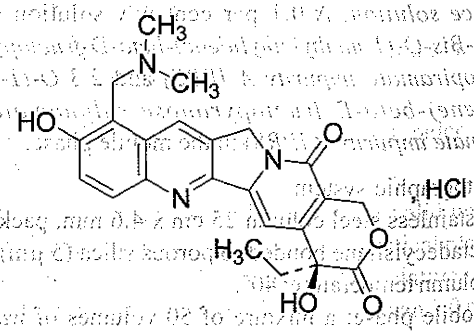
Reference solution. A 0.1 per cent w/v solution of *topiramate IPRS* in the mobile phase.

Inject the reference solution and the test solution.

Calculate the content of $C_{12}H_{21}NO_8S$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Topotecan Hydrochloride



$C_{23}H_{23}N_3O_5,HCl$ Mol. Wt. 457.9

Topotecan Hydrochloride is (4*S*)-10-[(dimethylamino) methyl]-4-ethyl-4,9-dihydroxy-1*H*-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-3,14(4*H*,12*H*)-dione hydrochloride.

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Topotecan Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{23}H_{23}N_3O_5$,HCl, calculated on the anhydrous basis.

Category. Anticancer.

Description. A light yellow to greenish yellow powder.

CAUTION — Topotecan Hydrochloride is cytotoxic; extra care is required to prevent inhaling particles and exposing the skin to it.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *topotecan hydrochloride IPRS* or with the reference spectrum of topotecan hydrochloride.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 3.5 to 4.5, determined in a 1.0 per cent w/v solution.

Specific optical rotation (2.4.22). +30.0° to +38.0°, determined in a 1.0 per cent w/v solution in *methanol*.

Total chloride. 7.6 per cent to 8.1 per cent.

Weigh 0.5 g, dissolve in 10 ml of *methanol*, add 20 ml of *water* and 20 ml of *glacial acetic acid*. Titrate with *0.1 M silver nitrate solution* using *eosin yellow solution* as indicator. Colour changes from orange to dark pink.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of Cl.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 30 volumes of *acetonitrile* and 70 volumes of a buffer solution prepared by diluting 1 ml of *trifluoroacetic acid* in 1000 ml of *water*.

Test solution. Dissolve 40 mg of the substance under examination in 100 ml of the solvent mixture.

Reference solution (a). A 0.04 per cent w/v solution of *topotecan hydrochloride IPRS* in the solvent mixture.

Reference solution (b). Dilute 1 ml of reference solution (a) to 100 ml with the solvent mixture.

Reference solution (c). A solution containing 0.006 per cent w/v each of *10-hydroxy comptothecin IPRS* and *camptothecin IPRS* in *N,N'-dimethylformamide*. Add 1.0 ml of the solution to a 0.04 per cent w/v solution of *topotecan hydrochloride IPRS* in the solvent mixture.

Use the chromatographic system described under Assay.

Inject reference solution (c). The test is not valid unless the relative retention time for the 10-hydroxy camptothecin and camptothecin is about 1.87 and 2.62 with respect to topotecan hydrochloride and the relative response factors for 10-hydroxy camptothecin and camptothecin are about 0.79 and 0.85 with respect to topotecan hydrochloride.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.15 times the area of the peak in the chromatogram obtained with reference solution (b) (0.15 per cent) and the sum of the areas of all the secondary peaks is not more than the area of the peak in the chromatogram obtained with reference solution (b) (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 13.0 per cent, determined on 0.1 g.

Bacterial endotoxins (2.2.3). Not more than 16 Endotoxin Units per mg of topotecan hydrochloride.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 10^2 CFU per g. 1 g is free from *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. 10 g is free from *Salmonella* and *Shigella*.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. a mixture of 30 volumes of *acetonitrile* and 70 volumes of a buffer solution, prepared by diluting 1 ml of *trifluoroacetic acid* to 1000 ml with *water*.

Test solution. Dissolve 10 mg of the substance under examination in 25.0 ml of the solvent mixture.

Reference solution. A 0.04 per cent w/v solution of *topotecan hydrochloride IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a solution of 1 ml of *trifluoroacetic acid* in 1000 ml of *water*,
  B. *acetonitrile*,
- flow rate: 1.2 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 260 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
28	70	30
38	70	30
43	85	15
48	85	15

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Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{23}N_3O_5,HCl$.

Storage. Store protected from light, at a temperature between 2° to 8°.

Topotecan Injection

Topotecan Hydrochloride Injection

Topotecan Injection is a sterile, stabilised solution of Topotecan Hydrochloride in Water for Injection.

Topotecan Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of the topotecan, $C_{23}H_{23}N_3O_5$.

Usual strength. 1 mg per ml.

Description. A clear, light yellow solution.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. It gives the reaction of chlorides (2.3.1).

Tests

pH (2.4.24). 2.5 to 3.5.

Bacterial endotoxins (2.2.3). Not more than 64.9 Endotoxin Unit per mg of topotecan.

Sterility (2.2.11). Complies with the test for sterility.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Accurately measure the volume of injection containing 2 mg of Topotecan, dilute to 50.0 ml with mobile phase.

Reference solution. Dissolve 10 mg of *topotecan hydrochloride IPRS* in 5 ml of *water* and dilute to 25.0 ml with mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 78 volumes of *water*, 22 volumes of *acetonitrile* and 1 volume of *1 M hydrochloric acid*,
- flow rate: 0.7 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

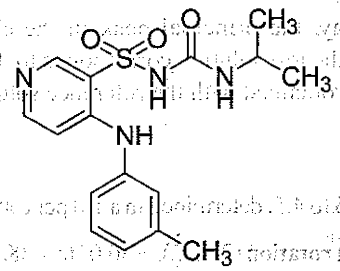
Inject the reference solution. The test is not valid unless the relative standard deviation is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{23}H_{23}N_3O_5$ in the injection.

Storage. Store protected from light, at a temperature not exceeding 2° to 8°.

Torsemide



$C_{16}H_{20}N_4O_3S$ Mol. Wt. 348.4

Torsemide is 3-pyridinesulfonamide, *N*-[[(1-methylethyl) amino]carbonyl]-4-[(3-methylphenyl)amino]-1-Isopropyl-3 [(4-*m*-toludino-3-pyridyl) sulfonyl] urea.

Torsemide contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{16}H_{20}N_4O_3S$, calculated on the anhydrous basis.

Category. Diuretic.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometery (2.4.6). Compare the spectrum with that obtained with *torsemide IPRS* or with the reference spectrum of torsemide.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 38 mg of the substance under examination in 30 ml of *methanol*, with the aid of ultrasound for 10 minutes. Add 45 ml of *0.02 M potassium phosphate*

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buffer pH 3.5 and dilute to 100.0 ml with the mobile phase, filter.

Reference solution (a). Dissolve 9.5 mg, each of, *torsemide related compound A IPRS, torsemiderelated compound B IPRS* and *torsemide related compound C IPRS* in 30 ml of *methanol*, with the aid of ultrasound about 10 minutes and add 45 ml of *0.02 M potassium phosphate buffer pH 3.5* and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 3 mg, each of, *torsemide IPRS* and *torsemide related compound A IPRS* in 30 ml of *methanol*, with the aid of ultrasound for 10 minutes and add 45 ml of *0.02 M potassium phosphate buffer pH 3.5* and dilute to 100.0 ml with the mobile phase.

Use the chromatographic system as described under Assay.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to torsemide and torsemide related compound A is not less than1.0, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 10.0 per cent for the torsemide peak.

Inject reference solution (a) and the test solution. Run the chromatogram 3 times the retention time of the principal peak for test solution. The area of any peak corresponding to torsemide related compound C is not more than 0.4 times the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any peak corresponding to torsemide related compound B is not more than 0.6 times the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.3 per cent), the area of any peak corresponding to torsemide related compound A is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent).

The area of any other secondary peak is not more than 0.1 per cent, the sum of the areas of all the secondary peaks excluding torsemide related compound A, B and C is not more than 0.2 per cent and the sum of the areas of all the secondary peaks is not more than 1.0 per cent, calculated by area normalization method.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 38 mg of the substance under examination in 30 ml of *methanol*, mix and sonicate to dissolve. Add 45.0 ml of *0.02 M phosphate buffer pH 3.5*, cool to room temperature and dilute to 100.0 ml with the mobile phase.

Reference solution. Dissolve 38 mg of *torsemide IPRS* in 30 ml of *methanol*, mix and sonicate to dissolve. Add 45 ml of *0.02 M phosphate buffer pH 3.5*, cool to room temperature and dilute to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with end-capped octadecylsilane bonded to porous silica (5µm),
- mobile phase: a mixture of 40 volumes of *methanol* and 60 volumes of *0.02 M phosphate buffer pH 3.5*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 288 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{20}N_4O_3S$.

Storage. Store protected from moisture.

Torsemide Tablets

Torsemide Tablets contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of tosemide, $C_{16}H_{20}N_4O_3S$.

Usual strengths. 5 mg; 10 mg; 20 mg; 100 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak of the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Buffer solution. Dissolve 2.72 g of *monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 4.0 with *orthophosphoric acid*.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

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Reference solution. A 0.055 per cent w/v solution of *torsemide IPRS* prepared by dissolving in *methanol* to fill 30 per cent of the volume of flask with the aid of ultrasound and add the buffer solution to fill 75 per cent of the volume of the flask. Dilute to volume with the mobile phase. Dilute the solution with the dissolution medium to obtain a solution having a final concentration similar to that of the test solution.

Use buffer solution and chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Q. Not less than 80 per cent of the stated amount of $C_{16}H_{20}N_4O_3S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing 40 mg of Torsemide in 30 ml of *methanol*, with the aid of ultrasound for 10 minutes. add 45 ml of *0.02 M potassium phosphate buffer* and dilute to 100.0 ml with the mobile phase, filter (*Prepare the solution immediately before use*).

Reference solution (a). Dissolve 10 mg of *torsemide related compound A IPRS* and 2 mg of *torsemide related compound E IPRS* in 30 ml of *methanol*, with the aid of ultrasound and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 40 mg of *torsemide IPRS* in 30 ml of *methanol*, with the aid of ultrasound. Add 45 ml of *0.02 M potassium phosphate buffer* and 4.0 ml of reference solution (a) and dilute to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 40 mg of *torsemide IPRS* in 30 ml of *methanol*, with the aid of ultrasound for 10 minutes. Add 45 ml of *0.02 M potassium phosphate buffer* and dilute to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 1.0 ml of reference solution (c) to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (e). Dilute 4.0 ml of reference solution (a) and 1.0 ml of reference solution (c) to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm),
- sample temperature: 6°,
- mobile phase: a mixture of 45 volumes of a solution containing 10 volumes of *acetonitrile* and 90 volumes of *methanol* and 55 volumes of a buffer solution prepared by dissolving 2.72 g of *monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 4.0 with *orthophosphoric acid*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 288 nm,
- injection volume: 20 µl.

Name	Relative retention time
Torsemide related compound A[1]	0.39
Torsemide related compoundE[2]	0.50
Torsemide related compound C[3]*	0.62
Torsemide related compound D[4]*	0.75
Torsemide	1.0
Torsemide related compound B[5]*	1.96

*Process-related impurity controlled in the drug substance

[1]4-[(3-methlphenyl)amino]-3-pyridinesulphonamide,

[2]4-(3-Tolyl)-2H-pyrido[4,3-e][1,2,4]thiadiazin-3(4H)-one 1,1-dioxide

[3]N-(Ethylcarbamoyl)-4-(3-tolylamino)pyridine-3-sulfonamide,

[4]Ethyl {[4-(3-tolylamino)pyridin-3-yl]sulfonyl}carbamate,

[5]N-(Butylcarbamoyl)-4-(3-tolylamino)pyridine-3-sulfonamide.

Inject reference solution (b), (d) and (e). The test is not valid unless the resolution between the peaks due to torsemide impurity A and torsemide related compound E is not less than 2.5, the tailing factor is not more than 2.0 for torsemide peak in the chromatogram obtained with reference solution (b), the relative standard deviation for replicate injections is not more than 5.0 per cent for torsemide peak in the chromatogram obtained with reference solution (e) and the signal-to-noise ratio is not less than 10 in the chromatogram obtained with reference solution (d).

Inject reference solution (e) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to torsemide related compound A is not more than 0.6 times the area of the corresponding peak in the chromatogram obtained with reference solution (e) (0.6 per cent), the area of any peak corresponding to torsemide related compound E is not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (e) (0.3 per cent), the area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 1.1 times the area of the principal peak in the chromatogram obtained with reference solution (e) (1.1 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), using the chromatographic conditions as described under Assay.

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Test solution. Disperse one tablet in *methanol* to fill 30 per cent of the volume of flask with the aid of ultrasound for 10 minutes. Add the buffer solution to fill 75 per cent of the volume of flask and dilute to 100.0 ml with the mobile phase, filter.

Reference solution. Dissolve a quantity of *torsemide IPRS* in *methanol* to fill 30 per cent of the volume of flask with the aid of ultrasound for 10 minutes and add buffer solution to fill 75 per cent of the volume of the flask and dilute to volume with the mobile phase to obtain a solution of the same concentration as that of the test solution.

Calculate the content of $C_{16}H_{20}N_4O_3S$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — The test solution is not stable at room temperature, it is stable for 15 hours at 6°.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 40 mg of Torsemide in *methanol* to fill 30 per cent of the volume of flask with the aid of ultrasound for 10 minutes. Add the buffer solution to fill 75 per cent of the volume of flask and dilute to 100.0 ml with the mobile phase, filter.

Reference solution. A 0.04 per cent w/v solution of *torsemide IPRS* prepared by dissolving in *methanol* to fill 30 per cent of the volume of flask with the aid of ultrasound for 10 minutes and add the buffer solution to fill 75 per cent of the volume of the flask and dilute to volume with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of solution containing 10 volumes of *acetonitrile* and 90 volumes of *methanol* and 50 volumes of a buffer solution prepared by dissolving 2.72 g of *monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 4.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 288 nm,
- injection volume: 20 µl.

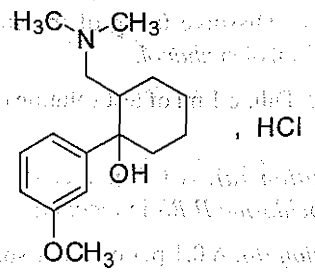
Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{20}N_4O_3S$ in the tablets.

Storage. Store protected from moisture at a temperature not exceeding 30°.

Tramadol Hydrochloride



$C_{16}H_{26}ClNO_2$ Mol. Wt. 299.8

Tramadol Hyrochloride is (1*RS*,2*RS*)-2-[(dimethylamino)-methyl]-1-(3-methoxyphenyl)cyclohexanol hydrochloride.

Tramadol Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{16}H_{26}ClNO_2$, calculated on the anhydrous basis.

Category. Analgesic.

Description. A white or almost white, crystalline powder.

Identification

Test B may be omitted if tests A and C are carried out: Test A may be omitted if tests B and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tramadol hydrochloride IPRS* or with the reference spectrum of tramadol hydrochloride.

B. In the test for impurity E, the principal spot in the chromatogram obtained with test solution (b) corresponds to the principal spot in the chromatogram obtained with reference solution (a).

C. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 5 per cent w/v solution is clear (2.4.1) and colourless (2.4.1).

Acidity. To 10 ml of 5 per cent w/v solution, add 0.2 ml of *methyl red solution* and 0.2 ml of *0.01 M hydrochloric acid.* The solution is red. Not more than 0.4 ml of *0.01 M sodium hydroxide* is required to change the colour of the indicator to yellow.

Optical rotation (2.4.22): −0.10° to +0.10°, determined on 5.0 per cent w/v solution.

Impurity E. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

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Mobile phase. A mixture of 1 volume of *concentrated ammonia*, 19 volumes of *2-propanol* and 80 volumes of *toluene*.

Test solution (a). Dissolve 0.1 g of the substances under examination in 2 ml of *methanol*.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol*.

Reference solution (a). A 0.5 per cent w/v solution of *tramadol hydrochloride IPRS* in *methanol*.

Reference solution (b). A 0.1 per cent w/v solution of *(2RS)-2-[(dimethylamino)methyl]cyclohexanone IPRS* (*tramadol impurity E IPRS*) in *methanol*. Dilute 1 ml of the solution to 10 ml with *methanol*.

Reference solution (c). A 0.5 per cent w/v solution of *(1RS,2SR)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl) cyclohexanol IPRS* (*tramadol impurity A IPRS*) in reference solution (a).

Saturate the plate for 20 minutes with *concentrated ammonia*. Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air, expose the plate to iodine vapour for 1 hour, and examine under ultraviolet light at 254 nm. The chromatogram obtained with reference solution (c) shows 2 clearly separated spots. In the chromatogram obtained with test solution (a), any secondary spot corresponding to tramadol impurity E is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.15 g of the substance under examination in 100.0 ml of the mobile phase.

Reference solution (a). Dilute 2.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *tramadol impurity A IPRS* in 4.0 ml of the test solution and dilute to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm packed with endcapped octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 29.5 volumes of *acetonitrile* and 70.5 volumes of a mixture of 0.2 ml of *trifluoroacetic acid* and 100 ml of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

The relative retention time with reference to tramadol for tramadol impurity A is about 0.85.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to tramadol impurity A and tramadol is not less than 2.0.

Inject reference solution (a) and the test solution. Run the chromatogram 4 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to tramadol impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any other secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.5 per cent, determined on 1.0 g.

Assay. Dissolve 0.18 g in 25 ml of *anhydrous acetic acid* and add 10 ml of *acetic anhydride*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02998 g of $C_{16}H_{26}ClNO_2$.

Storage. Store protected from light.

Tramadol Capsules

Tramadol Hydrochloride Capsules

Tramadol Capsules contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of tramadol hydrochloride, $C_{16}H_{25}NO_2,HCl$.

Usual Strengths. 50 mg.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 1 volume of *anhydrous formic acid*, 50 volumes of *acetone* and 50 volumes of *methanol*.

Test solution. Disperse the contents of capsules containing about 50 mg of Tramadol Hydrochloride in 25 ml of *methanol* and filter through a glass fiber filter (Such as Whatmann GF/A).

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Reference solution. A 0.2 per cent w/v solution of *tramadol hydrochloride IPRS* in *methanol*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and expose to iodine vapour until spots appear and examine in daylight. The principal spot in the chromatogram obtained with the test solution corresponds to spot obtained with the reference solution.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse the contents of capsules containing about 0.5 g of Tramadol Hydrochloride in 80 ml of the mobile phase with the aid of ultrasound and dilute to 100 ml with the mobile phase, filter through a glass fiber filter (Such as Whatmann GF/A).

Reference solution (a). Dilute 2 ml of the test solution to 100 ml with the mobile phase. Dilute 1 ml of the solution to 10 ml with the mobile phase.

Reference solution (b). A 0.0015 per cent w/v solution of *(1RS,2SR)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl) cyclohexanol IPRS (tramadol impurity A IPRS)* in the mobile phase.

Reference solution (c). A solution containing 0.0015 per cent w/v each of *tramadol hydrochloride IPRS* and *tramadol impurity A IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 1 volume of *trifluoroacetic acid*, 30 volumes of *acetonitrile* and 69 volumes of *water*,
- flow rate: 1ml per minute,
- spectrophotometer set at 271 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to tramadol impurity A and tramadol hydrochloride is not less than 3.0.

Inject reference solution (a), (b) and the test solution. Run the chromatogram four times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of peak corresponding to tramadol impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent), the area of secondary peak other than tramadol impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all other secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve a quantity of the mixed contents of 20 capsules containing about 50 mg of Tramadol Hydrochloride in 150 ml of the mobile phase with the aid of ultrasound and dilute to 200 ml with the mobile phase, filter.

Reference solution (a). A 0.025 per cent w/v solution of *tramadol hydrochloride IPRS* in the mobile phase.

Reference solution (b). A solution containing 0.0015 per cent w/v each of *tramadol hydrochloride IPRS* and *tramadol impurity A IPRS* in the mobile phase.

Use the chromatographic system as described under Related substances.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to tramadol impurity A and tramadol hydrochloride is not less than 3.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{16}H_{25}NO_2,HCl$ in the Capsules.

Tramadol Prolonged-release Tablets

Tramadol Sustained-release Tablets, Tramadol Extended-release Tablets

Tramadol Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Tramadol Prolonged-release Tablets contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of tramadol hydrochloride, $C_{16}H_{25}NO_2,HCl$.

Usual strengths. 50 mg; 100 mg.

Identification

A. Determine by thin-layer chromatography (2.4.17), using the plate coated with *silica gel GF254*.

Mobile phase. A mixture of 1 volume of *anhydrous formic acid*, 50 volumes of *acetone* and 50 volumes of *methanol*.

Test solution. Disperse a quantity of the powdered tablets containing 50 mg of tramadol hydrochloride with 25 ml of *methanol*, shake for 10 minutes, filter (such as Whatman GF/A).

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Reference solution. A 0.2 per cent w/v solution of *tramadol hydrochloride IPRS* in *methanol*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air and expose to iodine vapour until the spots appear and examine in daylight. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2). Complies with the test stated under Tablets.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 0.5 g of Tramadol Hydrochloride with 80 ml of mobile phase, mix with the aid of ultrasound, cool, add sufficient mobile phase to produce 100 ml and filter through a glass-fibre filter paper (such as Whatman GF/A).

Reference solution(a). Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase and further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A 0.0015 per cent w/v solution of *tramadol impurity A IPRS ((1RS,2SR)-2-[(dimethylamino) methyl]-1-(3-methoxyphenyl)cyclohexanol IPRS)* in the mobile phase.

Reference solution (c). A 0.0015 per cent w/v solution each of *tramadol hydrochloride IPRS* and *tramadol impurity A IPRS ((1RS,2SR)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol IPRS)* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 1 volume of *trifluoroacetic acid* , 30 volumes of *acetonitrile* and 69 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 271 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless, the resolution between tramadol hydrochloride and tramadol impurity A is not less than 3.0.

Inject reference solution (a), (b) and the test solution. Run the chromatogram 4 times the retention time of the principal peak for the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to tramadol impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14) as described under Related substances with the following modifications.

Test solution. Disperse a quantity of the powdered tablets containing 50 mg of tramadol hydrochloride with 150 ml of mobile phase, mix with the aid of ultrasound, cool, add sufficient mobile phase to produce 200.0 ml and filter through a glass-fibre filter paper (such as Whatman GF/A).

Reference solution. A 0.025 per cent w/v solution of *tramadol hydrochloride IPRS* in mobile phase.

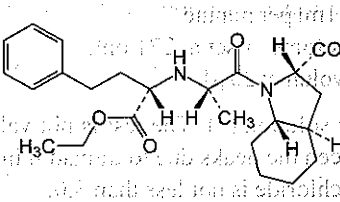
Inject reference solution (c). The test is not valid unless, the resolution between tramadol hydrochloride and tramadol impurity A is not less than 3.0.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{25}NO_2,HCl$ in the tablets.

Storage. Store protected from light and moisture, at temperature not exceeding 30°.

Trandolapril



$C_{24}H_{34}N_2O_5$ Mol. Wt. 430.5

Trandolapril is (2*S*,3a*R*,7a*S*)-1[(*S*)-2-[[(*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-1-oxo-propyl]octahydro-1*H*-indole-2-carboxylic acid.

Trandolapril contains not less than 98.5 per cent and not more than 101.5 per cent of $C_{24}H_{34}N_2O_5$, calculated on the dried basis.

Category. Antihypertensive.

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Description. A white to off white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *trandolapril IPRS* or with the reference spectrum of trandolapril.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). – 18.5° to – 16.5°, determined on 2.0 per cent w/v solution in *ethanol (95 per cent)*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in 100 ml of the mobile phase.

Reference solution. A 0.0005 per cent w/v solution of *trandolapril IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of buffer solution prepared by dissolving 6.71 g of *monobasic potassium orthophosphate* in 1000 ml of *water*, 35 volumes of *acetonitrile* and 5 volumes of *methanol*, adjusted to pH 4.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicates injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying at 50° for 3 hours under vacuum.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 10 mg of the substance under examination in 100.0 ml of the mobile phase.

Reference solution. A 0.01 per cent w/v solution of *trandolapril IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil C18),
- mobile phase: a mixture of 52 volumes of buffer solution prepared by dissolving 6.71 g of *monobasic potassium orthophosphate* in 1000 ml of *water*, 43 volumes of *acetonitrile* and 5 volumes of *methanol*, adjusted to pH 4.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{24}H_{34}N_2O_5$.

Storage. Store protected from light and moisture.

Trandolapril Tablets

Trandolapril Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of trandolapril, $C_{24}H_{34}N_2O_5$.

Usual strengths. 1 mg; 2 mg.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. When examined in the range of 200 nm to 400 nm (2.4.7), a 0.05 per cent w/v solution in *0.1 M hydrochloric acid* shows an absorption maximum as obtained with *trandolapril IPRS* of the same concentration.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium: 500 ml of *water*,
Speed and time: 50 rpm and 45 minutes.
Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

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Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve an weighed quantity of *trandolapril IPRS* in the mobile phase and dilute with dissolution medium to obtain a solution having a known concentration similar to the test solution.

Use chromatographic system as described under Assay using 100 µl injection volume:

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{24}H_{34}N_2O_5$.

Q. Not less than 80 per cent of the stated amount of $C_{24}H_{34}N_2O_5$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Use freshly prepared solutions.

Test solution. Disperse a quantity of powdered tablets containing about 10 mg of Trandolapril in 10.0 ml of the mobile phase, shake and filter.

Reference solution. A 0.001 per cent w/v solution of *trandolapril IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as ACE C8),
- mobile phase: a mixture of 60 volumes of buffer solution prepared by dissolving 6.71 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, 35 volumes of *acetonitrile* and 5 volumes of *methanol*, adjusted to pH 4.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. Run the chromatogram 5 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay with the following solutions.

Test solution. Disperse one tablet in the mobile phase, dilute to obtain a solution containing 0.005 per cent w/v of trandolapril in the mobile phase and filter.

Reference solution. Dissolve an weighed quantity of *trandolapril IPRS* in the mobile phase and dilute to obtain a solution having a known concentration similar to the test solution.

Calculate the content of $C_{24}H_{34}N_2O_5$ in the tablet.

Water (2.3.43). Not more than 7.0 per cent, determined on 0.5 g.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Transfer 10 intact tablets in 100.0-ml volumetric flask, and disperse in about 80 ml of mobile phase with the aid of ultrasound for about 30 minutes. Cool and dilute to volume with mobile phase. Mix well and filter. Dilute suitably with mobile phase to obtain a solution containing 0.005 per cent w/v of trandalapril.

Reference solution. A 0.005 per cent w/v solution of *trandolapril IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS-3),
- mobile phase: a mixture of 52 volumes of buffer solution prepared by dissolving 6.71 g of *monobasic potassium orthophosphate anhydrous* in 1000 ml of *water*, 43 volumes of *acetonitrile* and 5 volumes of *methanol*, adjusted to pH 4.0 with *orthophosphoric acid*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the theoretical plates of the principal peak is not less than 2000, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{24}H_{34}N_2O_5$ in the tablet.

Storage. Store at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the amount of Trandolapril.

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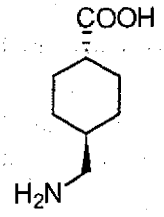
Tranexamic Acid



$C_8H_{15}NO_2$ Mol. Wt. 157.2

Tranexamic Acid is *trans*-4-(aminomethyl) cyclohexane-carboxylic acid.

Tranexamic Acid contains not less than 98.0 per cent and not more than 102.0 per cent of $C_8H_{15}NO_2$, calculated on the dried basis.

Category. Antifibrinolytic agent; Hemastatic.

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tranexamic acid IPRS* or with the reference spectrum of tranexamic acid.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in *water* and dilute to 10.0 ml with *water*.

Reference solution (a). A 0.005 per cent w/v solution of *tranexamic acid IPRS* in *water*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with *water*.

Reference solution (c). A solution containing 0.02 per cent w/v of *tranexamic acid IPRS* and 0.0002 per cent w/v of *tranexamic acid related compound C IPRS* in *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 18.3 g of *monobasic sodium phosphate* in 500 ml of *water*, add 8.3 ml of *triethylamine* and 2.3 g of *sodium dodecyl sulphate*, adjusted to pH 2.5 with *orthophosphoric acid*, dilute to 1000 ml with *water* and 40 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Tranexamic acid	1.0	—
Tranexamic acid related compound C[1]	1.1	0.005
Aminomethylbenzoic acid[2]	1.3	0.006
cis-Tranexamic acid[3]	1.5	1.21
Ditranexamic acid amine[4]	2.1	—

[1](RS)-4-(Aminomethyl)cyclohex-1-enecarboxylic acid,

[2]4-(Aminomethyl)benzoic acid,

[3]cis-4-(Aminomethyl)cyclohexanecarboxylic acid,

[4]*trans,trans*-4,4'-[Iminobis(methylene)]dicyclohexanecarboxylic acid.

Inject reference solution (a), (b) and (c). The test is not valid unless the resolution between the peaks due to tranexamic acid and tranexamic acid related compound C is not less than 2.0 in the chromatogram obtained with reference solution (c), the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (a) and signal-to-noise ratio is not less than 10 in the chromatogram obtained with reference solution (b).

Inject reference solution (a) and the test solution. Run the chromatogram 3 times the retention time of the principal peak for test solution. The area of any peak corresponding to tranexamic acid related compound C, aminomethylbenzoic acid and ditranexamic acid amine, each of, is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the area of any peak corresponding to *cis*-Tranexamic acid is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any other secondary peak is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent) and the sum of areas of all the secondary peaks is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in chromatogram obtained with reference solution (a) (0.025 per cent).

Chlorides (2.3.12). 1.8 g complies with the limit test for chlorides (140 ppm).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Assay. Determine by liquid chromatography (2.4.14).

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Test solution. Dissolve 50 mg of the substance under examination in *water* and dilute to 50.0 ml in *water*.

Reference solution. A 0.1 per cent w/v solution of *tranexamic acid IPRS* in *water*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 18.3 g of *monobasic sodium phosphate* in 500 ml of *water*, add 8.3 ml of *triethylamine* and 2.3 g of *sodium dodecyl sulphate*, adjusted to pH 2.5 with *orthophosphoric acid*, dilute to 1000 ml with *water* and 40 volumes of *methanol*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Run the chromatogram 2.5 times the retention time of the principal peak.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{15}NO_2$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Tranexamic Acid Injection

Tranexamic Acid Injection is a sterile solution of Tranexamic Acid in Water for Injections.

Tranexamic Acid Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of tranexamic acid, $C_8H_{15}NO_2$.

Usual strength. 100 mg per ml.

Identification

Transfer the content of injection into a beaker and heat on a hot plate at 100° for 30 minute, until a paste appears. Then heat it in an oven to complete dryness at 110° for about 30 minute.

A. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tranexamic acid IPRS* or with the reference spectrum of tranexamic acid.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 6.5 to 8.0.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dilute an accurately measured volume of injection with *water* to obtain a solution containing 1.0 per cent w/v of Tranexamic Acid.

Reference solution (a). A 0.005 per cent w/v solution of *tranexamic acid IPRS* in *water*.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 10.0 ml with *water*.

Reference solution (c). A solution containing 0.02 per cent w/v of *tranexamic acid IPRS* and 0.0002 per cent w/v of *tranexamic acid related compound C IPRS* in *water*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 18.3 g of *sodium dihydrogen orthophosphate* in 800 ml of *water*, add 8.3 ml of *triethylamine* and 2.3 g of *sodium dodecyl sulphate*, adjusted to pH 2.5 with *dilute orthophosphoric acid*, diluted to 1000 ml with *water* and 40 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Name	Relative retention time
Tranexamic acid	1.0
Tranexamic acid related compound C[1]*	1.1
Aminomethylbenzoic acid[2]*	1.3
cis-Tranexamic acid[3]*	1.5
Ditranexamic acid amine[4]*	2.1

*For identification only. These are process impurities monitored in the drug substance and are not included in the total impurities.

[1](RS)-4-(Aminomethyl)cyclohex-1-enecarboxylic acid.

[2]4-(Aminomethyl)benzoic acid.

[3]cis-4-(Aminomethyl)cyclohexanecarboxylic acid.

[4]*trans,trans*-4,4'-[Iminobis(methylene)]dicyclohexanecarboxylic acid.

Inject reference solution (a), (b) and (c). The test is not valid unless the resolution between the peaks due to tranexamic acid and tranexamic acid related compound C is not less than 2.0 in the chromatogram obtained with reference solution (c), the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (a) and signal-to-noise ratio is not less than 10 in the chromatogram obtained with reference solution (b). Inject reference solution (a) and the test solution. Run the chromatogram 3.3 times the retention time of the principal peak. The area of any secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Units per mg of Tranexamic Acid.

Sterility (2.2.11). Complies with the test for sterility.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Mix the contents of minimum 3 vials and dilute a suitable volume of the sample with *water* to obtain a solution containing 0.1 per cent w/v of Tranexamic acid.

Reference solution. A 0.1 per cent w/v solution of *tranexamic acid IPRS* in *water*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 18.3 g of *sodium dihydrogen orthophosphate* in 800 ml of *water*. add 8.3 ml of *triethylamine* and 2.3 g of *sodium dodecyl sulphate*, adjusted to pH 2.5 with *dilute orthophosphoric acid*, diluted to 1000 ml with *water* and 40 volumes of *methanol*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{15}NO_2$ in the injection.

Storage. Store at a temperature not exceeding 30°.

Tranexamic Acid Tablets

Tranexamic Acid Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of tranexamic acid, $C_8H_{15}NO_2$.

Usual strengths. 250 mg; 500 mg.

Identification

Shake a quantity of the powdered tablets containing 75 mg of Tranexamic Acid with 1 ml of *water*, mix on a vortex mixer for a few seconds, and sonicate for 1 minute. Pass the suspension through a suitable filter onto a suitable watchglass. Evaporate the filtrate in an oven at 60° for 2 hours, and then stir gently with a glass rod. Dry in an oven at 60°for another 1 hour.

A. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tranexamic acid IPRS* or with the reference spectrum of tranexamic acid.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a suitable quantity of *tranexamic acid IPRS* in the dissolution medium, to obtain a solution having known concentration similar to the test solution.

Use chromatographic system as described under Assay, with the following modifications.
- mobile phase: a mixture of 80 volumes of a buffer solution prepared by dissolving 10.5 g of *sodium dihydrogen phosphate monohydrate* in 1000 ml of *water*, add 8 ml of *triethylamine* and 2.3 g of *sodium dodecyl sulphate*, adjusted to pH 2.5 with *orthophosphoric acid* and 20 volumes of *acetonitrile*,

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{15}NO_2$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_8H_{15}NO_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 0.5 g of Tranexamic Acid in the mobile phase with

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the aid of ultrasound for 20 minutes with intermittent shaking and dilute to 50.0 ml with the mobile phase.

Reference solution (a). A 0.001 per cent w/v solution of *tranexamic acid IPRS* with the mobile phase.

Reference solution (b). A solution containing 0.002 per cent w/v of *tranexamic acid IPRS* and 0.0002 per cent w/v of *tranexamic acid related compound C IPRS* with the mobile phase.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm),
- mobile phase: a mixture of 85 volumes of a buffer solution prepared by dissolving 10.5 g of *sodium dihydrogen phosphate monohydrate* in 1000 ml of *water*, add 8 ml of *triethylamine* and 2.3 g of *sodium dodecyl sulphate*, adjusted to pH 2.5 with *orthophosphoric acid* and 15 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Name	Relative retention time
Tranexamic acid	1.0
Tranexamic acid related compound C[1*]	1.1
Tranexamic acid related compound D[2*]	1.2
Tranexamic acid related compound B[3]	1.5
Tranexamic acid related compound A[4]	2.3

*Process impurity controlled in the drug substance. It is included for identification purposes only and not be included in the total degradation products.

[1](RS)-4-(Aminomethyl)cyclohex-1-enecarboxylic acid,

[2]4-(Aminomethyl)benzoic acid,

[3]cis-4-(Aminomethyl)cyclohexanecarboxylic acid,

[4]*trans,trans*-4,4'-[Iminobis(methylene)]dicyclohexanecarboxylic acid.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to tranexamic acid and tranexamic acid related compound C is not less than 2.0 in the chromatogram obtained with reference solution (b), the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. Run the chromatogram 5.3 times the retention time of the principal peak. The area of any peak corresponding to tranexamic acid related compound B, multiplied with correction factor of 1.2, is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent), the area of any peak corresponding to tranexamic acid related compound A is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.65 g of Tranexamic Acid in *water*, with the aid of ultrasound for 20 minutes with intermittent shaking and dilute to 250.0 ml with *water*.

Reference solution. A 0.26 per cent w/v solution of *tranexamic acid IPRS* in *water*.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 85 volumes of a buffer solution prepared by dissolving 10.5 g of *sodium dihydrogen phosphate monohydrate* in 1000 ml of *water*, add 8 ml of *triethylamine* and 2.3 g of *sodium dodecyl sulphate*, adjusted to pH 2.5 with *orthophosphoric acid* and 15 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

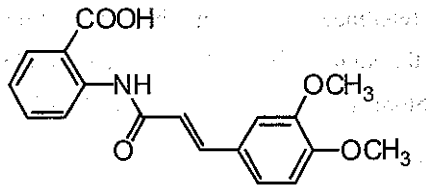
Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{15}NO_2$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Tranilast



$C_{18}H_{17}NO_5$ Mol Wt. 327.3

Tranilast is 2-((*E*)-3-(3,4-Dimethoxyphenyl)acrylamido)benzoic acid.

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Tranilast contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{18}H_{17}NO_5$, calculated on the dried basis.

Category. Antiallergic.

Description. A light yellow to green crystalline powder.

Identification

Melting range (2.4.21). 207° to 210°.

Tests

Related substances. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 30 ml of *methanol* and 70 ml of *benzene*.

Test solution. Dissolve about 1.0 g of the substance under examination in 5 ml of *dimethylformamide* and dilute to 100 ml with *ethanol (95 per cent)*.

Reference solution. Dilute 2.0 ml of the test solution to 100.0 ml with *ethanol (95 per cent)*.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 10 cm. Dry the plate in hot air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the principal spot in the chromatogram obtained with the reference solution (2.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

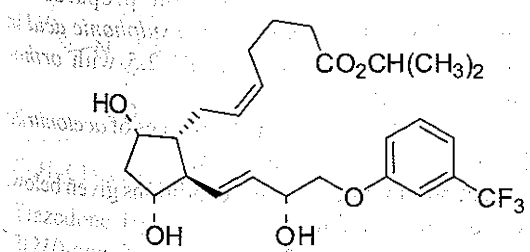
Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 100° for 3 hours.

Assay. Dissolve 0.3 g in 50.0 ml of *ethanol*. Titrate with *0.1 M sodium hydroxide*, using *phenolphthalein* as an indicator. Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.032734 g of $C_{18}H_{17}NO_5$.

Storage. Store protected from moisture.

Travoprost



$C_{26}H_{35}F_3O_6$ Mol. Wt. 500.6

Travoprost is (5*Z*)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(1*E*,3*R*)-3-hydroxy-4-[3-(trifluromethyl)phenoxy]-1-butenyl]-cyclopentyl]-5-heptenoic acid 1-methylethyl ester.

Travoprost contains not less than 96.0 per cent and not more than 102.0 per cent of $C_{26}H_{35}F_3O_6$, calculated on the anhydrous basis.

Category. Antiglaucoma.

Description. A colourless to yellowish oil.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *travoprost IPRS* or with the reference spectrum of travoprost.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Appearance of solution. A 1.0 per cent w/v solution in *ethanol (95 per cent)* is not more intensely coloured than reference solution YS7 (2.4.1).

Specific optical rotation (2.4.22). + 52° to + 58°, determined on 2.0 per cent w/v solution in *ethanol* at 365 nm.

Related substances. Not more than 3.5 per cent of 5,6-trans-travoprost.

Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in *acetonitrile* and dilute to 100.0 ml with *water*.

Reference solution. Dissolve 10 mg of *travoprost IPRS* in *acetonitrile* and dilute to 100.0 ml with *water*. Dilute 1.0 ml of the solution to 20.0 ml with *water*.

Use chromatographic system as described under Assay.

The relative retention time with reference to travoprost for 5,6-trans-travoprost is about 1.06.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than 12 times the area of the principal peak in the chromatogram obtained with the reference solution (6.0 per cent).

Water (2.3.43). Not more than 1.0 per cent, determined on 0.1 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in *acetonitrile* and dilute to 100.0 ml with *water*.

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Reference solution. Dissolve 10 mg of *travoprost IPRS* in *acetonitrile* and dilute to 10.0 ml with *water*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as Hypersil ODS),
- column temperature: 50°,
- mobile phase: A. a mixture of 66 volumes of buffer solution prepared by diluting 1 ml of *orthophosphoric acid* to 1000 ml with *water*, adjusted to pH 5.0 with *1 M sodium hydroxide*, 30 volumes of *acetonitrile* and 4 volumes of *propanol-2-ol*,
  B. a mixture of 80 volumes of *acetonitrile*, 4 volumes of *propanol-2-ol* and 16 volumes of *water*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent V/V)
0	100	0
5	80	20
50	80	20
70	0	100
80	0	100
82	100	0
90	100	0

Inject the reference solution. The test is not valid unless the resolution between the peaks due to travoprost and 5-trans-travoprost is not less than 1.5, the theoretical plates is not less than 1500, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{26}H_{35}F_3O_6$.

Storage. Store protected from moisture, at a temperature between 2° and 8°.

Travoprost Eye Drops

Travoprost Eye Drops contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of travoprost, $C_{26}H_{35}F_3O_6$.

Usual strength. 40 µg per ml.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 50 volumes of *benzene* and 40 volumes of *dioxane*.

Test solution. Extract eye drops containing about 0.8 mg of Travoprost with 20.0 ml of *chloroform* in a separating funnel. Collect the chloroform layer in a 250 ml beaker. Give three washings with chloroform. Evaporate the chloroform layer to dryness and add 3.0 ml of *methanol*.

Reference solution. Dissolve 20 mg of *travoprost IPRS* in 10 ml of *acetonitrile*, sonicate and dilute to 20.0 ml with *water*. Dilute 5.0 ml of the solution to 20.0 ml with *methanol*.

Apply to the plate 20 µl of each solution. Allow the mobile phase to rise 8 cm. Dry the plate and spray with 10 per cent w/v solution of *phosphomolybdic acid* in *ethanol*, heat the plate at 110° for 15 minutes and examine immediately. The principal spot in the chromatogram obtained with the test solution corresponds to the spot obtained with the reference solution.

Tests

pH (2.4.24). 5.7 to 6.3.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *water* and *acetonitrile*.

Test solution. Dilute the eye drops, if necessary with the solvent mixture, to produce a solution containing 0.004 per cent w/v of travoprost.

Reference solution (a). A 0.004 per cent w/v solution of *travoprost IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- column temperature: 35°,
- sample temperature: 10°,
- mobile phase A: a buffer solution prepared by dissolving 1.5 g of *1-octane sodium sulphonic acid* in 1000 ml of *water*, adjusted to pH 2.5 with *ortho-phosphosric acid*,
  B: a mixture of 80 volumes of *acetonitrile* and 20 volumes of mobile phase A,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 100 µl.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	50	50
50	42	58
52	50	50
60	50	50

Equilibrate the column for at least 60 minutes with the mobile phase at the initial composition.

The relative retention time with respect to travoprost for 5,6-trans travoprost is 1.1 [*NOTE — Travaprost IPRS* contains a small percentage of the 5,6- trans isomer].

Inject reference solution (a). The test is not valid unless the resolution obtained between travoprost and 5,6-trans travoprost is not less than 1.5. The column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (4.0 per cent) and the sum of areas of all the secondary peaks is not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (6.0 per cent).

Other tests. Comply with the tests stated under Eye Drops.

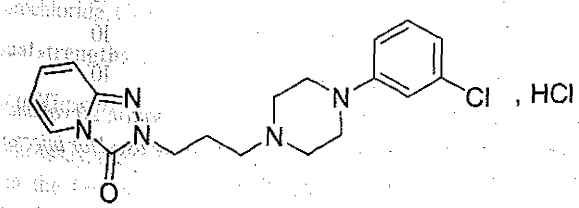
Assay. Determine by liquid chromatography (2.4.14) as described under test for Related substances.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{26}H_{35}F_3O_6$.

Storage. Store at a temperature not exceeding 30°.

Trazodone Hydrochloride



$C_{19}H_{22}ClN_5O,HCl$ Mol. Wt. 408.3

Trazodone Hydrochloride is 1,2,4-Triazolo[(4,3-a]pyridin-3(2*H*)-one, 2-[3-[4-(3-chlorophenyl)-1-piperazinyl]propyl]-, monohydrochloride.

Trazodone Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{19}H_{22}ClN_5O,HCl$, calculated on the dried basis.

Category. Antidepressant.

Description. A white to off white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *trazodone hydrochloride IPRS* or with the reference spectrum of trazodone hydrochloride.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of mobile phase A and 20 volumes of mobile phase B.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). A 0.0001 per cent w/v solution of *trazodone hydrochloride IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.0001 per cent w/v, each of, *trazodone related compound C IPRS* and *trazodone related compound D IPRS* in reference solution (a).

Chromatographic system

- a stainless steel column 7.5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm),
- mobile phase: A. a 0.01per cent v/v solution of *triethylamine* in *water*,
  B. a 0.01per cent v/v solution of *triethylamine* in *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
12	32	68
12.01	80	20
15	80	20

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Name	Relative retention time	Correction factor
Triazolopyridinone[1]	0.1	2.08
Trazodone *N*-oxide[2]	0.40	—
Deschloro trazodone[3]	0.65	1.41
Trazodone related compound C	0.96	—
Trazodone	1.0	—
Trazodone related compound D	1.1	—
4-Ethyl trazodone[4]	1.4	—
Trazodone isobutyl ether analog[5]	2.0	—
Bispiperazine analog[6]	2.1	0.77

[1][1,2,4]Triazolo[4,3-a]pyridin-3(2*H*)-one,

[2]4-(3-Chlorophenyl)-1-[3-(3-oxo-[1,2,4]triazolo[4,3-a]pyridin-2(3*H*)-yl)propyl]piperazine 1-oxide,

[3]2-[3-(4-Phenylpiperazin-1-yl)propyl]-[1,2,4]triazolo[4,3-a]pyridin-3(2*H*)-one,

[4]2-{3-[4-(3-Chloro-4-ethylphenyl)piperazin-1-yl]propyl}-[1,2,4]triazolo[4,3-a]pyridin-3(2*H*)-one,

[5]1-(3-Chlorophenyl)-4-(3-isobutoxypropyl)piperazine,

[6]1,3-Bis(4-(3-chlorophenyl)piperazin-1-yl)propane.

NOTE — A mixture of 40 volumes of acetonitrile, 30 volumes of 2-propanol, 30 volumes of acetone and 0.2 volume of formic acid may be used for injector wash to minimize sample carry-over.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks corresponding to trazodone related compound C and trazodone is not less than 1.5 and the peaks corresponding to trazodone and trazodone related compound D is not less than 2.8 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to triazolopyridinone, trazodone n-oxide, deschloro trazodone, trazodone related compound C, trazodone related compound D, 4-ethyl trazodone, trazodone isobutyl ether analog and bispiperazine analog, each of, is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Limit of Trazodone related compound F and Cyclophosphamide related compound A. Determine by liquid chromatography/ mass spectrometry (2.4.39)

NOTE — Perform this test only if trazodone related compound F and cyclophosphamide related compound A are known process impurities.

Solvent mixture. 10 volumes of *acetonitrile*, 90 volumes of *water* and 0.1 volume of *formic acid*.

Test solution. Dissolve 1g of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution . A solution containing 0.00025 per cent w/v, each of, *trazodone related compound F IPRS* and *cyclophosphamide related compound A IPRS* in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 7.5 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm),
- column temperature : 40°,
- mobile phase: A. a 0.005 *M ammonium bicarbonate* in *water*,

B. *acetonitrile*,
- a gradient programme using the conditions given below,
- MS/MS (tandom mass spectrophotometer) set as, Ionization: Triple quadrupole ionization in positive ion mode.
  Aquisition mode: Multiple reaction monitoring (MRM) of the following mass transitions; cyclophosphamide related compound A 142 → 63 trazodone related compound F 273 → 120
- flow rate: 1.5 ml per minute,
- flow rate to ion source: 0.5 ml per minute,
- injection volume: Adjust between 5 and 50 µl , depending on the mass spectrometer.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
6.5	20	80
6.51	90	10
10.0	90	10

NOTE — A mixture of 80 volumes of 2-propanol, 20 volumes of water and 0.1 volume of formic acid may be used for injector wash to minimize sample carry over.

The relative retention time with reference to trazodone related compound F for cyclophosphamide related compound A is about 0.4.

Inject the reference solution. The test is not valid unless the signal-to-noise ratio is not less than100 for the trazodone

related compound F peak, and not less than 50 for the cyclophosphamide related compound A peak and the relative standard deviation for replicate injections is not more than 15.0 per cent for each of trazodone related compound F and cyclophosphamide related compound A peak.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to trazodone related compound F and cyclophosphamide related compound A, each of, is not more than 0.01 times the area of the principal peak in the chromatogram obtained with the reference solution (0.00025 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°, at a pressure of 50 mm of Hg for 3 hours.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modifications.

Test solution. Weigh accurately 0.1g of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution. A 0.1 per cent w/v solution of *trazodone hydrochloride IPRS* in the solvent mixture.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{22}ClN_5O$, HCl.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Trazodone Tablets

Trazodone Hydrochloride Tablets

Trazodone Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of trazodone hydrochloride, $C_{19}H_{22}ClN_5O$, HCl.

Usual strengths. 25 mg; 50 mg; 100 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution .

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.01 M hydrochloric acid*,

Speed and time. 50 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *trazodone hydrochloride IPRS* in the dissolution medium and dilute with the same medium to obtain a solution of known concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 10 cm x 5 mm, packed with octadecylsilane bonded to porous silica (4 µm),
- mobile phase: a mixture of 25 volumes of a buffer solution prepared by dissolving 1.15 g of *ammonium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.0 with *sodium hydroxide* and 75 volumes of *methanol*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 246 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 900 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution. Run the chromatogram 4.5 times the retention time of the principal peak.

Calculate the content of $C_{19}H_{22}ClN_5O$, HCl in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{19}H_{22}ClN_5O$, HCl.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 65 volumes of *methanol*, 35 volumes of *water* and 0.3 volume of *hydrochloric acid*.

Test solution. Disperse a quantity of the powdered tablets containing 50 mg of trazodone in the solvent mixture, with the aid of ultrasound for 10 minutes and dilute to 100.0 ml with the solvent mixture.

Reference solution (a). An accurately weighed quantity of *trazodone hydrochloride IPRS* containing 0.0005 per cent w/v solution of trazodone in the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution (b). A solution containing 0.00007 per cent w/v of *trazodone hydrochloride IPRS* and 0.00015 per cent w/v of *trazodone related compound C IPRS* in the solvent mixture.

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Chromatographic system

- a stainless steel column 15 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- mobile phase: A. dissolve 6.75 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, add 1.0 ml of *triethylamine* and mix,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 0.7 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
5	90	10
30	60	40
35	60	40
60	42	58
63	30	70
78	30	70
78.1	90	10
90	90	10

Name	Relative retention time
Triazolopyridinone[1]*	0.1
Chlorophenylpiperazine[2]*	0.6
Hyroxypropyl chlorophenyl piperazine[3]*	0.7
Isotrazodone[4]*	0.8
Trazodone related compound C*	0.97
Trazodone hydrochloride	1.0
Trazodone dimer[5]*	1.5
Trazodone related compound F[6]*	1.6
Bispiperazine analog[7]*	1.8
Bis(3-chlorophenyl)piperazine[8]*	2.2

*Process impurity included for indentification only and not included in the calculation of total degradation products.

[1][1,2,4]Triazolo[4,3-a]pyridin-3(2*H*)-one,

[2]1-(3-Chlorophenyl)piperazine,

[3]3-[4-(3-chlorophenyl)piperazin-1-yl]propan-1-ol,

[4]1-{3-[4-(3-chlorophenyl)piperazin-1-yl]propyl}-[1,2,4]triazolo[4,3-a]pyridin-1-ium-3-olate,

[5]1,1-Bis{2-chloro-[4-(3-{1,2,4,-triazolo[4,3-a]pyridin-3-(2*H*)-on-2-yl}propyl)piperazine-1-yl]phenyl}ethane trihydrochloride,

[6]1-(3-chlorophenyl)-4-(3-chloropropyl)piperazine,

[7]1,3-Bis(4-(3-chlorophenyl)piperazin-1-yl)propane,

[8]1,4-bis(3-chlorophenyl)piperazine.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to trazodone related compound C and trazodone is not less than 2.5 in the chromatogram obtained with reference solution (b), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent) and the sum of the areas of all the secondary peaks is not more than 20 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14), as described under Dissolution with the following modifications.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of Trazodone Hydrochloride in *0.01M hydrochloric acid*, with the aid of ultrasound for 30 minutes and dilute to 50.0 ml with *0.01 M hydrochloric acid*. Dilute 5.0 ml of the solution to 50.0 ml with *0.01M hydrochloric acid*.

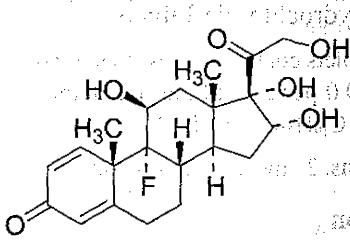
Reference solution. A 0.01 per cent w/v solution of *trazodone hydrochloride IPRS* in *0.01 M hydrochloric acid*.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{22}ClN_5O$, HCl in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Triamcinolone



$C_{21}H_{27}FO_6$ Mol. Wt. 394.4

Triamcinolone is 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione.

Triamcinolone contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{21}H_{27}FO_6$, calculated on the dried basis.

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Category. Corticosteroid.

Description. A white or almost white, crystalline powder; slightly hygroscopic.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *triamcinolone IPRS* or with the reference spectrum of triamcinolone.

B. When examined in the range 220 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution in *methanol* shows an absorption maximum at about 238 nm; absorbance at about 238 nm, about 0.76.

C. Dissolve 1 mg in 6 ml of *ethanol (95 per cent)*, add 5 ml of a 1 per cent w/v solution of *butylated hydroxytoluene* in *ethanol (95 per cent)* and 5 ml of *1 M sodium hydroxide* and heat on a water-bath under a reflux condenser for 20 minutes; a pinkish lavender colour is produced.

Tests

Specific optical rotation (2.4.22). +65.0° to +72.0°, determined in a 1.0 per cent w/v solution in *dimethylformamide*.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE— Prepare the following solutions immediately before use and protect from light.

Test solution. Dissolve 25 mg of the substance under examination in a mixture of equal volumes of *methanol* and *water* and dilute to 10 ml with the same solvent mixture.

Reference solution. Dilute 1 ml of the test solution to 100 ml with a mixture of equal volumes of *methanol* and *water*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with base-deactivated end-capped octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture prepared by mixing 525 ml of *methanol* with 400 ml of *water*, allowing to equilibrate, adjusting the volume to 1000.0 ml with *water* and mixing again,
- flow rate: 1 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution. Run the chromatogram 4.5 times the retention time of triamcinolone (about 11 minutes). In the chromatogram obtained with the test solution the area of any peak other than the principal peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1 per cent); not more than two such peaks have an area more than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent); the sum of the areas of all the peaks other than the principal peak is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (2 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Heavy metals (2.3.13). 0.8 g complies with the limit test for heavy metals, Method B (25 ppm).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Loss on drying (2.4.19). Not more than 2.0 per cent, determined on 1.0 g by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 3 hours.

Assay. Dissolve 25 mg in sufficient *ethanol (95 per cent)* to produce 100.0 ml and mix. Dilute 2.0 ml to 50.0 ml with *ethanol (95 per cent)* and measure the absorbance of the resulting solution at the maximum at about 238 nm (2.4.7).

Calculate the content of $C_{21}H_{27}FO_6$ taking 389 as the specific absorbance at 238 nm.

Storage. Store protected from light and moisture.

Triamcinolone Tablets

Triamcinolone Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of triamcinolone, $C_{21}H_{27}FO_6$.

Usual strengths. 2 mg; 4 mg; 8 mg.

Identification

A. Dissolve 1 mg of the residue obtained in the test for Related substances in 6 ml of *ethanol (95 per cent)*, add 5 ml of a 1 per cent w/v solution of *butylated hydroxytoluene* in *ethanol (95 per cent)* and 5 ml of *1 M sodium hydroxide* and heat on a water-bath under a reflux condenser for 20 minutes; a pinkish lavender colour is produced.

B. In the Assay, the chromatogram obtained with the principal peak obtained with the test solution corresponds to the peak due to triamcinolone in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium. 900 ml of *0.01 M hydrochloric acid,*

Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, dilute suitably if necessary with

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the medium, at the maximum at about 238 nm (2.4.7). Calculate the content of $C_{21}H_{27}FO_6$ in the medium from the absorbance obtained from a solution of known concentration of *triamcinolone IPRS* in the dissolution medium.

Q. Not less than 75 per cent of the stated amount of $C_{21}H_{27}FO_6$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets containing 15 mg of Triamcinolone with 15 ml of *ethanol* for 15 minutes, filter under reduced pressure through a fine filter paper (such as Whatman No 42) and evaporate the filtrate to dryness using a rotary evaporator. Reserve 1 mg of the residue for Identification test A. Dissolve the remainder of the residue in 15 ml of *methanol*.

Reference solution (a). Dilute 4 ml of the test solution to 100 ml with *methanol*.

Reference solution (b). Dilute 5 ml of the test solution to 50 ml with *methanol*. Dilute 5 ml of the resulting solution to 50 ml with the same solvent.

Chromatographic system

- a stainless steel column 20 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of *methanol* and *water*, adjusted so that the retention time of triamcinolone is about 5 minutes (approximately equal volumes of *methanol* and *water*),
- flow rate: 2 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 20 µl.

Inject reference solution (a). The test is not valid unless the column efficiency determined from the principal peak is not less than 2000 theoretical plates.

Inject reference solution (b). Run the chromatogram 4 times the retention time of the triamcinolone peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent) and not more than one such peak has an area more than that of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and the sum of the areas of any such peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Crush one tablet to a fine powder, add 100 ml of *ethanol (95 per cent)* and shake for 10 minutes. Filter, dilute 2.0 ml of the filtrate with sufficient *ethanol (95 per cent)* to produce a solution containing 0.002 per cent w/v of Triamcinolone and measure the absorbance of the resulting solution at the maximum at about 238 nm (2.4.7).

Calculate the content of $C_{21}H_{27}FO_6$ in the tablet taking 389 as the specific absorbance at 238 nm.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Internal standard solution. A 0.06 per cent w/v solution of *testosterone* in *methanol*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 2.5 mg of Triamcinolone in 5.0 ml of internal standard solution and dilute to 25.0 ml with 50 per cent v/v of *methanol*. Shake for 15 minutes, mix with the aid of ultrasound for 10 minutes, centrifuge and use the supernatant liquid.

Reference solution. Add 5.0 ml of internal standard solution to 5.0 ml of a 0.05 per cent w/v solution of *triamcinolone IPRS* in *methanol* and dilute to 25.0 ml with 50 per cent v/v of *methanol*.

Chromatographic system

- a stainless steel column 20 cm x 4 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 70 volumes of *methanol*, 30 volumes of *water* and 0.1 volume of *glacial acetic acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 20 µl.

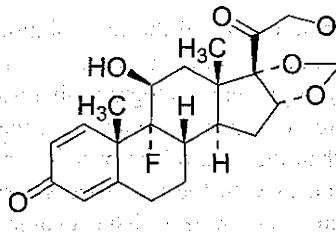
Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation of peak area ratio due to triamcinolone and internal standard for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{21}H_{27}FO_6$ in the tablets, using peak area ratio of triamcinolone and internal standard.

Storage. Store protected from moisture.

Triamcinolone Acetonide



$C_{24}H_{31}FO_6$ Mol. Wt. 434.5

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Triamcinolone Acetonide is 9α-fluoro-11β,21-dihydroxy-16α,17α-isopropylidenedioxy-1,4-pregnadiene-3,20-dione.

Triamcinolone Acetonide contains not less than 96.0 per cent and not more than 104.0 per cent of $C_{24}H_{31}FO_6$, calculated on the anhydrous basis.

Category. Corticosteroid.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Test C may be omitted if tests A and B are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *triamcinolone acetonide IPRS.*

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G.*

Solvent mixture. 90 volumes of *acetone* and 10 volumes of *formamide.*

Mobile phase. A mixture of 115 volumes of *cyclohexane*, 56 volumes of *chloroform* and 29 volumes of *toluene.*

Test solution. Dissolve 25 mg of the substance under examination in 10 ml of the solvent mixture.

Reference solution (a). Dissolve 25 mg of *triamcinolone acetonide IPRS* in 10 ml of the solvent mixture.

Reference solution (b). Mix equal volumes of the test solution and reference solution (a).

Place the dry plate in a tank containing a shallow layer of the solvent mixture, allow the solvent mixture to ascend to the top, remove the plate from the tank and allow the solvent to evaporate. Use within 2 hours, with the flow of the mobile phase in the direction in which the aforementioned treatment was done.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of warm air, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulphuric acid (20 per cent v/v).* Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

C. Heat 0.5 ml of *chromic-sulphuric acid* in a test-tube (5 cm × about 6 mm) in a naked flame until white fumes are evolved; the solution wets the sides of the tube readily and there is no greasiness. Add about 2 mg of the substance under examination and again heat in a naked flame until white fumes appear; the solution does not wet the sides of the tube and does not pour easily from the tube.

Tests

Light absorption (2.4.7). When examined in the range 230 nm to 360 nm, a 0.001 per cent w/v solution in *ethanol (95 per cent)* shows an absorption maximum at about 239 nm; absorbance at about 239 nm, 0.34 to 0.37.

Specific optical rotation (2,4,22). + 100° to + 107°, determined in a 1.0 per cent w/v solution in *dioxan.*

Related substances. Determine by liquid chromatography (2.4.14).

NOTE – Carry out the test protected from light.

Test solution. Dissolve 25 mg of the substance under examination in 7 ml of *methanol* and dilute to 10 ml with *water.*

Reference solution (a). Dissolve 2 mg of *triamcinolone acetonide IPRS* and 2 mg of *triamcinolone* in the mobile phase and dilute to 100 ml with the mobile phase.

Reference solution (b). Dilute 1 ml of the test solution to 100 ml with the mobile phase.

Chromatographic system

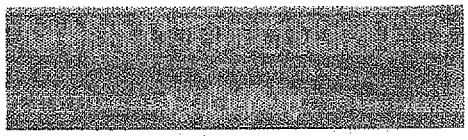
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture prepared by mixing 525 ml of *methanol* with 400 ml of *water*, allowing to equilibrate, adjusting the volume to 1000.0 ml with *water* and mixing again,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Equilibrate the column with the mobile phase for about 10 minutes.

Inject reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder.

Inject reference solution (a). The retention times are; triamcinolone, about 5 minutes and triamcinolone acetonide about 17 minutes. The test is not valid unless the resolution between the peaks corresponding to triamcinolone and triamcinolone acetonide is at least 15; if necessary, adjust the concentration of *methanol* in the mobile phase.

Inject reference solution (b) and the test solution. Continue the chromatography for 3.5 times the retention time of the principal peak in the chromatogram obtained with the test solution. In the chromatogram obtained with the test solution the area of any peak other than the principal peak is not more than 0.25 times the area of the principal peak in the



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chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks other than the principal peak is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak due to the solvent and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). Not more than 2.0 per cent, determined on 0.2 g.

Assay. Dissolve 25 mg in sufficient *ethanol* to produce 100.0 ml and mix. Dilute 5.0 ml to 100.0 ml with *ethanol (95 per cent)* and measure the absorbance of the resulting solution at the maximum at about 239 nm (2.4.7).

Calculate the content of $C_{24}H_{31}FO_6$ taking 354 as the specific absorbance at 239 nm.

Storage. Store protected from light and moisture.

Triamcinolone Acetonide Injection

Triamcinolone Acetonide Injection is a sterile suspension of Triamcinolone Acetonide in very fine particles in Water for Injections containing suitable dispersing agents.

Triamcinolone Acetonide Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of triamcinolone acetonide, $C_{24}H_{31}FO_6$.

Usual strength. 40 mg per ml.

Identification

Extract a volume containing about 50 mg of Triamcinolone Acetonide with two quantities, each of 10 ml, of *peroxide-free ether* and discard the ether extracts. Filter the aqueous layer through a sintered-glass filter, wash the residue with four quantities, each of 5 ml *of water* and dry at 105° for 1 hour. The residue complies with the following tests.

Test A may be omitted if tests B and C are carried out. Test C may be omitted if tests A and B are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *triamcinolone acetonide IPRS* or with the reference spectrum of triamcinolone acetonide.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Solvent mixture. 90 volumes of *acetone* and 10 volumes of *formamide*.

Mobile phase. A mixture of 115 volumes of *cyclohexane*, 56 volumes of *chloroform* and 29 volumes of *toluene*.

Test solution. Dissolve 25 mg of the substance under examination in 10 ml of the solvent mixture.

Reference solution (a). Dissolve 25 mg of *triamcinolone acetonide IPRS* in 10 ml of the solvent mixture.

Reference solution (b). Mix equal volumes of the test solution and reference solution (a).

Place the dry plate in a tank containing a shallow layer of the solvent mixture, allow the solvent mixture to ascend to the top, remove the plate from the tank and allow the solvent to evaporate. Use within 2 hours, with the flow of the mobile phase in the direction in which the aforementioned treatment was done.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of warm air, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulphuric acid (20 per cent v/v)*. Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

C. Heat 0.5 ml of *chromic-sulphuric acid* in a test-tube (5 cm × about 6 mm) in a naked flame until white fumes are evolved; the solution wets the sides of the tube readily and there is no greasiness. Add about 2 mg of the substance under examination and again heat in a naked flame until white fumes appear; the solution does not wet the sides of the tube and does not pour easily from the tube.

Tests

pH (2.4.24). 5.0 to 7.5.

Bacterial endotoxins (2.2.3). Not more than 4.4 Endotoxin Units per mg of triamcinolone acetonide.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Shake and mix contents of 10 containers. Dissolve a volume of the freshly mixed injection with 0.01 per cent w/v solution of prednisolone in *methanol* (internal standard) to obtain a final concentration of 0.02 per cent w/v of triamcinolone acetonide, shake and filter.

Reference solution (a). A solution containing 0.02 per cent w/v of *triamcinolone acetonide IPRS* in internal standard.

Reference solution (b). Prepare in the same manner as test solution but omitting the internal standard.

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Chromatographic system
- a stainless steel column 25 cm x 4 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 56 volumes of *methanol* and 44 volumes of *water*,
- flow rate: 1.4 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (b) for identification of peak due to triamcinolone acetonide.

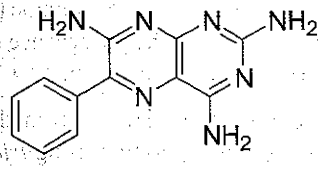
Inject reference solution (a). Adjust the flow rate of the mobile phase such that the separation of the triamcinolone acetonide and internal standard is optimised with a retention time of about 15 minutes for triamcinolone acetonide.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{24}H_{31}FO_6$ in the injection.

Storage. Store protected from light.

Triamterene



$C_{12}H_{11}N_7$ Mol. Wt. 253.3

Triamterene is 2,4,7-triamino-6-phenylpteridine.

Triamterene contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{12}H_{11}N_7$, calculated on the dried basis.

Category. Potassium sparing diuretic.

Description. A yellow, crystalline powder.

Identification

A. When examined in the range 250 nm to 380 nm (2.4.6), a 0.001 per cent w/v solution in a mixture of 9 volumes of *ethanol (95 per cent)* and 1 volume of *1 M hydrochloric acid* shows absorption maxima at about 262 nm and 360 nm, and a shoulder at about 285 nm.

B. A 0.1 per cent w/v solution in *anhydrous formic acid*, when examined under ultraviolet light at 365 nm shows an intense blue fluorescence. Solutions in other acids also exhibit a blue fluorescence.

Tests

Acidity. Boil 1.0 g with 20 ml of *water* for 5 minutes, cool, filter and wash the filter with three quantities, each of 10 ml, of *water*. Combine the filtrate and washings and add 0.3 ml of *phenolphthalein solution*. Not more than 1.5 ml of *0.01 M sodium hydroxide* is required to change the colour of the solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 10 mg of the substance under examination in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A 0.0005 per cent w/v solution of *nitrosotriaminopyrimidine IPRS (triamterene impurity A IPRS)* in the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve a suitable quantity of *2,7-diamino-6-phenylpteridin-4-ol IPRS (triamterene impurity B IPRS)* in 0.5 ml of the mobile phase with the aid of ultrasound. To this solution, add 0.5 ml of the test solution.

Chromatographic system
- a stainless steel column 25 cm x 4 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 0.2 volume of *butylamine*, 20 volumes of *acetonitrile*, 20 volumes of *methanol* and 60 volumes of *water*, adjusted to pH 5.3 with *acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 320 nm and 355 nm,
- injection volume: 50 µl.

The relative retention time with reference to triamterene for triamterene impurity A is about 0.6, for triamterene impurity B is about 0.8 and for triamterene impurity C is about 1.7.

Inject reference solution (b) and (c). The test is not valid unless the resolution between the peaks due to triamterene impurity B and triamterene at 355 nm is not less than 1.5 in the chromatogram obtained with reference solution (c) if necessary, increase the quantity of water in the mobile phase and signal-to- noise ratio for the principal peak in the chromatogram obtained with reference solution (b) at 320 nm is not less than 10. Calculate the contents of the impurities in the test solution using the correction factors, 1.8 for triamterene impurity B and 1.5 for triamterene impurity C.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to triamterene impurity A at 320 nm is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (50 ppm). The area of any peak corresponding to triamterene impurities

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B and C at 355 nm is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the area of any other secondary peak at 355 nm is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks at 355 nm is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Ignore any peak at 355 nm with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.15 g in 5 ml of *anhydrous formic acid*, add 100 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02533 g of $C_{12}H_{11}N_7$.

Storage. Store protected from light and moisture.

Triamterene Capsules

Triamterene Capsules contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of triamterene, $C_{12}H_{11}N_7$.

Usual strength. 50 mg.

Identification

The final solution obtained in the Assay has a bluish fluorescence and when examined in the range 250 nm to 380 nm (2.4.7), shows an absorption maximum at about 360 nm.

Tests

Dissolution (2.5.2),

Apparatus No. 2 (Paddle),

Medium. 900 ml of 1 per cent w/v solution of *polysorbate 20* in *0.1 M acetic acid*,

Speed and time. 100 rpm and 120 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, dilute suitably if necessary with the medium, at the maximum at about 357 nm (2.4.7). Calculate the content of $C_{12}H_{11}N_7$ in the medium from the absorbance obtained from a solution of known concentration of *triamterene IPRS* in the dissolution medium.

Q. Not less than 80 per cent of the stated amount of $C_{12}H_{11}N_7$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 90 volumes of *ethyl acetate*, 10 volumes of *18 M ammonia* and 10 volumes of *methanol*.

Test solution. Dissolve a quantity of the contents of the capsules containing 0.1 g of Triamterene in sufficient *dimethyl sulphoxide* to produce 20 ml and dilute 2 ml to 50 ml with *methanol*.

Reference solution. Dilute 1 volume of the test solution to 200 volumes with *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air until the odour of solvent is no longer detectable and examine under ultraviolet light at 365 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Other tests. Comply with the tests stated under Capsules.

Assay. Weigh a quantity of the contents of 20 capsules containing about 0.1 g of Triamterene, dissolve in 50 ml of a mixture of equal volumes of *glacial acetic acid* and *water* with the aid of gentle heat, cool and add sufficient *water* to produce 500.0 ml. Dilute 5.0 ml of the solution to 100.0 ml with *1 M acetic acid* and measure the absorbance of the resulting solution at the maximum at about 360 nm (2.4.7).

Calculate the content of $C_{12}H_{11}N_7$ from the absorbance obtained by repeating the operation using *triamterene IPRS* in place of the contents of the capsules.

Storage. Store protected from moisture.

Triamterene and Hydrochlorothiazide Tablets

Triamterene and Hydrochlorothiazide Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of triamterene, $C_{12}H_{11}N_7$ and hydrochlorothiazide, $C_7H_8ClN_3O_4S_2$.

Usual strength. Triamterene, 50 mg and Hydrochlorothiazide, 25 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

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Medium: 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 75 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a suitable quantity of *triamterene IPRS* and *hydrochlorothiazide IPRS* in the dissolution medium to obtain a solution of known concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 25cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 80 volumes of a buffer solution prepared by dissolving 6.9 g of *sodium dihydrogen phosphate* and 1.43 g of *propylamine hydrochloride* in 1000 ml of *water*, adjusted to pH 5.5 with *1M sodium hydroxide* and 20 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

The relative retention times with reference to triamterene for hydrochlorothiazide is about 0.65.

Inject the reference solution. The test is not valid unless the resolution between the hydrochlorothiazide and triamterene peaks is not less than 3.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent for both the peaks.

Inject the reference solution and the test solution.

Calculate the content of $C_{12}H_{11}N_7$ and $C_7H_8ClN_3O_4S_2$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{12}H_{11}N_7$ and $C_7H_8ClN_3O_4S_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture A. A buffer solution prepared by dissolving 6.8 g of *sodium acetate trihydrate* in 1000 ml of *water*, adjusted to pH 5.0 with *glacial acetic acid*.

Solvent mixture B. 75 volumes of *acetonitrile* and 25 volumes of *methanol*.

Test solution. Disperse a quantity of the powdered tablets containing 150 mg of Hydrochlorothiazide in 60 ml of *acetonitrile* and 6 ml of *glacial acetic acid*, with the aid of ultrasound for 10 minutes. Cool and dilute to 100.0 ml with *water*.

Reference solution. A 0.015 per cent w/v solution of *benzothiadiazine related compound A (4-Amino-6-chloro-1,3-benzenedisulphonamide) IPRS* in *acetonitrile*. Transfer 10.0 ml of the solution to 100-ml volumetric flask, add 50 ml of *acetonitrile* and 6 ml of *glacial acetic acid* and dilute with *water* to volume.

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 10 volumes of solvent mixture B and 90 volumes of solvent mixture A,
- flow rate: 2 ml per minute,
- spectrophotometer set 273 nm,
- injection volume: 10 µl.

The relative retention times with reference to benzothiadiazine related compound A for hydrochlorothiazide and triamterene are 1.5 and 10, respectively.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area any peak corresponding to benzothiadiazine related compound A is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent).

Other tests. Comply with the tests stated under tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 35 volumes of *acetonitrile*, 3.5 volumes of *glacial acetic acid* and 65 volumes of *water*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 250 mg of hydrochlorothiazide in the solvent mixture, with the aid of ultrasound for 30 minutes and dilute to 500.0 ml with the solvent mixture and filter. Dilute 3.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (a). A solution containing 0.3 per cent w/v of *triamterene IPRS* and 0.15 per cent w/v of *hydrochlorothiazide IPRS* in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.225 per cent w/v of *triamterene IPRS*, 0.15 per cent w/v of *hydrochlorothiazide IPRS* and 0.00075 per cent w/v of *benzothiadiazine related compound A IPRS* in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of a buffer solution prepared by dissolving 0.82 g of *sodium acetate*

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anhydrous in 1000 ml of *water*, adjusted to pH 4.5 with *glacial acetic acid* and 35 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 272 nm,
- injection volume: 20 µl.

The relative retention time with reference to hydrochlorothiazide for benzothiadiazine related compound A, is about 0.93.

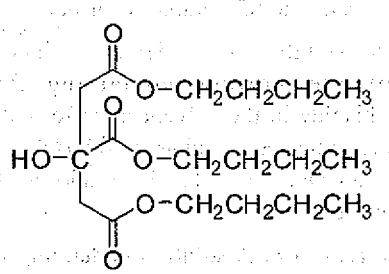
Inject reference solution (a) and reference solution (b). The test is not valid unless the resolution between the peaks due to hydrochlorothiazide and benzothiadiazine related compound A is not less than 1.5 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 2.0 per cent for both the peaks in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution. Run the chromatogram twice the retention time of hydrochlorothiazide peak.

Calculate the content of $C_{12}H_{11}N_7$ and $C_7H_8ClN_3O_4S_2$ in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Tributyl Citrate



$C_{18}H_{32}O_7$ Mol. Wt. 360.5

Tributyl Citrate contains not less than 99.0 per cent of $C_{18}H_{32}O_7$, calculated on the anhydrous basis.

Category. Excipient.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tributyl citrate IPRS* or with the reference spectrum of tributyl citrate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Weight per ml (2.4.29). 1.037 to 1.045.

Refractive index (2.4.27). 1.443 to 1.445.

Acidity. Dissolve 32.0 g in 30 ml of *ethanol (95 per cent)*, previously neutralised to *bromothymol blue*, add *bromothymol blue solution* and titrate with *0.1 M sodium hydroxide* to a faint blue endpoint; not more than 1.0 ml is required.

Water (2.3.43). Not more than 0.2 per cent.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Assay. Determine by gas chromatography (2.4.13).

Test solution. Dissolve about 300 mg of substance under examination in 10 ml of *toluene*.

Reference solution. A solution containing 3.0 per cent w/v of *tributyl citrate IPRS* and *acetyltributyl citrate IPRS* in *toluene*.

Chromatographic system
- a glass or stainless steel column 30 m x 0.32 mm, packed with bonded with a 0.5-µm layer of phase G42,
- temperature:
  column. 80° to 220° from 0.5 to 10 minutes,
  inlet port. 85° to 250° from 0.5 to 10 minutes (increased @ 20° per minute) and detector 275°,
- a flame ionisation detector,
- flow rate: 2.3 ml per minute of the nitrogen as the carrier gas.

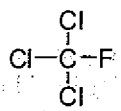
Inject 1 µl of the reference solution. The test is not valid unless the relative retention times with reference to acetyltributyl citrate for tributyl citrate is about 0.9, the resolution between the peaks corresponding to tributyl citrate and acetyltributyl citrate is not less than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject 1 µl of the reference solution and the test solution.

Calculate the content $C_{18}H_{32}O_7$.

Storage. Store protected from moisture.

Trichloromonofluoromethane



CCl_3F Mol. Wt. 137.4

Trichloromonofluoromethane contains not less than 99.6 per cent and not more than 100.0 per cent of CCl_3F, calculated on the anhydrous basis.

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Category. Pharmaceutical aid (Excipient).

Identification

Determine by infrared absorption spectrophotometry (2.4.6), compare the spectrum with the obtained with *trichloromonofluromethane IPRS* or with the reference spectrum of trichloromonofluromethane.

Tests

Boiling temperature. Transfer a 100 ml sample at about 24° to a tared, pear-shaped, 100-ml centrifuge tube containing a few boiling stones, and weigh. Suspend a thermometer in the liquid, and place the tube in a medium maintained at a temperature of 32° above the expected boiling temperature. When the thermometer reading becomes constant, record as the boiling temperature the thermometer reading after at least 5per cent of the substance under examination has distilled. Retain the remainder of the substance for the determination of High-Boiling Residues.

Water (2.3.43). Not more than 0.001 per cent (Method 3) with the following modifications (a) provide the closed-system titrating vessel with an opening through which passes a coarse-porosity gas dispersion tube connected to a sample cylinder; (b) dilute the reagent with anhydrous methanol to give a water equivalence factor of between 0.2 and 1.0 mg per ml, age this diluted solution for not less than 16 hours before sanitation; (c) obtain a 100 g sample as directed under inhalation preparation, and introduce the same into the titration vessel through the gas dispersion tube at a rate of about 100 ml of gas per minute.

High-boiling residues. Not more than 0.01 per cent, Allow 85 ml of the sample to distill as directed in the test for Approximate Boiling Temperature, and transfer the centrifuge tube containing the remaining 15 ml of substance to a medium maintained at a temperature 10° above the boiling temperature. After 30 minutes, remove the tube from the water-bath, blot dry, and weigh. Calculate the weight of the residue.

Inorganic chlorides. Place 5 ml of anhydrous *methanol* in a test tube, add 3 drops of a saturated solution of silver nitrate in anhydrous *methanol*, shake, and add 7 g of Trichloromono-fluoromethane; no opalescence or turbidity is produced.

Assay. Determine by gas chromatography (2.4.13).

Test solution. Introduce the liquid phase of Trichloromono-fluoromethane into an evacuated headspace vial

Reference solution. Introduce a liquid-phase mixture of dichlorodifluoromethane, dichlorotetrafluoroethane, and trichloromonofluoromethane into an evacuated headspace vial.

Chromatographic system
- a steel column 1.8 m x 2 mm, packed with support S12, containing 1 per cent phase G25,
- temperature:
  column 70° to 170° @10° per minute and 170° for 5 minutes,
  inlet port at 110° and detector at 200°,
- a flame ionisation detector,
- flow rate: 20 ml per minute of the nitrogen as carrier gas

Head-space conditions which may be used:
- bath temperature 100°,
- valve/loop temperature 105°,
- sampling time is 3 seconds.

The relative retention time with reference to trichloromonofluoromethane for dichlorodifluoromethane is about 0.5 and for dichlorotetrafluoroethane is about 0.8.

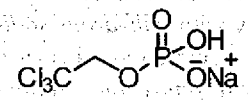
Inject the reference solution. The sum of these two peak areas is not more than 0.2 per cent of the total of all peak areas and the sum of the areas of all peaks other than that for trichloromonofluoromethane is not more than 0.4 per cent of the total of all peak areas.

Inject the reference solution and the test solution. The test is not valid unless the resolution between the peaks due dichlorotetrafluoroethane and trichloromonofluoromethane is not less than 2.0.

Calculate the content of CCl_3F.

Storage. Store protected from moisture and prevent exposure to excessive heat.

Triclofos Sodium



$C_2H_3Cl_3NaO_4P$ Mol. Wt. 251.4

Triclofos Sodium is sodium 2,2,2-trichloroethyl hydrogen orthophosphate.

Triclofos Sodium contains not less than 41.3 per cent and not more than 43.2 per cent of Cl and not less than 97.0 per cent and not more than 102.0 per cent of $C_2H_3Cl_3NaO_4P$, both calculated on the dried basis.

Category. Hypnotic.

Description. A white or almost white powder; hygroscopic.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *triclofos sodium IPRS* or with the reference spectrum of triclofos sodium.

B. It gives the reactions of sodium salts (2.3.1).

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C. Heat 0.1 g with 1 g of *anhydrous sodium carbonate* to a dull red heat and maintain for 10 minutes, cool, extract the residue with *water* and filter. The filtrate gives the reactions of chlorides (2.3.1) and of phosphates (2.3.1).

Tests

pH (2.4.24). 3.0 to 4.5, determined on a 2.0 per cent w/v solution.

Appearance of solution. A 2.0 per cent w/v solution is clear (2.4.1).

Chlorides (2.3.12). Dissolve 0.5 g in 50 ml of *water*. 15 ml of the solution complies with the limit test for chlorides (0.17 per cent).

Phosphates. Not more than 1.0 per cent, calculated as PO_4, when determined by dissolving 25 mg in 10 ml of *water*, add 4 ml of *1 M sulphuric acid*, 1 ml of *ammonium molybdate solution* and 2 ml of *methylaminophenol-sulphite reagent* and allow to stand for 15 minutes and dilute to 25 ml with *water*, allow to stand for a further 15 minutes and measure the absorbance of the solution at 730 nm (2.4.7). Calculate the content of phosphate from a calibration curve prepared by treating suitable volumes of a 0.00143 per cent w/v solution of *potassium dihydrogen orthophosphate* in the same manner.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1 g by drying at 100°, at a pressure not exceeding 0.7 kPa for 3 hours.

Assay. *For Cl* — Mix 0.25 g with 1 g of *anhydrous sodium carbonate* in a nickel crucible about 3 cm in diameter, fill the crucible completely with *anhydrous sodium carbonate* and invert into a nickel crucible about 4 cm in diameter; cover the smaller crucible with *anhydrous sodium carbonate*, well pressed down, using 25 g of *anhydrous sodium carbonate* in all. Heat for 30 minutes at a dull red heat, cool, transfer to a 400 ml beaker, add 150 ml of *water* and boil gently for 10 minutes. Filter through absorbent cotton into a 600 ml beaker, washing the residue with hot *water*, until 400 ml of filtrate has been collected. Cool, cautiously add *nitric acid* until the solution is neutral to *litmus paper* and add 3 ml of *nitric acid* in excess. Add 50 ml of *0.1 M silver nitrate*, allow to stand until precipitation is complete, filter, wash the precipitate with *water* and titrate the combined filtrate and washings with *0.1 M ammonium thiocyanate* using *ammonium iron(III) sulphate solution* as indicator.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of Cl.

For $C_2H_3Cl_3NaO_4P$ — Heat 0.2 g in a Kjeldahl flask with 2 ml of *sulphuric acid* and 2.5 ml of *nitric acid* until brown fumes cease to be evolved, cool, add 1 ml of *nitric acid* and heat again. Continue adding *nitric acid* and heating until brown fumes are no longer evolved and the solution is colourless when cold. Heat until dense, white fumes are evolved, cool, transfer the solution to a flask with the aid of 150 ml of *water*, add 50 ml of *citric-molybdic acid solution* and heat slowly to boiling. Swirling the flask continuously, add 25 ml of *quinoline solution* at first drop wise and then in a steady stream, heat on a water-bath for 5 minutes and cool. Filter, wash the precipitate with *water* until free from acid, transfer the precipitate to a flask with the aid of 100 ml of *water*, add 50 ml of *0.5 M sodium hydroxide* and shake until dissolved. Titrate the excess of alkali with *0.5 M hydrochloric acid* using *phenolphthalein-thymol blue solution* as indicator.

1 ml of *0.5 M sodium hydroxide* is equivalent to 4.835 mg of $C_2H_3Cl_3NaO_4P$. Correct the result for the content of phosphate, as determined by the test described above; 1 mg of PO_4 is equivalent to 2.65 mg of $C_2H_3Cl_3NaO_4P$.

Triclofos Oral Solution

Triclofos Oral Solution is a solution of Triclofos Sodium in a suitable flavoured vehicle.

Triclofos Oral Solution contains not less than 95.0 per cent and not more than 105.0 per cent of $C_2H_3Cl_3NaO_4P$.

Identification

Disperse a quantity of the oral solution containing 0.25 g of Triclofos Sodium with 40 ml of *water*, add 5 ml of *1 M sulphuric acid* and extract with 25 ml of *isoamyl alcohol*. The extract complies with the following tests.

A. Evaporate 5 ml to dryness, heat the residue with 1 ml of a 50 per cent v/v solution of *sulphuric acid* and 1 ml of a 5.0 per cent w/v solution of *potassium permanganate* in a water-bath for 5 minutes, add 7 ml of *water* and decolourise the solution with 1 ml of a 5.0 per cent w/v solution of *oxalic acid*. To 1 ml, add 1 ml of *pyridine* and 1 ml of *2 M sodium hydroxide*, heat in a water-bath for 1 minute, stirring continuously; a pink colour is produced in the pyridine layer.

B. Evaporate 10 ml to dryness, heat the residue with 1 g of *anhydrous sodium carbonate* at a dull red heat for 10 minutes, cool, extract the residue with *water* and filter. The residue gives the reactions of chlorides (2.3.1) and of phosphates (2.3.1).

Tests

Other tests. Comply with the tests stated under Oral Liquids.

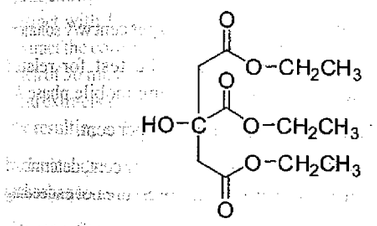
Assay. To a quantity containing 16 mg of Triclofos Sodium, add 15 ml of *water*, 1 ml of *1 M sodium hydroxide* and 15 ml of *ether*, shake for 1 minute, allow to separate, wash the ether layer with 1 ml of *water* and discard the ether solution. Add

2.5 ml of *1 M sulphuric acid* to the combined aqueous layer and washings, extract with four 10-ml quantities of *isoamyl alcohol*, allowing each extract to stand until good separation is achieved, combine the isoamyl alcohol extracts, remove any further water that separates and dilute to 50 ml with *isoamyl alcohol*. Transfer 10 ml of the solution to a 20 ml glass ampoule, add 10 ml of *alcoholic potassium hydroxide solution*, seal the ampoule, mix well and heat in an autoclave at 120° for 2 hours. Allow the ampoule to cool, transfer the contents to a flask with the aid of 20 ml of *2 M nitric acid* and add 25 ml of *0.02 M silver nitrate*. Titrate with *0.02 M ammonium thiocyanate* using *ammonium iron(III) sulphate solution* as indicator. Repeat the titration using 10 ml of *alcoholic potassium hydroxide solution* in place of the contents of the ampoule. The difference between the titrations represents the amount of silver nitrate required.

1 ml of *0.02 M silver nitrate* is equivalent to 1.676 mg of $C_2H_3Cl_3NaO_4P$.

Determine the weight per ml of the oral solution (2.4.29) and calculate the content of $C_2H_3Cl_3NaO_4P$.

Triethyl Citrate



$C_{12}H_{20}O_7$ Mol. Wt. 276.3

Triethyl Citrate is triethyl 2-hydroxypropane-1, 2, 3-tricarboxylate.

Triethyl Citrate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{12}H_{20}O_7$, calculated on the anhydrous basis.

Category. Excipient.

Description. A clear, viscous, colourless or almost colourless, hygroscopic liquid.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *triethyl citrate IPRS* or with the reference spectrum of triethyl citrate.

B. To 0.5 ml add 5 ml of *ethanol (95 per cent)* and 4 ml of *dilute sodium hydroxide solution*. Boil under reflux for about 10 minutes, 2 ml of the solution gives the reactions of citrates (2.3.1).

C. It gives the reactions of esters (2.3.1).

Tests

Appearance of solution. The substance under examination is clear (2.4.1) and not more intensely coloured than reference solution BYS6 (2.4.1).

Acidity. Dilute 10 g with 10 ml of previously neutralized *ethanol (95 per cent)*. Add 0.5 ml of *bromothymol blue solution*. Not more than 0.3 ml of *0.1 M sodium hydroxide* is required to change the color of the indicator to blue.

Refractive index (2.4.27). 1.440 to 1.446.

Related substances. Determine by gas chromatography (2.4.13).

Test solution. Disperse 1.0 ml of the substance under examination in 50.0 ml of *dichloromethane*.

Reference solution. Disperse 1.0 ml of the substance under examination and 0.5 ml of *methyl tridecanoate* in 50.0 ml of *dichloromethane*.

Chromatographic system
- a fused silica column 30 m x 0.32 mm, packed with poly (dimethyl) siloxane (5 µm),
- temperature:
  column 200°,
  injection port and detector at 220°,
- a flame ionization detector,
- linear velocity about 26 cm per second using nitrogen as carrier gas.

Inject 1 µl of the reference solution. The test is not valid unless the resolution between the peaks due to triethyl citrate and methyl tridecanoate is not less than 1.5.

Inject 1 µl each of the test solution and the reference solution. Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.2 per cent the area of the principal peak in the chromatogram obtained with the reference solution. The sum of the areas of all the secondary peaks is not more than 0.5 per cent the area of the principal peak in the chromatogram obtained with the reference solution. Ignore any peak with an area less than 0.04 per cent the area of the principal peak in the chromatogram obtained with the reference solution.

Heavy metals (2.3.13). Dissolve 4.0 g in 8 ml of *ethanol (95 per cent)* and dilute to 20 ml with *water*. 12 ml of the resulting solution complies with the limit test for the heavy metals,

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method D (5 ppm). Prepare the standard solution using *lead standard solution (1 ppm Pb)* obtained by diluting *lead standard solution (100 ppm Pb)* with a mixture of equal volumes of *ethanol (95 per cent)* and *water*.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

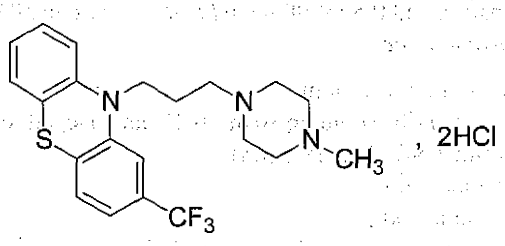
Water (2.3.43). Not more than 0.25 per cent, determined on 1.0 g.

Assay. To 1.5 g, add 25 ml of *2-propanol*, 50 ml of *water*, 25.0 ml of *1 M sodium hydroxide* and a few glass beads into a 250-ml borosilicate-glass flask fitted with a reflux condenser. Heat under a reflux condenser for 1 hour and allow to cool. Titrate with *1 M hydrochloric acid*, using 1 ml of *phenolphthalein solution* as indicator. Carry out a blank titration.

1 ml of *1 M sodium hydroxide* is equivalent to 0.0921 g of $C_{12}H_{20}O_7$.

Storage. Store protected from moisture.

Trifluoperazine Hydrochloride



, 2HCl

$C_{21}H_{24}F_3N_3S,2HCl$ Mol. Wt. 480.4

Trifluoperazine hydrochloride is 10-[3-(4-methylpiperazin-1-yl)propyl]-2-trifluoromethylphenothiazine dihydrochloride.

Trifluoperazine Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{21}H_{24}F_3N_3S$, 2HCl, calculated on the dried basis.

Category. Antipsychotic; antiemetic.

Description. A white to pale yellow, crystalline powder; slightly hygroscopic.

NOTE — In the following procedures, the test and standard specimens and the solutions containing them should be protected by carrying out the tests without delay and in subdued light.

Identification

A. Dissolve 20 mg in 10 ml of *water*, make the solution alkaline to *litmus paper* with *5 M sodium hydroxide* and extract with two quantities, each of 20 ml, of *light petroleum (60° to 80°)*. Combine the extracts, wash with 10 ml of *water*, shake with 5 g of *anhydrous sodium sulphate*, filter and evaporate the filtrate carefully to dryness.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *trifluoperazine hydrochloride IPRS* treated in the same manner or with the reference spectrum of trifluoperazine.

B. Complies with the test for identification of phenothiazines (2.3.3), using as reference solution a 0.2 per cent w/v solution of *trifluoperazine hydrochloride IPRS* in *chloroform*.

C. When examined in the range 280 nm to 360 nm (2.4.7), a 0.01 per cent w/v solution in *0.1 M hydrochloric acid* measured immediately after preparation shows an absorption maximum at about 305 nm. Dilute 10 ml of the solution to 100 ml with *0.1 M hydrochloric acid*. When examined in the range 230 nm to 280 nm, the resulting solution shows an absorption maximum at about 256 nm; absorbance at about 256 nm, about 0.65.

D. Dissolve 5 mg in 2 ml of *sulphuric acid* and allow to stand for 5 minutes; an orange colour is produced.

E. A 10 per cent w/v solution gives the reactions of chlorides (2.3.1).

Tests

pH (2.4.24). 1.7 to 2.6, determined in a 10.0 per cent w/v solution.

Related substances. Complies with the test for related substances in phenothiazines (2.3.5), using mobile phase A.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° at a pressure not exceeding 0.7 kPa.

Assay. Dissolve 0.4 g in 50 ml of *anhydrous glacial acetic acid* add 10 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02402 g of $C_{21}H_{24}F_3N_3S$, 2HCl.

Storage. Store protected from light and moisture.

Trifluoperazine Injection

Trifluoperazine Hydrochloride Injection

Trifluoperazine Injection is a sterile solution of Trifluoperazine Hydrochloride in Water for Injections.

Trifluoperazine Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of trifluoperazine, $C_{21}H_{24}F_3N_3S$.

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Usual strength. The equivalent of 1 mg of trifluoperazine per ml.

Description. A clear, colourless solution.

NOTE — In the following procedures, the test and standard specimens and the solutions containing them should be protected by carrying out the tests without delay and in subdued light.

Identification

When examined in the range 230 nm to 360 nm (2.4.7), the final solution obtained in Assay shows an absorption maximum at about 256 nm.

Tests

pH (2.4.24). 4.0 to 5.0.

Bacterial endotoxins (2.2.3). Not more than 172.0 Endotoxin Units per mg of trifluoperazine.

Other tests. Comply with the tests stated under Parenteral preparations (Injections).

Assay. To an measured volume of the injection containing about 50 mg of trifluoperazine add 10 ml of *2 M sulphuric acid* and extract with three quantities, each of 25 ml, of *carbon tetrachloride*. Discard the carbon tetrachloride extract after each extraction. Add 10 ml of *strong ammonia solution* and extract with five quantities, each of 50 ml, of *cyclohexane*. Extract the combined cyclohexane extracts with five quantities, each of 50 ml, of *0.1 M hydrochloric acid*. Dilute the combined acid extracts to 500.0 ml and mix. Measure the absorbance of the resulting solution at the maximum at about 256 nm (2.4.7).

Calculate the content of $C_{21}H_{24}F_3N_3S$ taking 743 as the specific absorbance at 256 nm.

Storage. Store protected from light.

Labelling. The label states the strength in terms of the equivalent amount of trifluoperazine per ml.

Trifluoperazine Tablets

Trifluoperazine Hydrochloride Tablets

Trifluoperazine Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of trifluoperazine, $C_{21}H_{24}F_3N_3S$. The tablets are coated.

Usual strengths. The equivalent of 1 mg and 5 mg of trifluoperazine.

NOTE — In the following procedures, the test and standard specimens and the solutions containing them should be protected by carrying out the tests without delay and in subdued light.

Identification

A. Shake a quantity of the powdered tablets containing 20 mg of trifluoperazine with 30 ml of *1 M hydrochloric acid* for 10 minutes, filter, make the filtrate alkaline to litmus paper with *5 M sodium hydroxide* and extract with two quantities, each of 20 ml, of *light petroleum (60° to 80°)*. Combine the extracts, wash with 10 ml of *water*, shake with 5 g of *anhydrous sodium sulphate*, filter and evaporate the filtrate carefully to dryness.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *trifluoperazine hydrochloride IPRS* treated in the same manner or with the reference spectrum of trifluoperazine.

B. Extract a quantity of the powdered tablets containing 5 mg of trifluoperazine with 5 ml of *acetone*, filter and evaporate the filtrate to dryness. Add 2 ml of *sulphuric acid* to the residue and allow to stand for 5 minutes; an orange colour is produced.

Tests

Dissolution (2.5.2).

Apparatis No. 1 (Basket),
Medium. 900 ml of *0.1 M hydrochloric acid,*
Speed and time. 50 rpm, 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, dilute suitably if necessary with the medium, at the maximum at about 255 nm and at about 278 nm (2.4.7). Calculate the content of $C_{21}H_{24}F_3N_3S$ in the medium from the difference in absorbance at 255 nm and at 278 nm, obtained from a solution of known concentration of *trifluoperazine hydrochloride IPRS* in the dissolution medium.

Q. Not less than 75 per cent of the stated amount of $C_{21}H_{24}F_3N_3S$.

Uniformity of content. Complies with the test stated under Tablets.

Place one tablet in a 100-ml volumetric flask, add 50 ml of a mixture of 19 volumes of *water* and 1 volume of *hydrochloric acid*, shake until the tablet has completely disintegrated, dilute to volume with the same mixture, mix and filter, rejecting the first few ml of the filtrate. Dilute suitably, if necessary with the same solvent mixture and measure the absorbance of the resulting solution at the maximum at about 256 nm (2.4.7). Calculate the content of $C_{21}H_{24}F_3N_3S$ taking 743 as the specific absorbance at 256 nm.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 5 mg of trifluoperazine, shake for 15 minutes with 400 ml of a mixture of 19 volumes of *water* and

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1 volume of *hydrochloric acid*, dilute to 500.0 ml with the same solvent mixture, mix and filter. Measure the absorbance of the filtrate at the maximum at about 256 nm (2.4.7).

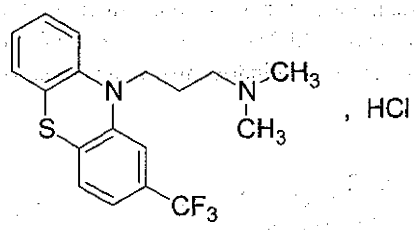
Calculate the content of $C_{21}H_{24}F_3N_3S$ taking 743 as the specific absorbance at 256 nm.

Storage. Store protected from light and moisture.

Labelling. The label states the strength in terms of the equivalent amount of trifluoperazine.

Triflupromazine Hydrochloride

Flupromazine Hydrochloride



$C_{18}H_{19}F_3N_2S, HCl$ Mol. Wt. 388.9

Triflupromazine Hydrochloride is 10-[3-(dimethylamino)-propyl)]-2-trifluoromethylphenothiazine hydrochloride.

Triflupromazine Hydrochloride contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{18}H_{19}F_3N_2S$, HCl, calculated on the dried basis.

Category. Antipsychotic; antiemetic.

Description. A white to pale yellowish brown, crystalline powder.

NOTE — In the following procedures, the test and standard specimens and the solutions containing them should be protected by carrying out the tests without delay and in subdued light.

Identification

A. Dissolve 20 mg in 10 ml of *water*, make the solution alkaline to *litmus paper* with *5 M sodium hydroxide* and extract with two quantities, each of 20 ml, of *light petroleum (60° to 80°)*. Combine the extracts, wash with 10 ml of *water*, shake with 5 g of *anhydrous sodium sulphate*, filter and evaporate the filtrate carefully to dryness.

The residue complies with the following test. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *triflupromazine hydrochloride IPRS*, treated in the same manner or with the reference spectrum of triflupromazine hydrochloride.

B. Complies with the test for identification of phenothiazines (2.3.3), using as reference solution a 0.2 per cent w/v solution of *triflupromazine hydrochloride IPRS* in *chloroform*.

C. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *0.1 M hydrochloric acid* shows an absorption maximum at about 256 nm and a less well-defined maximum at about 305 nm.

D. A 10 per cent w/v solution gives the reactions of chlorides (2.3.1).

Tests

Related substances. Complies with the test for Related substances in phenothiazines (2.3.3), using *mobile phase A*.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Assay. Dissolve 0.8 g in 50 ml of *anhydrous glacial acetic acid* add 10 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03889 g of $C_{18}H_{19}F_3N_2S$, HCl.

Storage. Store protected from light and moisture.

Triflupromazine Injection

Triflupromazine Hydrochloride Injection; Flupromazine Hydrochloride Injection; Flupromazine Injection

Triflupromazine Injection is a sterile solution of Triflupromazine Hydrochloride in Water for Injections.

Triflupromazine Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of triflupromazine hydrochloride, $C_{18}H_{19}F_3N_2S$, HCl.

Usual strength. 10 mg per ml.

Description. A clear, almost colourless solution.

NOTE — In the following procedures, the test and standard specimens and the solutions containing them should be protected by carrying out the tests without delay and in subdued light.

Identification

A. To an appropriate quantity of the injection add sufficient *0.1 M hydrochloric acid* to produce a solution containing 0.001 per cent w/v solution of Triflupromazine Hydrochloride.

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When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows an absorption maximum at about 256 nm.

B. To a volume containing about 100 mg of Triflupromazine Hydrochloride add 5 ml of *8 M nitric acid* and mix; a pink to amber colour develops which quickly turns dark brown and then changes to a clear solution having a yellow tint.

Tests

pH (2.4.24). 3.5 to 5.2.

Bacterial endotoxins (2.2.3). Not more than 5.8 Endotoxin Units per mg of triflupromazine hydrochloride.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. To an measured volume of the injection containing about 20 mg of Triflupromazine Hydrochloride add sufficient of a mixture of 19 volumes of *water* and 1 volume of *hydrochloric acid* to produce 100.0 ml and mix. Dilute 5.0 ml of the solution to 100.0 ml with the same mixture and mix. Measure the absorbance of the resulting solution at the maximum at about 256 nm (2.4.7).

Calculate the content of $C_{18}H_{19}F_3N_2S$, HCl taking 700 as the specific absorbance at 256 nm.

Storage. Store protected from light.

Triflupromazine Tablets

Triflupromazine Hydrochloride Tablets; Flupromazine Hydrochloride Tablets; Flupromazine Tablets

Triflupromazine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of triflupromazine hydrochloride, $C_{18}H_{19}F_3N_2S$, HCl.

Usual strengths. 10 mg; 25 mg.

NOTE — In the following procedures, the test and standard specimens and the solutions containing them should be protected by carrying out the tests without delay and in subdued light.

Identification

A. Shake a quantity of the powdered tablets containing 20 mg of Triflupromazine Hydrochloride with 30 ml of *1 M hydrochloric acid* for 10 minutes, filter, make the filtrate alkaline to *litmus paper* with *5 M sodium hydroxide* and extract with two quantities, each of 20 ml, of *light petroleum (boiling range 60° to 80°)*. Combine the extracts, wash with 10 ml of water, shake with 5 g of *anhydrous sodium sulphate*, filter and evaporate the filtrate carefully to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *triflupromazine hydrochloride IPRS*, treated in the same manner or with the reference spectrum of triflupromazine hydrochloride.

B. Extract a quantity of the powdered tablets containing 5 mg of Triflupromazine Hydrochloride with 5 ml of *acetone*, filter and evaporate the filtrate to dryness.

When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *0.1 M hydrochloric acid* shows an absorption maximum at about 256 nm.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),
Medium. 900 ml of *0.01 M hydrochloric acid*,
Speed and time. 100 rpm, 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, dilute suitably if necessary with the medium, at the maximum at about 305 nm (2.4.7). Calculate the content of $C_{18}H_{19}F_3N_2S$,HCl in the medium from the absorbance obtained from a solution of known concentration of *triflupromazine hydrochloride IPRS* in the dissolution medium.

Q. Not less than 75 per cent of the stated amount of $C_{18}H_{19}F_3N_2S$.HCl.

Uniformity of content. Complies with the test stated under Tablets.

Crush one tablet and transfer to a 100-ml volumetric flask with the aid of a mixture of 19 volumes of *water* and 1 volume of *hydrochloric acid*. Shake well, dilute to volume with the same mixture and complete the procedure described under the Assay beginning at the words "mix and filter,...".

Calculate the content of $C_{18}H_{19}F_3N_2S$, HCl in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 50 mg of Triflupromazine Hydrochloride and shake for 15 minutes with 200 ml of a mixture of 19 volumes of *water* and 1 volume of *hydrochloric acid*. Dilute to 500.0 ml with the same solvent mixture, mix and filter, rejecting the first few ml of the filtrate. Dilute 10.0 ml to 100.0 ml with the same solvent mixture. Measure the absorbance of the filtrate at the maximum at about 256 nm (2.4.7).

Calculate the content of $C_{18}H_{19}F_3N_2S$, HCl taking 700 as the specific absorbance at 256 nm.

Storage. Store protected from light and moisture.

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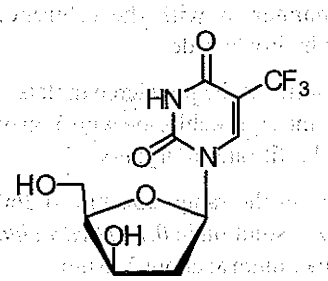
Trifluridine

Triflurothymidene



$C_{10}H_{11}F_3N_2O_5$ Mol.Wt. 296.2

Trifluridine is 5-Trifluoromethyl-2'-deoxyuridine.

Trifluridine contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{10}H_{11}F_3N_2O_5$, calculated on the dried basis.

Category. Pyrimidine nucleoside analogue; antiviral (herpes viruses).

Description. A white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *trifluridine IPRS* or with the reference spectrum of trifluridine.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). +47.0° to +51.0°, determined in 3.0 per cent w/v solution in *water*.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 5 volumes of *acetonitrile* and 95 volumes of a *0.05 M sodium dihydrogen orthophosphate*, previously adjusted to pH 6.0 with *ammonia*.

Test solution. Dissolve 20 mg of substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS-2),
- mobile phase: A. *0.05M sodium dihydrogen orthophosphate*, adjusted to pH 6.0 with *ammonia*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 263 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
3	95	5
10	85	15
15	85	15
16	95	5
25	95	5

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plate and the tailing factor is not more than 2.0.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying under vacuum at 105° for 4 hours.

Assay. Determine by liquid chromatography (2.4.14), as described under test for Related substances with the following modifications.

Reference solution. A 0.02 per cent w/v solution of *trifluridine IPRS* in the solvent mixture.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{11}F_3N_2O_5$.

Storage. Store protected from light.

Trifluridine Eye Drops

Trifluridine Eye Drops are a sterile solution of Trifluridine in Purified Water.

Trifluridine Eye Drops contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of trifluridine, $C_{10}H_{11}F_3N_2O_5$.

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Usual strength. 1.0 per cent w/v.

Identification

A. Evaporate a volume of the eye drops containing 0.1 g of Trifluridine to dryness using a rotary evaporator and dry at 60° under reduced pressure. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *trifluridine IPRS* treated in the same manner or with the reference spectrum of trifluridine.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.0 to 6.5.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 5 volumes of *acetonitrile* and 95 volumes of a *0.05M sodium dihydrogen orthophosphate*, adjusted to pH 6.0 with *ammonia*.

Test solution. Dilute the eye drops if necessary to obtain a solution containing 0.02 per cent w/v of Trifluridine in the solvent mixture.

Reference solution (a). Dilute 1 volume of the test solution to 100 volumes in the solvent mixture.

Reference solution (b). Dilute 1 volume of reference solution (a) to 10 volumes in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Inertsil ODS 2),
- mobile phase: A. *0.05M sodium dihydrogen orthophosphate*, adjusted to pH 6.0 with *ammonia*.
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 263 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
3	95	5
10	85	15
15	85	15
16	95	5
25	95	5

Inject reference solution (b). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore any peak with an area less than 0.5 times the area of the peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Other tests. Comply with the tests stated under Eye Drops.

Assay. Determine by liquid chromatography (2.4.14) as described under test for Related substances with the following modifications.

Reference solution. A 0.02 per cent w/v solution of *trifluridine IPRS* in the solvent mixture.

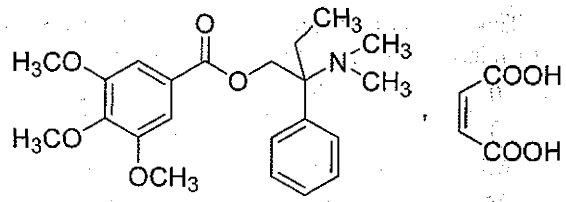
Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{11}F_3N_2O_5$ in the eye drops.

Storage. Store protected from light, at a temperature between 2° to 8°.

Trimebutine Maleate



$C_{26}H_{33}NO_9$ Mol Wt. 503.5

Trimebutine MaleateArterolane Maleate is (2*RS*)-2-(dimethylamino)-2-phenylbutyl 3,4,5-trimethoxybenzoate (*Z*)-butenedioate.

Trimebutine MaleateArterolane Maleate contains not less than 99.0 per cent and not more than 101.5 per cent of $C_{26}H_{33}NO_9$, calculated on the dried basis.

Category. Treatment of irritable bowel syndrome.

Description. A white or almost white, crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *trimebutine maleate IPRS* or with the reference spectrum of trimebutine maleate.

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Tests

Appearance of solution. A 0.5 per cent w/v solution in *acetone* is clear (2.4.1) and colourless (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 0.24 g of *anhydrous sodium dihydrogen phosphate* in 180 ml of *water*, adjusted to pH 2.5 with *dilute orthophosphoric acid*, dilute to 200 ml with *water*. Add 50 ml of *acetonitrile* and mix.

Test solution. Dissolve 10 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. dissolve 3.6 g of *anhydrous sodium dihydrogen phosphate* in 990 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and dilute to 1000.0 ml with *water*;

  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	78	22
3	78	22
6.5	65	35
15	60	40
35	60	40
40	78	22

Name	Relative retention time
Maleic acid	0.17
Trimebutine maleate impurity E[1]	0.9
Trimebutine maleate (Retention time about 12 minutes)	1.0
Trimebutine maleate impurity D[2]	1.3
Trimebutine maleate impurity C[3]	1.4

[1](2*RS*)-2-(methylamino)-2-phenylbutyl 3,4,5-trimethoxybenzoate,

[2](1*RS*)-1-[(dimethylamino)methyl]-1-phenylpropyl 3,4,5-trimethoxybenzoate,

[3]methyl 3,4,5-trimethoxybenzoate.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak due to trimebutine maleate impurity E is not more than 6 times the area of the principal peak in the chromatogram obtained with the reference solution (0.6 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.1per cent) and the sum of the areas of all the secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent) and any peak due to maleic acid.

Heavy metals (2.3.13). *Solvent mixture.* 15 volumes of *water* and 85 volumes of *dioxan.*

Dissolve 2 g in 20 ml of a solvent mixture. 12 ml of the solution complies with the limit test for heavy metals, Method D (10 ppm), using 10 ml of *lead standard solution (1 ppm pb)* in the same solvent mixture.

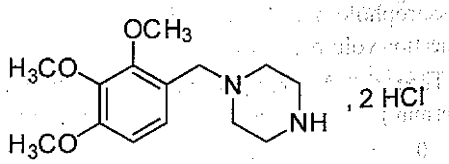
Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1 g by drying in an oven at 105°.

Assay. Dissolve 0.3 g in 40 ml of *anhydrous acetic acid.* Titrate with *0.1 M perchloric acid.* Determine the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.05035 g of $C_{26}H_{33}NO_9$.

Trimetazidine Hydrochloride



$C_{14}H_{22}N_2O_3, 2HCl$ Mol. Wt. 339.3

Trimetazidine Hydrochloride is 1-[(2,3,4-trimethoxyphenyl)-methyl]piperazine dihydrochloride.

Trimetazidine Hydrochloride contains not less than 98.5 per cent and not more than 101.5 per cent, calculated on the dried basis.

Category. Antiischemic metabolic agent.

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Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *trimetazidine dihydrochloride IPRS* or with the reference spectrum of trimetazidine dihydrochloride.

B. It gives reaction (A) for chlorides (2.3.1).

Tests

Appearance of solution. A 10 per cent w/v solution is clear (2.4.1) and not more intensely coloured than reference solution BYS6 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.2 g of the substance under examination in 50.0 ml of *water*.

Reference solution. Dilute 2.0 ml of the test solution to 100.0 ml with *water*. Dilute 5.0 ml of the solution to 100.0 ml with *water*.

Chromatographic system

- a stainless steel column15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 30°,
- mobile phase: A. a mixture of 35.7 volumes of *methanol* and 64.3 volumes of a 0.29 per cent w/v solution of *sodium heptanesulphonate*, adjusted to pH 3.0 with *orthophosphoric acid*,
  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
50	75	25
52	95	5

Equilibrate the column for not less than 1 hour using 95 volumes of mobile phase A and 5 volumes of mobile phase B.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates. The relative retention time with reference to trimetazidine for (2,3,4-trimethoxyphenyl)methanol (trimetazidine impurity D) is about 0.2, for 2,3,4-trimethoxybenzaldehyde (trimetazidine impurity C) is about 0.4, for ethyl 4-(2,3,4-trimethoxybenzyl)piperazine-1-carboxylate (trimetazidine impurity H) is about 0.6, for 1-(3,4,5-trimethoxybenzyl)piperazine (trimetazidine impurity A) and *N*-methyltrimetazidine (trimetazidine impurity I) is about 0.9, for 1-(2,4,5-trimethoxybenzyl)piperazine (trimetazidine impurity E) is about 0.95, for 1-(2,4,6-trimethoxybenzyl) piperazine (trimetazidine impurity F) is about 1.4 and for 1,4-bis(2,3,4-trimethoxybenzyl) piperazine (trimetazidine impurity B) is about 1.8.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of each peak due to trimetazidine impurity A, B, C, D, E, F, H, I multiplied with correction factor 0.55 for impurity B, 0.37 for impurity C and 0.71 for impurity F is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Impurity G. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 20 volumes of *concentrated ammonia* and 80 volumes of *ethanol (95 per cent)*.

Test solution. Dissolve 0.1 g of the substance under examination in 10.0 ml of *methanol*.

Reference solution. Dissolve 22.6 mg of *piperazine hydrate* (trimetazidine impurity G) in 100 ml of *methanol*. Dilute 10 ml of the solution to 100 ml with *methanol*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 8 cm. Dry the plate at 105° for 30 minutes and spray with *iodoplatinate reagent*. Any secondary spot corresponding to trimetazidine impurity G is not more intense than the spot in the chromatogram obtained with the reference solution (0.1 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 2.5 per cent, determined on 1.0 g by drying in an oven at 105 ° over *diphosphorus pentoxide* at a pressure not exceeding 15 kPa.

Assay. Dissolve 0.12 g in 50.0 ml of *water*. Add 1 ml of *nitric acid* and titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.01696 g of $C_{14}H_{24}Cl_2N_2O_3$.

Storage. Store protected from moisture.

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Trimetazidine Tablets

Trimetazidine Hydrochloride Tablets

Trimetazidine Hydrochloride Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of trimetazidine dihydrochloride, $C_{14}H_{22}N_2O_3,2HCl$.

Usual strength. 20 mg.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. *Solvent mixture.* Mix 3 volumes of *ethanol (95 per cent)* and 1 volume of *water*.

Triturate a quantity of the powdered tablets containing 10 mg of Trimetazidine Hydrochloride with 10 ml of solvent mixture, filter and evaporate the filtrate to a low volume. Add 2 ml of *water* to residue and shake. To 1 ml of the solution add 1 ml of *p-benzoquinone*, boil gently for 2 to 3 minutes and cool. A red colour is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *water*,

Speed and time. 50 rpm and 45 minutes.

Determine by liquid chromatography (2.4.14).

Test solution. Withdraw a suitable volume of the medium and filter. Dilute 4.0 ml of the filtrate to 25.0 ml with the *water*, further dilute 5.0 ml to 10.0 ml with *0.1 M hydrochloric acid*.

Reference solution. A solution containing 0.017 per cent w/v of *trimetazidine dihydrochloride IPRS* in *water*, further dilute 5 ml to 50.0 ml with *water*, further dilute 5.0 ml to 25.0 ml with *water*, further dilute 5.0 ml to 10.0 ml with *0.1 M hydrochloric acid*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 850 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 1000 ml of *water* and adjusted to pH 3.0 with *ortho phosphoric acid* and 150 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 5000 theoretical plates and the relative standard deviation for replicate injections is not more than 1.5 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{14}H_{22}N_2O_3,2HCl$.

Q. Not less than 80 per cent of the stated amount of $C_{14}H_{22}N_2O_3,2HCl$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 50 volumes of *0.1 M hydrochloric acid* and 50 volumes of *ethanol* (95 per cent).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 15 mg of Trimetazidine Hydrochloride in about 75 ml of solvent mixture sonicate for 20 minutes and dilute to 100.0 ml with the solvent mixture and centrifuge. Dilute 5.0 ml of the solution to 50.0 ml with *0.1 M hydrochloric acid*.

Reference solution. Dilute 5.0 ml of 0.015 per cent w/v solution of *trimetazidine dihydrochloride IPRS* in the solvent mixture to 50.0 ml with *0.1 M hydrochloric acid*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 850 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 150 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 10 µl.

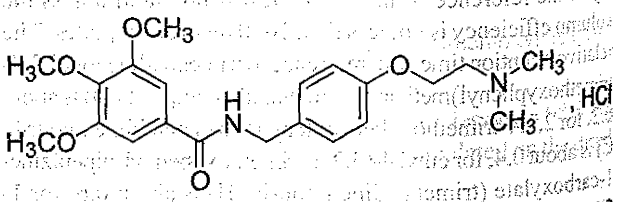
Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{14}H_{22}N_2O_3,2HCl$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Trimethobenzamide Hydrochloride



$C_{21}H_{29}ClN_2O_5$ Mol Wt. 424.9

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Trimethobenzamide Hydrochloride is *N*-{[4-(2-dimethylamino-ethoxy)phenyl]methyl}-3,4,5-trimethoxybenzamide hydrochloride.

Trimethobenzamide Hydrochloride contains not less than 98.5 per cent and not more than 100.5 per cent of $C_{21}H_{29}ClN_2O_5$, calculated on the dried basis.

Category. Antiemetic

Description. A white to off-white powder.

Identification

In the Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 50 volumes of *water* and 50 volumes of *acetonitrile*.

Test solution. Dissolve about 50 mg of the substance under examination in solvent mixture and diluted to 50.0 ml with solvent mixture.

Reference solution. A 0.1 per cent w/v solution of *trimethobenzamide hydrochloride IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. 0.1 per cent v/v solution of *trifluoroacetic acid* in *water*
  B. 0.1 per cent v/v solution of *trifluoroacetic acid* in *acetonitrile*,
- a linear gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
30	20	80
35	80	20
40	80	20

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and tailing factor is not more than 2.0.

Inject the test solution. In the chromatogram obtained with test solution the per cent area of any secondary peak is not more than 0.2 per cent and the total area per cent of peaks other than the principal peak is not more than 1.0 per cent, calculated by area normalisation.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

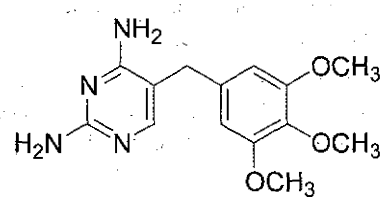
Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 4 hours.

Assay. Dissolve 0.4 g in 50.0 ml of *glacial acetic acid*, add 15 ml of *mercuric acetate solution* and titrate with *0.1 M perchloric acid* determining the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.04249 g of $C_{21}H_{29}ClN_2O_5$

Trimethoprim



$C_{14}H_{18}N_4O_3$ Mol. Wt. 290.3

Trimethoprim is 5-(3,4,5-trimethoxybenzyl)pyrimidine-2,4-diamine.

Trimethoprim contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{14}H_{18}N_4O_3$, calculated on the dried basis.

Category. Antibacterial.

Description. A white or yellowish white powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *trimethoprim IPRS* or with the reference spectrum of trimethoprim.

B. Dissolve 25 mg in 25 ml of *ethanol (95 per cent)* and dilute 2.0 ml to 100 ml with *0.1 M sodium hydroxide*.

When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows an absorption maximum at about 287 nm; absorbance at about 287 nm, about 0.49.

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C. Dissolve about 25 mg in 5 ml of *0.005 M sulphuric acid*, with heating if necessary, add 2 ml of a 1.6 per cent w/v solution of *potassium permanganate* in *0.1 M sodium hydroxide*. Heat to boiling and to the hot solution add 0.4 ml of *formaldehyde solution*. Mix, add 1 ml of *0.5 M sulphuric acid*, mix and heat to boiling. Cool and filter. Add 2 ml of *chloroform* to the filtrate and shake vigorously. The chloroform layer exhibits a green fluorescence when examined under ultraviolet light at 365 nm.

Tests

Appearance of solution. A 5.0 per cent w/v solution in a mixture of 10 volumes of *chloroform*, 9 volumes of *methanol* and 2 volumes of *water* is not more intensely coloured than reference solution BYS7 (2.4.1).

Related substances. A. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 20.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4 mm, packed with base deactivated octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 30 volumes of *methanol* and 70 volumes of a 0.14 per cent w/v solution of *sodium perchlorate*, adjusted to pH 3.6 with *orthophosphoric acid*,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Trimethoprim impurity C[1]	0.8	—
Trimethoprim impurity E[2]	0.9	0.53
Trimethoprim (Retention time: about 5 minutes)	1.0	—
Trimethoprim impurity A[3]	1.5	—
Trimethoprim impurity D[4]	2.0	—
Trimethoprim impurity G[5]	2.1	—
Trimethoprim impurity B[6]	2.3	0.43
Trimethoprim impurity J[7]	2.7	0.66
Trimethoprim impurity F[8]	4.0	—

[1](*RS*)-(2,4-diaminopyrimidin-5-yl)(3,4,5-trimethoxyphenyl) methanol,

[2]4-amino-5-(3,4,5-trimethoxybenzyl)pyrimidin-2-ol,

[3]*N*²-methyl-5-(3,4,5-trimethoxybenzyl)pyrimidine-2,4-diamine,

[4]2-amino-5-(3,4,5-trimethoxybenzyl)pyrimidin-4-ol,

[5]5-(4-ethoxy-3,5-dimethoxybenzyl)pyrimidine-2,4- diamine,

[6](2,4-diaminopyrimidin-5-yl)(3,4,5-trimethoxyphenyl)methanone,

[7]3,4,5-trimethoxybenzoic acid,

[8]5-(3-bromo-4,5-dimethoxybenzyl)pyrimidine-2,4-diamine.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram 11 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent). Ignore any peak with an area less than 0.04 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent) and peak corresponding to trimethoprim impurity H at relative retention time of about 10.3.

B. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.005 per cent w/v each of *trimethoprim IPRS* and *trimethoprim impurity B IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with nitrile bonded to porous silica (5 µm),
- mobile phase: dissolve 1.14 g of *sodium hexane sulphonate* in 600 ml of a 1.36 per cent w/v solution of *potassium dihydrogen phosphate*, adjusted to pH 3.1 with *orthophosphoric acid* and mix with 400 ml of *methanol*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Trimethoprim (Retention time: about 4 minutes)	1.0	—
Trimethoprim impurity H[1]	1.8	0.5
Trimethoprim impurity I[2]	4.9	0.28

[1]methyl 3,4,5-trimethoxybenzoate,

[2]3-(phenylamino)-2-(3,4,5-trimethoxybenzyl)prop-2-enenitrile.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to trimethoprim and trimethoprim impurity B is not less than 2.0.

Inject reference solution (a) and the test solution. Run the chromatogram 6 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of all the secondary peaks is not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Ignore any peak with an area less than 0.04 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent) and any peak due to trimethoprim impurity B at relative retention time of about 1.3.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.25 g in 50 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02903 g of $C_{14}H_{18}N_4O_3$.

Storage. Store protected from light.

Trimethoprim Tablets

Trimethoprim Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of trimethoprim, $C_{14}H_{18}N_4O_3$.

Usual strengths. 100 mg; 200 mg.

Identification

A. Shake a quantity of the powdered tablets containing 0.1 g of Trimethoprim with 10 ml of *chloroform*, filter and evaporate the filtrate to dryness.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *trimethoprim IPRS* or with the reference spectrum of trimethoprim.

B. Shake a quantity of the powdered tablets containing 0.1 g of Trimethoprim with 60 ml of *0.1 M hydrochloric acid* for 20 minutes, add sufficient *0.1 M hydrochloric acid* to produce 100 ml, filter and dilute 5 ml of the filtrate to 250 ml with *0.1 M sodium hydroxide*. When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows an absorption maximum at about 287 nm.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.01 M hydrochloric acid*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, dilute suitably if necessary with the medium, at the maximum at about 271 nm (2.4.7). Calculate the content of $C_{14}H_{18}N_4O_3$ in the medium from the absorbance obtained from a solution of known concentration of *trimethoprim IPRS* in the dissolution medium.

Q. Not less than 75 per cent of the stated amount of $C_{14}H_{18}N_4O_3$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets containing 0.2 g of Trimethoprim with 50 ml of the mobile phase, filter and use the filtrate.

Reference solution. Dilute 1 volume of the test solution to 100 volumes with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 5 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: 0.14 per cent w/v solution of *sodium perchlorate* in *methanol (60 per cent)* adjusted to pH 3.1 with *0.1 M hydrochloric acid*,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

The column efficiency, determined using the peak due to trimethoprim in the chromatogram obtained with reference solution, should be at least 8,000 theoretical plates per metre.

In the chromatogram obtained with the test solution the sum of the areas of any secondary peaks is not greater than the area of the principal peak in the chromatogram obtained with reference solution.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.1 g of Trimethoprim, add 100 ml of *glacial acetic acid*, shake for 20 minutes, dilute to 200.0 ml with *glacial acetic acid* and filter. To 5.0 ml of the filtrate add 15 ml of *glacial acetic acid* and dilute to 100.0 ml with *water*.

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Measure the absorbance of the resulting solution at the maximum at about 271 nm (2.4.7).

Calculate the content of $C_{14}H_{18}N_4O_3$ taking 204 as the specific absorbance at 271 nm.

Storage. Store protected from moisture.

Trimethoprim and Sulphamethoxazole Oral Suspension

Sulphamethoxazole and Trimethoprim Oral Suspension; Co-trimoxazole Oral Suspension; Co-trimoxazole Mixture

Trimethoprim and Sulphamethoxazole Oral Suspension is a suspension of Trimethoprim and Sulphamethoxazole in a suitable flavoured vehicle. It contains 5 parts of Sulphamethoxazole for 1 part, by weight, of Trimethoprim.

Trimethoprim and Sulphamethoxazole Oral Suspension contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of trimethoprim, $C_{14}H_{18}N_4O_3$, and sulphamethoxazole, $C_{10}H_{11}N_3O_3S$.

Category. Antibacterial.

Usual strengths. Trimethoprim 40 mg and Sulphamethoxazole 200 mg in 5 ml; Trimethoprim 80 mg and Sulphamethoxazole 400 mg in 5 ml.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 20 volumes of *chloroform*, 2 volumes of *methanol* and 1 volume of *dimethylformamide*.

Test solution. Add 20 ml of *methanol* (or a suitable volume of *methanol* to yield 0.4 per cent w/v solution of Trimethoprim) to 5 ml of the suspension, mix, shake with 10 g of *anhydrous sodium sulphate*, centrifuge and use the supernatant liquid.

Reference solution (a). A 2.0 per cent w/v solution of *sulphamethoxazole IPRS* in *methanol*.

Reference solution (b). A 0.4 per cent w/v solution of *trimethoprim IPRS* in *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air, spray with *dilute potassium iodobismuthate solution*. One of the principal spots in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a) and the other corresponds to that in the chromatogram obtained with reference solution (b).

Tests

pH (2.4.24). 5.0 to 6.5.

Other tests. Comply with the tests stated under Oral liquids.

Assay. Determine by liquid Chromatography (2.4.14).

Test solution. Dilute a volume of oral suspension containing about 80 mg of sulphamethoxazole with 30 ml of *methanol* and sonicate for 10 minutes and dilute to 50.0 ml with *methanol* and filter. Dilute a volume of filtrate to obtain a solution containing 0.016 per cent w/v of sulphamethoxazole with the mobile phase.

Reference solution. Dilute 5 ml of a solution containing 0.032 percent w/v of *trimethoprim IPRS* and 0.16 percent w/v of *sulphamethoxazole IPRS* in *methanol* to 50.0 ml with mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: mix 700 ml of *water*, 200 ml of *acetonitrile*, and 1.0 ml of *triethylamine*, adjusted to pH 5.9 with *0.2 M sodium hydroxide* or *dilute glacial acetic acid* (1.0 per cent v/v) and dilute to 1000.0 ml with *water*, filter,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention time for sulphamethoxazole with reference to trimethoprim is about 1.8.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to sulphamethoxazole and trimethoprim is not less than 5.0, the relative standard deviation for replicate injections is not more than 2.0 per cent and the tailing factor is not more than 2.0 for sulphamethoxazole and trimethoprim peaks.

Inject the reference solution and the test solution.

Calculate the content of $C_{14}H_{18}N_4O_3$ and $C_{10}H_{11}N_3O_3S$ in the oral suspension.

Storage. Store protected from light and moisture. The suspension should not be allowed to freeze.

Labelling. The label states (1) the content of Trimethoprim and of Sulphamethoxazole in each 5 ml of the suspension; (2) that the contents should be shaken before use; (3) that a suspension containing 40 mg of Trimethoprim and 200 mg of Sulphamethoxazole in 5 ml is meant for paediatric use.

Trimethoprim and Sulphamethoxazole Tablets

Sulphamethoxazole and Trimethoprim Tablets; Co-trimoxazole Tablets

Trimethoprim and Sulphamethoxazole Tablets contain 5 parts of Sulphamethoxazole for 1 part, by weight, of Trimethoprim.

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Trimethoprim and Sulphamethoxazole Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amounts of trimethoprim, $C_{14}H_{18}N_4O_3$, and sulphamethoxazole, $C_{10}H_{11}N_3O_3S$.

Category. Antibacterial.

Usual strengths. Trimethoprim 20 mg and Sulpha- methoxazole 100 mg; Trimethoprim 80 mg and Sulphamethoxazole 400 mg; Trimethoprim 160 mg and Sulphamethoxazole 800 mg.

Identification

A. To a quantity of the powdered tablets containing 50 mg of Trimethoprim add 30 ml of *0.1 M sodium hydroxide* and extract with two quantities, each of 50 ml, of *chloroform*. Wash the combined chloroform extracts with two quantities, each of 10 ml, of *0.1 M sodium hydroxide* and then with 10 ml of *water*. Combine the aqueous extract and washings (solution A) and reserve for test B. Shake with 5 g of *anhydrous sodium sulphate*, filter and evaporate to dryness using a rotary evaporator.

The residue complies with the following test. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *trimethoprim IPRS* or with the reference spectrum of trimethoprim.

B. Filter solution A, add, dropwise, sufficient *2 M hydrochloric acid* to the filtrate to make it just acidic and extract with 50 ml of *ether*. Wash the ether layer with 10 ml of *water*, shake with 5 g of *anhydrous sodium sulphate*, filter and evaporate the filtrate to dryness using a rotary evaporator. Dissolve the residue in the minimum volume of a 5 per cent w/v solution of *sodium carbonate*, add *1 M hydrochloric acid* dropwise until precipitation is complete and filter. Wash the residue sparingly with water and dry at 105°.

The residue complies with the following test. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *sulphamethoxazole IPRS* or with the reference spectrum of sulphamethoxazole.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 20 volumes of *chloroform*, 2 volumes of *methanol* and 1 volume of *dimethylformamide*.

Test solution. Shake a quantity of the powdered tablets containing 0.4 g of Sulphamethoxazole with 20 ml of *methanol* and filter.

Reference solution (a). A 2.0 per cent w/v solution of *sulphamethoxazole IPRS* in *methanol*.

Reference solution (b). A 0.4 per cent w/v solution of *trimethoprim IPRS* in *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air, spray with *dilute potassium iodobismuthate solution*. One of the principal spots in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a) and the other corresponds to that in the chromatogram obtained with reference solution (b).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 75 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a suitable quantity of *sulphamethoxazole IPRS* and *trimethoprim IPRS* in the dissolution medium to obtain a solution having the same concentration as that to the test solution.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent for both the peaks.

Calculate the content of $C_{14}H_{18}N4O_3$, and $C_{10}H_{11}N_3O_3S$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{14}H_{18}N4O_3$, and $C_{10}H_{11}N_3O_3S$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid Chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 160 mg of sulphamethoxazole with 50 ml of *methanol* and sonicate with intermittent shaking for 5 minutes and dilute to 100.0 ml with *methanol* and filter. Dilute a volume of filtrate to obtain a solution containing 0.016 per cent w/v of sulphamethoxazole with the mobile phase.

Reference solution. Dilute 5 ml of a solution containing 0.032 percent w/v of *trimethoprim IPRS* and 0.16 percent w/v of *sulphamethoxazole IPRS* in *methanol* to 50.0 ml with mobile phase.

Chromatographic system
– a stainless steel column 25 cm x 4.6 mm packed with *octyldecylsilane* bonded to porous silica (5 µm),

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– mobile phase: mix 700 ml of *water*, 200 ml of *acetonitrile*, and 1.0 ml of *triethylamine*, adjusted to pH 5.9 with *0.2 M sodium hydroxide* or *dilute glacial acetic acid* (1.0 per cent v/v) and dilute to 1000 ml with *water*, filter,
– flow rate: 2 ml per minute,
– spectrophotometer set at 254 nm,
– injection volume: 20 µl.

The relative retention time for sulphamethoxazole with reference to trimethoprim is about 1.8.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to sulphamethoxazole and trimethoprim is not less than 5.0, the relative standard deviation for replicate injections is not more than 2.0 per cent and the tailing factor is not more than 2.0 for sulfamethoxazole and trimethoprim peaks.

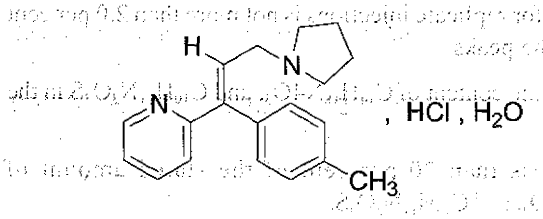
Inject the reference solution and the test solution.

Calculate the content of $C_{14}H_{18}N_4O_3$ and $C_{10}H_{11}N_3O_3S$ in the tablets.

Storage. Store protected from light and moisture.

Labelling. The label states the quantities of Trimethoprim and of Sulphamethoxazole in each tablet.

Triprolidine Hydrochloride



, HCl , H_2O

$C_{19}H_{22}N_2,HCl,H_2O$ Mol. Wt. 332.9

Triprolidine Hydrochloride is (*E*)-2-(3-pyrrolidin-1-yl-1-(*p*-tolyl)prop-1-enyl)pyridine hydrochloride monohydrate.

Triprolidine Hydrochloride contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{19}H_{22}N_2$, HCl, calculated on the anhydrous basis.

Category. Histamine H_1-receptor antagonist.

Description. A white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *triprolidine hydrochloride IPRS* or with the reference spectrum of triprolidine hydrochloride.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution shows absorption maxima at about 230 nm and 276 nm; absorbance at about 230 nm, about 0.50 and at about 276 nm, about 0.25.

C. When examined in the range 230 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution in *0.05 M sulphuric acid* shows an absorption maximum at about 290 nm; absorbance at about 290 nm, about 0.6.

D. Dissolve 0.1 g in 2 ml of *2 M hydrochloric acid* and add 0.5 ml of *potassium mercuri-iodide solution*; a pale yellow precipitate is produced.

E. A 5 per cent w/v solution gives the reactions of chlorides (2.3.1).

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Mobile phase. A mixture of equal volumes of *2-butanone* and *dimethylformamide*.

Test solution. Dissolve 0.1 g of the substance under examination in 10 ml of *methanol*.

Reference solution (a). Dissolve 10 mg of the substance under examination in 100 ml of *methanol*.

Reference solution (b). A 0.02 per cent w/v solution of *Z-triprolidine IPRS* in *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. In the chromatogram obtained with the test solution any spot corresponding to Z-triprolidine is not more intense than the spot in the chromatogram obtained with reference solution (b) and any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (a).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). 4.0 to 6.0 per cent, determined on 0.4 g.

Assay. Dissolve 0.25 g, in a mixture of 50 ml of *anhydrous glacial acetic acid*, 0.5 ml of *acetic anhydride* and add 15 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01574 g of $C_{19}H_{22}N_2$, HCl.

Storage. Store protected from light and moisture.

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Triprolidine Tablets

Triprolidine Hydrochloride Tablets

Triprolidine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of triprolidine hydrochloride, $C_{19}H_{22}N_2,HCl,H_2O$.

Usual strength. 10 mg.

Identification

A. Extract a quantity of the powdered tablets containing 10 mg of Triprolidine Hydrochloride with *ether*, filter, discard the ether extract and dry the residue. Extract the residue with *chloroform*, filter and evaporate the filtrate to dryness. Add 0.1 ml of *ether*, stir and allow to evaporate.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *triprolidine hydrochloride IPRS* or with the reference spectrum of triprolidine hydrochloride.

B. In the test for Related substances, the principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (b).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket).

Medium. 500 ml of acetate buffer pH 4.0 prepared by dissolving 2.45 g of *sodium acetate trihydrate* and 4.9 g of *glacial acetic acid* in 1000 ml of *water*.

Speed and time. 50 rpm, 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtrate, dilute suitably if necessary with the medium, at the maximum at about 277 nm (2.4.7). Calculate the content of $C_{19}H_{22}N_2.HCl.H_2O$ in the medium from the absorbance obtained from a solution of known concentration of *triprolidine hydrochloride IPRS* in the dissolution medium.

Q. Not less than 80 per cent of the stated amount of $C_{19}H_{22}N_2.HCl.H_2O$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Mobile phase. A mixture of equal volumes of *2-butanone* and *dimethylformamide*.

Test solution. Extract a quantity of powdered tablets containing 10 mg of Triprolidine Hydrochloride with *methanol*, filter, evaporate to dryness and dissolve the residue in 1 ml of *methanol*.

Reference solution (a). A 0.02 per cent w/v solution of *Z-triprolidine IPRS* in *methanol*.

Reference solution (b). A 1.0 per cent w/v solution of *triprolidine hydrochloride IPRS* in *methanol*.

Reference solution (c). A 0.01 per cent w/v solution of *triprolidine hydrochloride IPRS* in *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. In the chromatogram obtained with the test solution any spot corresponding to Z-triprolidine is not more intense than the spot in the chromatogram obtained with reference solution (a) and any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (c).

Uniformity of content. Complies with the test stated under Tablets.

Take one tablet and transfer to a 50-ml volumetric flask, add 30 ml of water and sonicate for 15 minutes, make up to volume with water, mix and filter, rejecting the first few ml of filtrate. Pipette 25 ml of the filtrate and carry out the procedure mentioned under Assay begining at the words "add 1 g of sodium chloride ....."

Calculate the content of $C_{19}H_{22}N_2,HCl,H_2O$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 7.5 mg of Triprolidine Hydrochloride, add 15 ml of *water* and 1 g of *sodium chloride*, shake for 2 to 3 minutes and add sufficient *5 M sodium hydroxide* to make it alkaline. Extract with four quantities, each of 20 ml, of *ether* and wash the combined extracts with two quantities, each of 5 ml, of a mixture of equal volumes of a saturated solution of *sodium chloride* and *water*. Extract the ether solution with 20 ml of *0.1 M hydrochloric acid*, wash the ether with two quantities, each of 5 ml, of *water* and add the washings to the acid extract. Heat on a water-bath for 30 minutes, cool and add sufficient *water* to produce 50.0 ml. Dilute 10.0 ml to 100.0 ml with *0.1 M hydrochloric acid*. Measure the absorbance of the resulting solution at the maximum at about 290 nm (2.4.7).

Calculate the content of $C_{19}H_{22}N_2,HCl,H_2O$ taking 290 as the specific absorbance at 290 nm.

Storage. Store protected from light and moisture.

Trisodium Edetate Concentrate for Injection

Trisodium Edetate Concentrate for Injection is a sterile solution in Water for Injections containing 20 per cent w/v solution of trisodium edetate prepared by the interaction of

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Disodium Edetate and Sodium Hydroxide. It should be diluted with a suitable diluent in accordance with the manufacturer's instructions.

Trisodium Edetate Concentrate for Injection contains not less than 19.0 per cent w/v and not more than 21.0 per cent w/v solution of trisodium edetate, $C_{10}H_{13}N_2Na_3O_8$.

Category. Used in the treatment of hypercalcaemia.

Description. A colourless solution.

Identification

A. Dissolve 2 g in 25 ml of *water*, add 6 ml of *lead nitrate solution*, shake and add 3 ml of *potassium iodide solution*; no yellow precipitate is produced. Make alkaline to red litmus paper with *2 M ammonia* and add 5 ml of *ammonium oxalate solution*; no precipitate is produced.

B. Dissolve 0.5 g in 10 ml of *water*, add 0.5 ml of a 10 per cent w/v solution of *calcium chloride*, make alkaline to red litmus paper with *2 M ammonia* and add 3 ml of *ammonium oxalate solution*; no precipitate is produced.

C. Evaporate to dryness and ignite. The residue gives the reactions of sodium salts (2.3.1).

Tests

pH (2.4.24). 7.0 to 8.0.

Bacterial endotoxins (2.2.3). If necessary, dilute the infusion with *water BET* to give a solution containing 10 mg per ml. The solution contains not more than 1.4 Endotoxin Unit per ml.

Other tests. Comply with the tests stated under Parenteral Preparations (Concentrated Solutions for Injection).

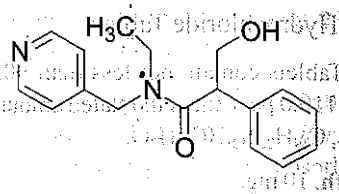
Assay. Dilute 10.0 ml to 100.0 ml with *water* and use the solution to titrate a mixture of 25.0 ml of *0.05 M magnesium sulphate* and 10 ml of *ammonia buffer pH 10.9* using *mordant black II mixed triturate* as indicator.

1 ml of *0.05 M magnesium sulphate* is equivalent to 0.01791 g of $C_{10}H_{13}N_2Na_3O_8$.

Storage. Store in hermetically-sealed, lead-free glass containers.

Labelling. The label states (1) the strength in terms of anhydrous trisodium edetate contained in a suitable dose-volume; (2) 'Trisodium Edetate Concentrate for Injection'; (3) that the solution must be diluted with either Sodium Chloride Intravenous Infusion or Dextrose Intravenous Infusion before administration.

Tropicamide



$C_{17}H_{20}N_2O_2$ Mol. Wt. 284.4

Tropicamide is (*RS*)-*N*-ethyl-3-hydroxy-2-phenyl-*N*-(pyrid-4-ylmethyl)propionamide

Tropicamide contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{17}H_{20}N_2O_2$, calculated on the dried basis.

Category. Mydriatic; cycloplegic.

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tropicamide IPRS* or with the reference spectrum of tropicamide.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.003 per cent w/v solution in *0.1 M hydrochloric acid* shows an absorption maximum only at about 254 nm; absorbance at about 254 nm, about 0.54.

C. Dissolve 5 mg in 3 ml of a mixture of 9 ml of *acetic anhydride*, 1 ml of *6 M acetic acid* and 0.1 g of *citric acid* and heat on a water-bath for 5 to 10 minutes; a reddish yellow colour is produced.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 3 ml of *acetonitrile* and dilute to 50.0 ml with *water*.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *water*. Dilute 1.0 ml of the solution to 10.0 ml with *water*.

Reference solution (b). Dissolve 5 mg each of tropicamide impurity A, *tropicamide impurity C IPRS* and *tropicamide impurity D IPRS* in 2 ml of *acetonitrile* and dilute to 50.0 ml with *water*. Dilute 1.0 ml of the solution to 10.0 ml with *water*.

Reference solution (c). Dilute 1.5 ml of reference solution (b) to 10.0 ml with *water*.

Chromatographic system

– a stainless steel column 15 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (3 µm),

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— column temperature: 40°,
— mobile phase: a mixture of 73 volumes of solution prepared by dissolving 0.135 g of *sodium dodecyl sulphate* and 3.4 ml of *orthophosphoric acid* in 950 ml of *water*, adjusted to pH 3.0 with *sodium hydroxide solution* and dilute to 1000 ml with *water* and 27 volumes of *acetonitrile*,
— flow rate: 0.8 ml per minute,
— spectrophotometer set at 210 nm and 254 nm,
— injection volume: 15 µl.

Name	Relative retention time	Correction factor
Tropicamide impurity C[1]	0.4	—
Tropicamide impurity A[2]	0.5	0.8
Tropicamide impurity D[3]	0.8	—
Tropicamide (Retention time: about 11 minutes)	1.0	—
Tropicamide impurity B[4]	2.3	0.6

[1]tropic acid,
[2]*N*-(pyridin-4-ylmethyl)ethanamine,
[3]phenylacetic acid,
[4]*N*-ethyl-2-phenyl-*N*-(pyridin-4-ylmethyl)propenamide.

Inject reference solution (b). The test is not valid unless the resolution at 210 nm between the peaks due to impurities C and A and impurities A and D is not less than 2.0.

Inject reference solution (a), (c) and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to tropicamide impurity B at 254 nm is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). The area of any peak corresponding to tropicamide impurity A at 254 nm is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent). The area of any peak corresponding to tropicamide impurity C at 210 nm is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.15 per cent). The area of any peak corresponding to tropicamide impurity D at 210 nm is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.15 per cent), the area of any other secondary peak at 254 nm is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks other than tropicamide impurities C and D at 254 nm is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak at 254 nm, with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 80° at a pressure not exceeding 0.7 kPa for 4 hours.

Assay. Dissolve 0.5 g in 80 ml of *anhydrous glacial acetic acid*. Titrate with *0.1 M perchloric acid*, using *1-naphthol-benzein* solution as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02844 g of $C_{17}H_{20}N_2O_2$.

Storage. Store protected from light and moisture.

Tropicamide Eye Drops

Tropicamide Eye Drops are a sterile solution of Tropicamide in Purified Water. They may contain stabilisers, suitable antimicrobial agents and suitable substances to increase the viscosity of the solution.

Tropicamide Eye Drops contain not less than 95.0 per cent and not more than 110.0 per cent of the stated amount of tropicamide, $C_{17}H_{20}N_2O_2$.

Usual strengths. 0.5 per cent w/v; 1.0 per cent w/v.

Identification

A. Shake a volume containing 20 mg of Tropicamide with 10 ml of *chloroform*, dry the chloroform layer over *anhydrous sodium sulphate*, filter and evaporate the filtrate to dryness. Dissolve the residue in minimum quantity of *chloroform*, add dropwise to finely powdered *potassium bromide IR*, mix and dry at 60°.

On the residue determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tropicamide IPRS* or with the reference spectrum of tropicamide.

B. When examined in the range 230 nm to 360 nm (2.4.7), the final solution obtained in the Assay shows an absorption maximum at about 254 nm.

Tests

pH (2.4.24). 4.0 to 5.8.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 95 volumes of *chloroform*, 5 volumes of *methanol* and 0.5 volume of *strong ammonia solution*.

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Test solution. Extract a volume containing 50 mg of tropicamide with 10 ml of *chloroform,* filter through *sodium sulphate anhydrous* and dilute to 10.0 ml with *chloroform.*

Reference solution (a). Dilute 1 volume of the eye drops to 200 volumes with *water.*

Reference solution (b). Dilute 1 volume of the eye drops to 500 volumes with *water.*

Apply to the plate 40 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b).

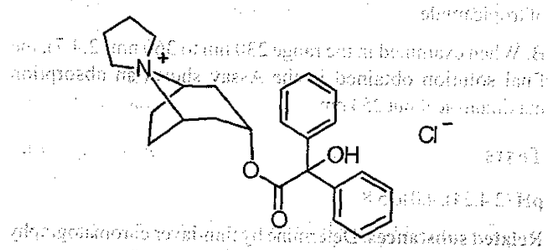
Other tests. Comply with the tests stated under Eye Drops.

Assay. To a volume containing about 30 mg of Tropicamide add sufficient *water* to produce 100.0 ml. To 10.0 ml of the resulting solution add 2 ml of a 10 per cent w/v solution of *anhydrous sodium carbonate* and extract with four quantities, each of 20 ml, of *chloroform.* Wash the combined *chloroform* extracts with 25 ml of *phosphate buffer pH 6.5.* Wash the aqueous layer with 10 ml of *chloroform,* combine the chloroform extracts and shake with four quantities, each of 20 ml, of *0.5 M sulphuric acid.* Combine the acid extracts, dilute to 100.0 ml with *0.5 M sulphuric acid* and measure the absorbance of the resulting solution at the maximum at about 254 nm (2.4.7).

Calculate the content of $C_{17}H_{20}N_2O_2$ taking 172 as the specific absorbance at 254 nm.

Storage. Store in a refrigerator (8° to 15°). It should not be allowed to freeze.

Trospium Chloride



$C_{25}H_{30}ClNO_3$ Mol.Wt. 428.0

Trospium Chloride is 3-(2-Hydroxy-2,2-diphenylacetoxy)spiro [bicyclo[3.2.1]octane-8,1'-pyrrolidin]-1'-ium chloride.

Trospium Chloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{25}H_{30}ClNO_3$, calculated on the dried basis.

Category. Anticholinergic.

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *trospium chloride IPRS* or with the reference spectrum of trospium chloride.

B. It gives reaction (A) of chlorides (2.3.1).

Tests

Solution A. A 10 per cent w/v solution in *carbon dioxide-free water.*

Appearance of solution. Solution A is clear (2.4.1) and not more intensely coloured than reference solution BS7 (2.4.1).

pH (2.4.24). 5.0 to 7.0, dilute 5.0 ml of solution A to 50.0 ml with *carbon dioxide-free water.*

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 30 mg of the substance under examination in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). A 0.03 per cent w/v solution of *trospium impurity A IPRS* in the mobile phase.

Reference solution (b). A 0.15 per cent w/v solution of *trospium impurity B IPRS* in the mobile phase.

Reference solution (c). Dilute a mixture of 0.3 ml of the test solution, 1.0 ml of reference solution (a) and 1.0 ml of reference solution (b) to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 1 volume of *triethylamine* and 3 volumes of *orthophosphoric acid* with 700 volumes of *water* and add 300 volumes of *acetonitrile,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Name	Relative retention time
Trospium impurity B[1]	0.7
Trospium (Retention time about 10 minutes)	1.0
Trospium impurity A[2]	1.9

[1](1R,3r,5S)-8'-azabicyclo[3.2.1]oct-3-yl hydroxydiphenylacetate,

[2]hydroxydiphenylacetic acid (benzilic acid).

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to trospium impurity B and trospium is not less than 3.0.

Inject reference solution (c) and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak due to trospium impurity B is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.5 per cent), the area of any peak due to trospium impurity A is not more than 3 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.3 per cent), the area of any other secondary peak is not more than 0.2 times the area of the peak due to trospium impurity B in the chromatogram obtained with reference solution (c) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the peak due to trospium impurity B in the chromatogram obtained with reference solution (c) (1.0 per cent). Ignore any peak with an area less than 0.1 times the area of peak due to trospium impurity B in the chromatogram obtained with reference solution (c) (0.05 per cent).

Impurity C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G.*

Mobile phase. A mixture of 1 volume of *glacial acetic acid* and 3.5 volumes of *hydrochloric acid* and 45 volumes of *acetonitrile.*

Test solution. Dissolve 0.2 g of the substance under examination in 2.0 ml of *methanol.*

Reference solution (a). Dissolve 1 mg of *trospium impurity C IPRS ((1R,3r,5S)-3-hydroxyspiro[8-azoniabicyclo[3.2.1] octane8,1-pyrrolidinium] IPRS)* in 2.0 ml of *methanol.*

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with *methanol.* To 50 µl of the solution, add 1 ml of reference solution (a).

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in a warm air until the odour of *acetic acid* is no longer perceptible. Spray the plate with *potassium iodobismuthate solution* and subsequently with a 0.5 per cent w/v solution of *sodium nitrite.* The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly visible and separated spots. Any spot corresponding to impurity C is not more intense than the area of the principal spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.3 g in 50.0 ml of *water.* Titrate with *0.1 M silver nitrate* determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.0428 g of $C_{25}H_{30}ClNO_3$.

Storage. Store protected from light.

Trospium Chloride Tablets

Trospium Chloride Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of trospium chloride, $C_{25}H_{30}ClNO_3$.

Usual strength. 20 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 1000 ml of *0.1 M hydrochloric acid,*
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter through 0.45 µm pore size filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate and dilute, if necessary, with the dissolution medium.

Reference solution. A 0.05 per cent w/v solution of *trospium chloride IPRS* in the mobile phase. Dilute 4.0 ml of the solution to 100.0 ml with dissolution medium.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 350 volumes of *acetonitrile,* 1 volume of *triethylamine,* 3 volumes of *orthophosphoric acid* and 650 volumes of *water,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

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Calculate the content of $C_{25}H_{30}ClNO_3$.

Q. Not less than 80 per cent of the stated amount of $C_{25}H_{30}ClNO_3$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Transfer a quantity of powder containing 150 mg of Trospium Chloride to 50.0 ml volumetric flask. Add about 30.0 ml of mobile phase and disperse with the aid of ultrasound for 30 minutes. Dilute to volume with the mobile phase. Filter this solution through 0.45 µm pore size filter.

Reference solution (a). A solution containing each of 0.001 per cent w/v of *trospium chloride IPRS, 0.0003 per cent w/v of trospium chloride impurity A IPRS* and 0.0015 per cent w/v of *trospium chloride impurity B IPRS* in the mobile phase.

Reference solution (b). A solution containing 0.0003 per cent w/v of *trospium chloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octaylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 300 volumes of *acetonitrile*, 1 volume of *triethylamine*, 3 volumes of *orthophosphoric acid* and 700 volumes of *water*,
- column temperature: 40°,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Nortropane benzilate (trospium chloride impurity B)	0.7	1.0
Trospium	1.0	—
Benzilic acid (trospium chloride impurity A)[1]	1.9	0.59

[1](1*R*,3r,5*S*)-8-azabicyclo[3.2.1]octan-3-ylhydroxydiphenylacetate.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to trospium and trospium chloride impurity B is not less than 3.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to trospium chloride impurity B and trospium chloride impurity A are not more than 2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 100 mg of Trospium Chloride in 75 ml of mobile phase, sonicate for about 30 minutes and dilute to 100.0 ml with the mobile phase. Further dilute with mobile phase to obtain a solution containing 0.01 per cent w/v of trospium chloride in the mobile phase, mix well and filter, rejecting the first few ml of the filtrate. Dilute 5.0 ml of the filtrate to 50.0 ml with mobile phase and mix well.

Reference solution. A 0.01 per cent w/v solution of *trospium chloride IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 350 volumes of *acetonitrile*, 1 volume of *triethylamine*, 3 volumes of *orthophosphoric acid* and 650 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

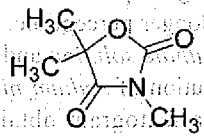
Inject the reference solution and the test solution.

Calculate the content of $C_{25}H_{30}ClNO_3$ in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Troxidone

Trimethadione



$C_6H_9NO_3$ Mol. Wt. 143.1

Troxidone is 3,5,5-trimethyloxazolidine-2,4-dione.

Troxidone contains not less than 98.0 per cent and not more than 102.0 per cent of $C_6H_9NO_3$, calculated on the dried basis.

Category. Anticonvulsant.

Description. Colourless or almost colourless crystals.

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Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *troxidone IPRS* or with the reference spectrum of troxidone.

B. To 2 ml of a 5.0 per cent w/v solution in *carbon dioxide-free water* (solution A) add 1 ml of *barium hydroxide solution*; a white precipitate is produced which dissolves on the addition of 1 ml of *2 M hydrochloric acid*.

C. Dissolve 0.3 g in a mixture of 5 ml of *ethanolic potassium hydroxide solution* and 5 ml of *ethanol (95 per cent)* and allow to stand for 10 minutes. Add 0.05 ml of *phenolphthalein solution* and carefully add *hydrochloric acid* until the solution is neutral. Evaporate to dryness on a water-bath, shake the residue with four quantities, each of 5 ml, of *ether*, filter the combined ether extracts and evaporate the filtrate to dryness. The residue, after recrystallisation from 5 ml of *toluene* and drying, melts at about 80° (2.4.21).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. To 10 ml of solution A add 0.1 ml of *methyl red solution*. Not more than 0.1 ml of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the solution.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying over *anhydrous silica gel* for 6 hours.

Assay. Determine by gas chromatography (2.4.13).

Test solution. Weigh 0.1 g of the substance under examination and dissolve in 10 ml of the solutions prepared by dissolving 0.125 g of *1-decanol* (internal standard) in sufficient *ethanol* to produce 25.0 ml (solution B).

Reference solution. Dissolve 0.1 g of *troxidone IPRS* in sufficient solution B to produce 10.0 ml.

Chromatographic system
- a stainless steel column 0.75 m x 3 mm, packed with porous polymer beads (120 to 150 µm),
- temperature:
  column 210°,
  inlet port at 240° and detector at 270°,
- flow rate: 30 ml per minute, using nitrogen as the carrier gas.

Inject 1 µl of the test solution and the reference solution.

Calculate the content of $C_6H_9NO_3$.

Storage. Store protected from light and moisture.

Troxidone Capsules

Trimethadione Capsules

Troxidone Capsules contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of troxidone, $C_6H_9NO_3$.

Usual strength. 250 mg.

Identification

To a quantity of the contents of the capsules containing 1 g of Troxidone add 25 ml of *ether*, set aside in a stoppered flask for 20 minutes, decant the ether through a filter, and if an insoluble residue remains, digest it with another 10-ml portion of *ether* as before, and filter into the first ether filtrate. Evaporate the ether extracts to dryness with the aid of a current of air and dry the residue at a pressure of 2 kPa for 2 hours. The residue complies with the following tests.

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *troxidone IPRS* or with the reference spectrum of troxidone.

B. To 2 ml of a 5.0 per cent w/v solution in *carbon dioxide-free water* (solution A) add 1 ml of *barium hydroxide solution*; a white precipitate is produced which dissolves on the addition of 1 ml of *2 M hydrochloric acid*.

C. Dissolve 0.3 g in a mixture of 5 ml of *ethanolic potassium hydroxide solution* and 5 ml of *ethanol (95 per cent)* and allow to stand for 10 minutes. Add 0.05 ml of *phenolphthalein solution* and carefully add *hydrochloric acid* until the solution is neutral. Evaporate to dryness on a water-bath, shake the residue with four quantities, each of 5 ml, of *ether*, filter the combined ether extracts and evaporate the filtrate to dryness. The residue, after recrystallisation from 5 ml of *toluene* and drying, melts at about 80° (2.4.21).

Tests

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by gas chromatography (2.4.13).

Test solution. To a quantity of the contents of the capsules containing about 1.0 g of Troxidone add 25 ml of a 0.5 per cent w/v solution of *1-decanol* (internal standard) in *ethanol*

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(solution B), shake for 30 minutes, add sufficient of internal standard solution to produce 100.0 ml, mix and centrifuge. Use the supernatant liquid.

Reference solution. Dissolve 0.1 g of *troxidone IPRS* in sufficient solution B to produce 10.0 ml.

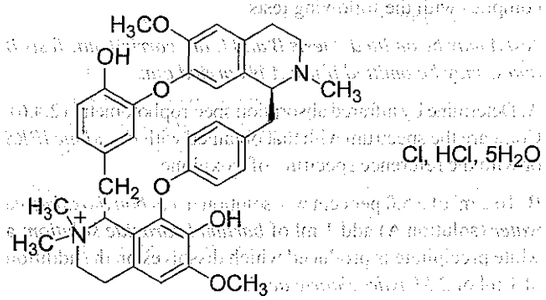
Chromatographic system
- a stainless steel column 0.75 m × 3 mm, packed with porous polymer beads (120 to 150 µm),
- temperature:
  column 210°,
  inlet port at 240° and detector at 270°,
- flow rate: 30 ml per minute, using nitrogen as the carrier gas.

Inject 1 µl of the test solution and the reference solution.

Calculate the content of $C_6H_9NO_3$ in the capsules.

Storage. Store protected from moisture.

Tubocurarine Chloride



$C_{37}H_{41}ClN_2O_6,HCl,5H_2O$ Mol. Wt. 771.7

Tubocurarine Chloride is 7′,12′-dihydroxy-6,6′-dimethoxy-2,2′,2′-trimethyltubocuraranium chloride hydrochloride pentahydrate.

Tubocurarine Chloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{37}H_{41}ClN_2O_6,HCl$, calculated on the anhydrous basis.

Category. General anaesthetic.

Description. A white or yellowish white, crystalline powder.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests B, C and D may be omitted if tests A and E are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tubocurarine chloride IPRS* or with the reference spectrum of tubocurarine chloride.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.005 per cent w/v solution shows an absorption maximum at about 280 nm and a minimum at about 255 nm, absorbance at about 280 nm is 0.56 to 0.62, calculated on the anhydrous basis.

C. To 1 ml of a 2.5 per cent w/v solution add 0.2 ml of *ferric chloride solution* and heat in a water-bath for 1 minute; a green colour is produced; 1 ml of *water* treated in the same manner gives a brown colour.

D. To 20 ml of a 0.05 per cent w/v solution add 0.2 ml of *sulphuric acid* and 2 ml of a 1 per cent w/v solution of *potassium iodate*, mix and warm on a water-bath for 30 minutes; a yellow colour is produced.

E. It gives reaction (A) of chlorides and the reactions of alkaloids (2.3.1).

Tests

Appearance of solution. Dissolve 0.5 g in sufficient *carbon dioxide-free water* to produce 50.0 ml (solution A). Solution A is clear (2.4.1), and not more intensely coloured than reference solution YS6 (2.4.1).

pH (2.4.24). 4.0 to 6.0, determined in Solution A.

Specific optical rotation (2.4.22). + 210° to + 222°, determined in solution A 3 hours after preparation.

Chloroform-soluble substances. Not more than 2 per cent, determined by the following method. Dissolve 0.25 g in 150 ml of *water* contained in a separating funnel with a grease-free stopcock. Add 5 ml of a saturated solution of *sodium bicarbonate* and extract with three quantities, each of 20 ml, of *chloroform*. Wash the combined chloroform extracts with 10 ml of *water*, filter the *chloroform* solution into a tared beaker and wash the filter with two successive quantities, each of 5 ml, of *chloroform*. Add the washings to the filtrate. Remove the chloroform on a water-bath, dry the residue at 105° for 1 hour, cool and weigh. The residue does not dissolve in 10 ml of *water* but dissolves on the addition of 1 ml of *2 M hydrochloric acid*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*

Mobile phase. The lower layer of a mixture of equal volumes of *chloroform*, *methanol* and a 12.5 per cent w/v solution of *trichloroacetic acid*.

Test solution. Dissolve 0.25 g of the substance under examination in 10 ml of *water*.

Reference solution (a). A 0.0375 per cent w/v solution of the substance under examination in *water*.

Reference solution (b). A 0.01875 per cent w/v solution of the substance under examination in *water*.

Apply to the plate 5 µl of each solution. After development, dry the plate in a current of cold air and spray with a mixture of 1 volume of *potassium ferricyanide solution*, 1 volume of *water* and 2 volumes of *ferric chloride solution*, prepared immediately before use. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b).

Sulphated ash (2.3.18). Not more than 0.25 per cent, determined on 0.2 g.

Water (2.3.43). 9.0 to 12.0 per cent, determined on 0.3 g.

Assay. Dissolve 25 mg in sufficient *water* to produce 500.0 ml and measure the absorbance of the resulting solution at the maximum at about 280 nm (2.4.7).

Calculate the content of $C_{37}H_{41}ClN_2O_6,HCl$ from the absorbance obtained by repeating the operation using 25 mg, weighed, of *tubocurarine chloride IPRS* in place of the substance under examination.

Storage. Store protected from moisture.

Tubocurarine Injection

Tubocurarine Chloride Injection

Tubocurarine Injection is a sterile solution of Tubocurarine Chloride in Water for Injections. It may contain suitable buffering agents.

Tubocurarine Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of tubocurarine chloride, $C_{37}H_{41}ClN_2O_6,HCl,5H_2O$.

Usual strength. 10 mg per ml.

Description. A colourless or faintly coloured solution.

Identification

A. Mix a volume containing 15 mg of Tubocurarine Chloride with 5 ml of *acetone*, evaporate the liquid at a pressure of 2 kPa and add successive quantities of 2 ml of *acetone*, evaporating each quantity at a pressure of 2 kPa until a dry residue is obtained.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *tubocurarine chloride IPRS* or with the reference spectrum of tubocurarine chloride.

B. Dilute 1 ml to 30 ml with *water*. To 1 ml of the resulting solution add 0.5 ml of *mercuric nitrate solution*; a cherry-red colour slowly develops.

Tests

pH (2.4.24). 4.0 to 6.0.

Optical rotation (2.4.22). + 0.172° to + 0.206° for each mg of tubocurarine chloride, $C_{37}H_{41}ClN_2O_6,HCl,5H_2O$ per ml stated on the label.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*

Mobile phase. The lower layer of a mixture of equal volumes of *chloroform*, *methanol* and a 12.5 per cent w/v solution of *trichloroacetic acid*.

Test solution. A volume of the injection containing 10 mg of Tubocurarine Chloride diluted to 1 ml.

Reference solution (a). Dilute 3 volumes of test solution to 200 volumes with *water*.

Reference solution (b). Dilute 1 volume of reference solution (a) to 2 volumes with *water*.

Apply to the plate 10 µl of each solution. After development, dry the plate in a current of cold air and spray with a mixture of 1 volume of *potassium ferricyanide solution*, 1 volume of *water* and 2 volumes of *ferric chloride solution*, prepared immediately before use. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b).

Bacterial endotoxins (2.2.3). Not more than 10.0 Endotoxin Units per mg of tubocurarine chloride.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Dilute a volume containing about 50 mg of Tubocurarine Chloride to 1000.0 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 280 nm (2.4.7).

Calculate the content of $C_{37}H_{41}ClN_2O_6,HCl,5H_2O$ taking 105 as the specific absorbance at 280 nm.

Storage. Store protected from moisture.

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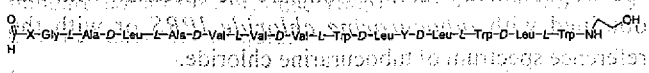
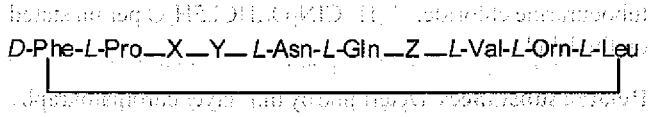
Tyrothricin



Gramicidin	X	Y	Mol. Formula	Mol. Wt.
A1	*L*-Val	*L*-Trp	$C_{99}H_{140}N_{20}O_{17}$	1882
A2	*L*-Ile	*L*-Trp	$C_{100}H_{142}N_{20}O_{17}$	1896
C1	*L*-Val	*L*-Tyr	$C_{97}H_{139}N_{19}O_{18}$	1859
C2	*L*-Ile	*L*-Tyr	$C_{98}H_{141}N_{19}O_{18}$	1873



Tyrocidine	X	Y	Z	Mol. Formula	Mol. Wt.
A	*L*-Phe	*D*-Phe	*L*-Tyr	$C_{66}H_{87}N_{13}O_{13}$	1271
B	*L*-Trp	*D*-Phe	*L*-Tyr	$C_{68}H_{89}N_{14}O_{13}$	1311
C	*L*-Trp	*D*-Trp	*L*-Tyr	$C_{70}H_{90}N_{15}O_{13}$	1350
D	*L*-Trp	*D*-Trp	*L*-Trp	$C_{72}H_{91}N_{16}O_{12}$	1373
E	*L*-Trp	*D*-Phe	*L*-Phe	$C_{66}H_{88}N_{13}O_{12}$	1255

Tyrothricin is a mixture of antimicrobial linear and cyclic polypeptides, obtained from the fermentation broth of *Brevibacillus brevis* Dubos. It consists mainly of gramicidins and tyrocidins, other related compounds may be present in similar amount.

Tyrothricin has potency not less than 180 Units per mg and not more than 280 Units per mg, calculated on the dried basis.

Category. Polypeptide antibacterial.

Description. A white or almost white powder.

Identification

Test A may be omitted if a test B is carried out. Tests B may be omitted if test A is carried out.

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel F254*.

Mobile phase. A mixture of 2.5 volumes of *methanol*, 7.5 volumes of *butanol*, 20 volumes of *acetic acid*, 40 volumes of *butyl acetate* and 12 volumes of *water*.

Test solution. Dissolve 5 mg of the substance under examination in 4 ml of *ethanol (95 per cent)*.

Reference solution. Dissolve 5 mg of the *tyrothricin IPRS* in 4 ml of *ethanol (95 per cent)*.

Apply to the plate 1 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spots in the chromatogram obtained with the test solution correspond to that in the chromatogram obtained with reference solution.

Spray the plate with *dimethylaminobenzaldehyde solution*, heat the plate in warm air until spots appear and examine. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

The upper group corresponds to gramicidins, the lower group to tyrocidins.

B. Composition (as described under Tests).

Tests

Composition. Determine by liquid chromatography (2.4.14).

NOTE— Use freshly prepared solution and use immediately.

Test solution. Dissolve 5 mg of the substance under examination in 2 ml *methanol* and dilute to 5.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of the *tyrothricin IPRS* in 2 ml of *methanol* and dilute to 5.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 50 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 60°,
- mobile phase: a mixture of 25 volumes of *ammonium sulphate* by dissolving 0.79 g in 1000 ml and 75 volumes of *methanol*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 25 µl.

Name	Relative retention time
gramicidine C1	0.8
gramicidine C2	0.9
gramicidine A1 (retention time about 10 minutes)	1.0
gramicidine A2	1.1
tyrocidins	1.5 to 6

Inject reference solution (a). Run the chromatogram 6 times the retention time of principal peak of gramicidin A1.

Inject reference solution (b) and the test solution. The peaks to H_p and H_v valley ratio is not less than 3.0, where H_p = height

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above the baseline of the peak due to gramicidin A2 and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to gramicidin A1.

The sum of area of peaks for gramicidin other than principal peak in the chromatogram obtained with test solution is not less than 25.0 per cent and not greater than 50.0 per cent and sum of all the peaks for tyrocidins is not less than 50.0 per cent and not greater than 70.0 per cent and sum of the all peaks is not less than 85.0 per cent. Ignore any peak with the sum of the area due to gramicidins in the chromatogram obtained with reference solution (b).

Sulphated ash (2.3.18). Not more than 1.5 per cent, determined on 1.0 g.

Loss on drying (2.4.19). Not more than 4.0 per cent, determined on 1.0 g by drying in an oven at 60° under high vacuum for 3 hours.

Assay. Determine by the microbiological assay of antibiotics, Method B (2.2.10). Use *gramicidin IPRS* as reference standard and prepare a test solution of tyrothricin containing about the same amount of gramicidin as the corresponding solution of *gramicidin IPRS* i.e. 5 times more concentrated.

Storage. Store protected from light and moisture.

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U

Udenafil 3895
Ulipristal Acetate 3896
Undecenoic Acid 3897
Urea 3897
Urea Cream 3898
Urokinase 3899
Ursodeoxycholic Acid 3900
Ursodeoxycholic Acid Tablets 3902

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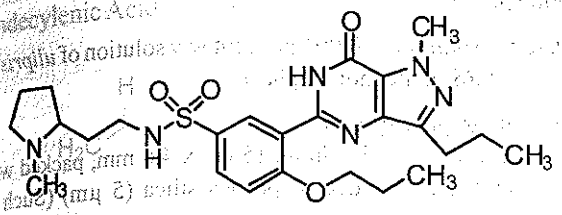
Udenafil



$C_{25}H_{36}N_6O_4S$ Mol Wt. 516.7

Udenafil is 5-{2-Propoxy-5-[*N*-2-(1-methylpyrolidin-2-yl)sulphamoyl]phenyl}-1-methyl-3-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-d]pyrimidin-7-one

Udenafil contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{25}H_{36}N_6O_4S$, calculated on the dried basis.

Category. Erectile Dysfunction.

Description. A white to off white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *udenafil IPRS* or with the reference spectrum of udenafil.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 40 volumes of mobile phase A and 60 volumes of mobile phase B.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with solvent mixture.

Reference solution. A 0.0003 per cent w/v solution of *udenafil IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. Dissolve 2.3 g of *ammonium dihyrogen orthophosphate* and 0.66 g of *diammonium hyrogen orthophosphate* in 1000 ml of *water*, add 1.0 ml of *triethylamine*, adjusted to pH 6.5 with *orthophosphoric acid*,

  B. a mixture of 85volumes of *acetonitrile* and 15volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 15 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	65	35
20	65	35
27	37	63
50	37	63
52	65	35
60	65	35

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.3 per cent and the sum of areas of all the secondary peaks is not more than 1.0 per cent, calculated by area normalization.

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 40 volumes of mobile phase A and 60 volumes of mobile phase B.

Test solution. Dissolve 50 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *udenafil IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. dissolve 2.3 g of *ammonium dihyrogen orthophosphate* and 0.66 g of *diammonium hyrogen orthophosphate* in 1000 ml of *water*, add 1.0 ml of *triethylamine*, adjusted to pH 6.5 with *orthophosphoric acid*,

  B. a mixture of 85volumes of *acetonitrile* and 15volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 295 nm,
- injection volume: 15 µl.

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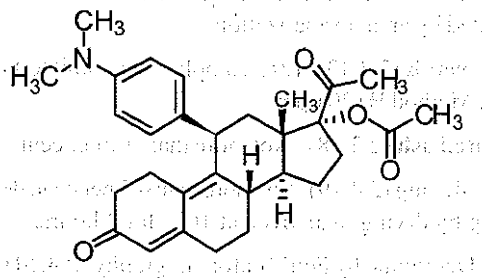
Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0.01	65	35
20	65	35
23	20	80
30	20	80
32	65	35
40	65	35

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates , the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{25}H_{36}N_6O_4S$.

Ulipristal Acetate



$C_{30}H_{37}NO_4$ Mol Wt. 475.6

Ulipristal Acetate is 17α-acetoxy-11β-(4-dimetylaminophenyl)-3,20-dioxo-19-nor-4,9-pregnadiene.

Ulipristal Acetate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{30}H_{37}NO_4$, calculated on dried basis.

Category. Selective progesterone receptor modulator (SPRM).

Description. A white to yellow powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ulipristal acetate IPRS* or with the reference spectrum of ulipristal acetate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution. A 0.001 per cent w/v solution of *ulipristal acetate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm) (Such as inertsil),
- mobile phase: a mixture of 30 volumes of *water* and 70 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 307 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the test solution. The area of any secondary peak is not more than 1.0 per cent and the sum of areas of all the secondary peaks is not more than the 2.0 per cent, calculated by area normalization.

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 60° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with mobile phase. Dilute 2.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution. A 0.002 per cent w/v solution of *ulipristal acetate IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 25°,
- mobile phase: a mixture of 30 volumes of *water* and 70 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 307 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{30}H_{37}NO_4$.

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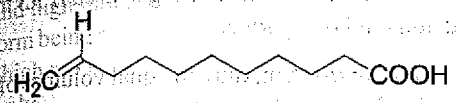
Undecenoic Acid

Undecylenic Acid



$C_{11}H_{20}O_2$ Mol. Wt. 184.3

Undecenoic Acid is 10-undecenoic acid.

Undecenoic Acid contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{11}H_{20}O_2$.

Category. Antifungal (topical).

Description. A white or very pale yellow, crystalline mass or colourless or pale yellow liquid.

Identification

A. Dissolve 0.1 g in a mixture of 2 ml of *1 M sulphuric acid* and 5 ml of *glacial acetic acid* and add dropwise 0.25 ml of *potassium permanganate solution*; the colour of the permanganate solution is discharged.

B. Boil 2 g under a reflux condenser with 2 ml of freshly distilled *aniline* for 10 minutes, allow to cool, add 30 ml of *ether* and extract with three quantities, each of 20 ml, of *2 M hydrochloric acid* and then with 20 ml of *water*. Evaporate the organic layer to dryness on a water-bath. The residue, after recrystallising twice from *ethanol (70 per cent)* and drying over *phosphorus pentoxide* at a pressure of 1.5 to 2.5 kPa for 3 hours melts at 66° to 68°.

Tests

Congealing range (2.4.10). 21° to 24°.

Refractive index (2.4.27). 1.447 to 1.450.

Peroxide value (2.3.35). Not more than 10.

Iodine value (2.3.28). 131 to 140.

Fixed and mineral oils. Boil 1.0 g with 25 ml of *water* and 5 ml of *sodium carbonate solution* for 3 minutes. The hot solution is not more opalescent than opalescence standard OS2 (2.4.1).

Water-soluble acids. Shake 2.0 g with 20 ml of warm *water*, allow to separate and filter the aqueous layer through a moistened filter paper. To 5 ml of the filtrate add 0.01 ml of *dilute phenolphthalein solution*. Not more than 0.1 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

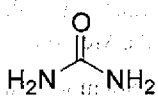
Sulphated ash (2.3.18). Not more than 0.15 per cent.

Assay. Dissolve 0.75 g in 10 ml of *ethanol (95 per cent)* and titrate with *0.5 M sodium hydroxide* using 0.1 ml of *dilute phenolphthalein solution* as indicator.

1 ml of *0.5 M sodium hydroxide* is equivalent to 0.09214 g of $C_{11}H_{20}O_2$.

Storage. Store protected from light and moisture.

Urea



CH_4N_2O Mol. Wt. 60.1

Urea is the diamide of carbonic acid.

Urea contains not less than 99.0 per cent and not more than 101.0 per cent of CH_4N_2O, calculated on the dried basis.

Category. Keratolytic.

Description. A white, crystalline powder or transparent crystals; slightly hygroscopic.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *urea IPRS* or with the reference spectrum of urea.

B. Heat 0.5 g in a test-tube; it liquefies, and ammonia is evolved which is recognised by its characteristic odour. Heat further until the liquid is turbid, cool and dissolve in 10 ml of *water*. Add 1 ml of a 10 per cent w/v solution of *sodium hydroxide* and 0.05 ml of *copper sulphate solution*; a reddish violet colour is produced.

C. Dissolve 0.1 g in 1 ml of *water* and add 1 ml of *nitric acid*; a white, crystalline precipitate is produced.

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Alkalinity. To 10 ml of a 5.0 per cent w/v solution (solution A) add 0.1 ml of *methyl red solution* and 0.4 ml of *0.01 M hydrochloric acid*; the resulting solution is red to orange.

Biuret. Not more than 0.1 per cent, determined by the following method. To 10 ml of a 20 per cent w/v solution add 5 ml of

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water, 0.5 ml of a 0.5 per cent w/v solution of *copper sulphate* and 0.5 ml of *10 M sodium hydroxide* and allow to stand for 5 minutes. Any reddish violet colour obtained is not more intense than that in a standard prepared at the same time and in the same manner using 10 ml of a 0.02 per cent w/v solution of *biuret* in place of the substance under examination.

Ethanol-insoluble matter. Not more than 0.04 per cent, determined by the following method. Dissolve 5.0 g in 50 ml of warm *ethanol (95 per cent)*, filter through a tared filter, wash the filter with 20 ml of warm *ethanol (95 per cent)* and dry at 105° for 1 hour.

Heavy metals (2.3.13). Dissolve 1.0 g in 20 ml of *water* and 5 ml of *0.1 M hydrochloric acid*. The solution complies with the limit test for heavy metals, Method A (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 1 hour.

Assay. Dissolve 0.5 g in sufficient of a 10 per cent v/v solution of *sulphuric acid* to produce 100.0 ml and mix. Place 5.0 ml of the resulting solution in a long-necked flask, add 10 ml of *sulphuric acid* and heat gently until evolution of gas ceases. Boil gently for 10 minutes, cool, cautiously add 40 ml of *water*, cool again and place in a steam-distillation apparatus. Add 50 ml of *10 M sodium hydroxide* and distil immediately by passing steam through the mixture. Continue the distillation for 1 hour, collecting the distillate in 40 ml of a 4 per cent w/v solution of *boric acid*. Titrate with *0.1 M hydrochloric acid*, using 0.25 ml of *methyl red-methylene blue solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M hydrochloric acid* is equivalent to 0.003003 g of CH_4N_2O.

Storage. Store protected from moisture.

Urea Cream

Urea Cream contains Urea in a suitable cream base.

Urea Cream contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of urea, CH_4N_2O.

Usual strength. 10 per cent w/w.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. For the first development use *2,2,4-trimethylpentane*. Dry the plate in air. For the second development use a mixture of 99 volumes of *ethanol* and 1 volume of *strong ammonia solution*.

Test solution. Disperse with heating a quantity of the cream containing 50 mg of Urea in 1 ml of *water*, cool, add 4 ml of *acetone*, mix and filter.

Reference solution (a). Dissolve 50 mg of *urea IPRS* in 1 ml of *water* and add 4 ml of *acetone*.

Reference solution (b). A mixture of equal volumes of the test solution and reference solution (a).

Apply to the plate 10 µl of each solution. After development, dry the plate in air, spray with a solution containing 0.5 per cent w/v solution of *4-dimethylaminobenzaldehyde* and 0.5 per cent v/v of *sulphuric acid* in *ethanol*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The chromatogram obtained with reference solution (b) shows a single, compact spot.

B. To a quantity containing 0.1 g of Urea add 50 ml of *water* and heat until dispersed, cool in ice and filter through glass wool. Adjusted to pH 6.0 to 7.0 using *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* as necessary. To 5 ml add 5 ml of a 0.1 per cent w/v suspension of urease-active meal and allow to stand for 30 minutes in a stoppered flask at 37°. When the resulting solution is heated in a water-bath a vapour is produced that turns moist red *litmus paper* blue.

Tests

Other tests. Complies with the tests stated under Creams.

Assay. Weigh a quantity containing 42 mg of Urea, shake with 150 ml of hot *water* for 20 minutes, allow to cool and dilute to 500.0 ml with *water*. Filter through a fine glass microfibre filter paper or by any other means, transfer 1.0 ml of the filtrate to a 100-ml volumetric flask, add 2 ml of a 0.1 per cent w/v suspension of urease-active meal, stopper the flask and allow to stand for 15 minutes at 37°. Immediately add 25 ml of a solution containing 12 g of *sodium salicylate* and 0.24 g of *sodium nitroprusside* in 200 ml and 25 ml of a solution prepared by diluting a volume of *sodium hypochlorite solution* containing 0.66 g of available chlorine with *0.2 M sodium hydroxide* to 1000 ml. Mix well, allow to stand at 37° for 5 minutes and dilute to 100.0 ml with *water*. Measure the absorbance of the resulting solution at the maximum at about 665 nm (2.4.17), using as the blank a solution prepared in the same manner but using 1.0 ml of *water* in place of 1.0 ml of the filtrate.

Calculate the content of CH_4N_2O from the absorbance obtained by using 42 mg of urea RS in place of the substance under examination.

Storage. Store in accordance with the instructions of the manufacturer.

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Urokinase

Urokinase is an enzyme, obtained from human urine that activates plasminogen. It consists of a mixture of low molecular weight and high molecular weight forms, the high molecular weight form being predominant. The molecular weights of the low and high molecular weight forms are 33,000 and 54,000 respectively.

It is prepared in conditions designed to minimise microbial and viral contamination. In particular, adequate measures, such as heating the substance in solution at 60° for 10 hours, are taken to inactivate viruses.

Urokinase contains not less than 70,000 Units of urokinase activity per mg of protein.

Category. Fibrinolytic enzyme.

Description. A white or almost white, amorphous powder.

Identification

A. Place 0.5 ml of citrated human plasma and 0.5 ml of citrated bovine plasma in two separate haemolysis tubes maintained in a water-bath at 37°. To each tube add 0.1 ml of a solution of the substance under examination containing 1000 Units per ml in *phosphate buffer pH 7.4* and 0.1 ml of a solution of thrombin containing 20 Units per ml in *phosphate buffer pH 7.4* and shake immediately; in both tubes, a clot forms and lyses within 30 minutes.

B. Carry out a suitable immunodiffusion test.

Tests

Appearance of solution. A 0.1 per cent w/v solution in *water* is clear (2.4.1), and colourless (2.4.1).

Molecular fractions. Determine by size-exclusion chromatography (2.4.16).

Test solution. Dissolve 0.1 g of the substance under examination in 100.0 ml of *0.02 M phosphate buffer pH 8.0*.

Chromatographic system

- a column 90 cm × 16 mm, packed with a cross-linked dextran suitable for fractionation of proteins in the range of molecular weight from 4000 to 150,000 (Such as Sephadex G-100) at 5°,
- mobile phase: 1.75 per cent w/v solution of *sodium chloride* in *0.02 M phosphate buffer pH 8.0*,
- flow rate: 6 ml per hour,
- spectrophotometer set at 280 nm.

Apply 1 ml of the test solution to the column, rinse twice with 0.5 ml portions of the buffer and then carry out the elution. The eluate may be collected in 1 ml fractions. Plot the individual absorbances on a graph. Draw perpendicular lines towards the axis of the abscissae from the minima before the high molecular weight peak, between the high and the low molecular weight peaks, and after the low molecular weight peak. Combine separately the high and low molecular weight fractions and for each combined fraction determine the activity by the method described under Assay. The ratio of the activity in the combined high molecular weight fraction to that in the combined low molecular weight fraction is not less than 2.0.

Total protein. Determine by Method C for the determination of nitrogen (2.3.30), using 10 mg of the substance under examination and multiplying the result by 6.25 to obtain the content of protein.

Hepatitis-B surface antigen. Examine by a suitably sensitive method such as radio-immunoassay; hepatitis-B surface antigen is not detected.

Abnormal toxicity (2.2.1). Complies with the test, using a solution containing 2,500 Units in 0.5 ml of *saline solution*.

Thromboplastic contaminants. Dissolve suitable quantities of the substance under examination in *barbitone buffer pH 7.4* to obtain solutions containing 5000, 2500, 1250, 625 and 312 Units per ml. Into each of six haemolysis tubes, 1 cm in internal diameter, place 0.1 ml of citrated rabbit plasma. Add 0.1 ml of one of each of the solutions of the substance under examination to each of five of the tubes and 0.1 ml of *barbitone buffer pH 7.4* to the sixth (control). Incubate the six tubes at 25° ± 0.5° for 5 minutes and then add 0.1 ml of a 0.3675 per cent w/v solution of *calcium chloride*. Using a stop-watch, measure the clotting time for each solution and the control. Plot the shortening of the recalcification time (control clotting time minus clotting time for each solution) against log concentration. Extrapolate the best-fitting straight line through the five points until it reaches the log concentration axis. The urokinase activity at the intersection point represents the limit concentration for clotting activity (zero clotting activity). The zero clotting activity is not less than 150 Units per ml.

Vasoactive substances. Anesthetise a rabbit by intraperitoneal injection of 0.15 g of *phenobarbitone sodium* per kg of body weight. Dissolve in *normal saline solution* a sufficient quantity of the substance under examination to give a solution containing 40,000 Units per ml. Administer by intravenous infusion at a rate of 1 ml per minute a sufficient volume of the solution of the substance under examination such that the dose is 20,000 Units per kg of body weight. Measure the arterial pressure and heart rate at intervals of 15 minutes for 5 hours after the infusion. No significant and lasting alterations in arterial pressure or heart rate are produced, except those arising from the effects of the anaesthetic.

Assay. The potency of urokinase is determined by comparing its ability to activate human plasminogen to form plasmin with that of the Standard Preparation. The plasmin generated is

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determined by measurement of the time taken to lyse a fibrin clot under the conditions of a suitable method of Assay.

Standard Preparation

The Standard Preparation is the 1st International Reference Preparation for Urokinase, human, established in 1968, consisting of partially purified freeze-dried urokinase from human urine with lactose (supplied in ampoules containing 4800 Units of urokinase activity) or another suitable preparation the activity of which has been determined in relation to the International Reference Preparation.

Method

Unless otherwise prescribed, use *phosphate buffer pH 7.4* containing 3.0 per cent w/v solution of *bovine albumin* for the preparation of solutions and dilutions.

Prepare a solution of the Standard Preparation containing 1000 Units of urokinase activity per ml and prepare a solution of the preparation under examination expected to have the same concentration; keep the solutions in ice and use within 6 hours. Prepare three 1.5-fold serial dilutions of the solution of the Standard Preparation so that the longest clot-lysis time is less than 20 minutes and the shortest clot-lysis time is greater than 3 minutes. Prepare three similar dilutions of the solution of the preparation under examination. Keep the solutions in ice and use within 1 hour. Using 24 tubes 8 mm in diameter, label the tubes S_1, S_2, S_3 for the dilutions of the Standard Preparation and T_1, T_2, T_3 for the dilutions of the preparation under examination, allocating four tubes to each dilution. Place the tubes in ice. Into each tube introduce 0.2 ml of the appropriate dilution, 0.2 ml of *phosphate buffer pH 7.4* containing 3 per cent w/v solution of *bovine albumin* and 0.1 ml of a solution containing 20 Units of *thrombin* per ml. Place the tubes in a water-bath at 37° and allow to stand for 2 minutes to attain temperature equilibrium. Using an automatic pipette, introduce into the bottom of the first tube 0.5 ml of a 1.0 per cent w/v solution of *bovine euglobulin fraction* ensuring mixing. At 5-second intervals introduce successively into the remaining tubes 0.5 ml of a 1.0 per cent w/v solution of *bovine euglobulin fraction*. Using a stop-watch, measure for each tube the time in seconds that elapses between the addition of the euglobulin and the lysis of the clot.

Using the logarithms of the lysis times, calculate the result of the assay by standard statistical methods.

The estimated potency is not less than 90.0 per cent and not more than 111.0 per cent of the stated potency.

The fiducial limits of error are not less than 80.0 per cent and not more than 125.0 per cent of the stated potency.

Urokinase intended for use in the manufacture of parenteral preparations or ophthalmic preparations complies with the following additional requirements.

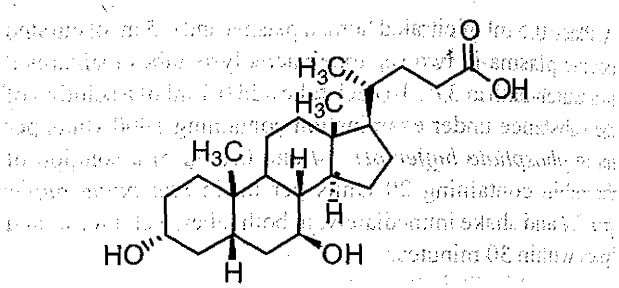
Bacterial endotoxins (2.2.3). Not more than 0.002 Endotoxin Unit per Unit of urokinase activity.

Sterility (2.2.11). Complies with the test for sterility, if the urokinase intended for use in the manufacture of parenteral preparations without a further appropriate sterilisation procedure.

Storage. Store protected from light in a refrigerator (2° to 8°). The containers should be sterile, tamper-evident and sealed so as to exclude microorganisms.

Labelling. The label states (1) the number of Units of urokinase activity in the container; (2) the number of Units of urokinase activity per mg of protein; (3) the storage conditions; (4) whether or not it is intended for use in the manufacture of parenteral preparations or ophthalmic preparations.

Ursodeoxycholic Acid



$C_{24}H_{40}O_4$ Mol. Wt. 392.6

Ursodeoxycholic Acid is 3α,7β-dihydroxy-5β-cholan-24-oic acid.

Ursodexoxycholic acid contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{24}H_{40}O_4$, calculated on the dried basis.

Category. Treatment of gallstones.

Description. A white or almost white powder.

Identification

Tests B and C may be omitted if test A is carried out. Test A may be omitted if tests B and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ursodeoxycholic acid IPRS* or with the reference spectrum of ursodeoxycholic acid.

B. In the test for Impurity C, the principal spot in the chromatogram obtained with test solution (b) corresponds to the principal spot in the chromatogram obtained with reference solution (a).

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C. Dissolve 10 mg in 1 ml of *sulphuric acid*. Add 0.1 ml of *formaldehyde solution* and allow to stand for 5 minutes, add 5 ml of *water*; the suspension obtained is greenish-blue.

Tests

Specific optical rotation (2.4.22). +58.0° to +62.0°, determined on 2.0 per cent w/v solution in *ethanol*.

Impurity C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel*.

Solvent mixture. 10 volumes of *water* and 90 volumes of *acetone*.

Mobile phase. A mixture of 1 volume of *glacial acetic acid*, 30 volumes of *acetone* and 60 volumes of *dichloromethane*.

Test solution (a). Dissolve 0.4 g of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10.0 ml with the solvent mixture.

Reference solution (a). A 0.4 per cent w/v solution of *ursodeoxycholic acid IPRS* in the solvent mixture.

Reference solution (b). A 0.2 per cent w/v solution of *lithocholic acid IPRS (ursodeoxycholic acid impurity C IPRS)* in the solvent mixture (Solution A). Dilute 2.0 ml of the solution to 100.0 ml with the solvent mixture.

Reference solution (c). To 5.0 ml of solution A, add 10 mg of *chenodeoxycholic acid IPRS (ursodeoxycholic acid impurity A IPRS)* and dilute to 50.0 ml with the solvent mixture.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate at 120° for 10 minutes and spray immediately with a 4.76 per cent w/v solution of *phosphomolybdic acid* in a mixture of 1 volume of *sulphuric acid* and 20 volumes of *glacial acetic acid* and heat at 120° until blue spots appear on a lighter background. In the chromatogram obtained with reference solution (c), the chromatogram shows 2 clearly separated spots. In the chromatogram obtained with test solution (a), any spot due to ursodeoxycholic acid impurity C is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 10 volumes of *methanol* and 90 volumes of the mobile phase.

Test solution. Dissolve 60 mg of the substance under examination in the solvent mixture and dilute to 20.0 ml with the solvent mixture.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 30 volumes of *acetonitrile*, 37 volumes of a 0.078 per cent w/v solution of *sodium dihydrogen phosphate*, adjusted to pH 3.0 with *orthophosphoric acid* and 40 volumes of *methanol*,
- flow rate: 0.8 ml per minute,
- refractometer detector at 35°,
- injection volume: 150 µl.

Name	Relative retention time
Ursodeoxycholic acid impurity H[1]	0.9
Ursodeoxycholic acid (Retention time: about 14 minutes)	1.0
Ursodeoxycholic acid impurity A[2]	2.8

[1] 3α,7α-dihydroxy-5α-cholan-24-oic acid,

[2] chenodeoxycholic acid.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. Run the chromatogram 4 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to ursodeoxycholic acid impurity A is not more than 10 times the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 15 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1 g by drying in an oven at 105°.

Assay. Dissolve 0.35 g in 50 ml of *ethanol (95 per cent)*, previously neutralised to 0.2 ml of *phenolphthalein solution*.

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Add 50 ml of *water* and titrate with *0.1 M sodium hydroxide* until a pink colour is obtained.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.03926 g of $C_{24}H_{40}O_4$.

Ursodeoxycholic Acid Tablets

Ursodeoxycholic Acid Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of ursodeoxycholic acid, $C_{24}H_{40}O_4$.

Usual strengths. 150 mg; 300 mg and 600 mg.

Identification

A. Extract a quantity of the powdered tablets containing 0.1 g of Ursodeoxycholic Acid with 10 ml of *ethanol* (*95 per cent*), centrifuge and evaporate the supernatant liquid to dryness in a stream of nitrogen in a water-bath at room temperature. Dry the residue at room temperature at a pressure of 0.7 kPa for 2 hours. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ursodeoxycholic acid IPRS* or with the reference spectrum of ursodeoxycholic acid.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of phosphate buffer prepared by using 0.68 per cent w/v *potassium dihydrogen orthophosphate*, adjusted to pH 8.0 using *2 M sodium hydroxide*,

Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve 17 mg of *ursodeoxycholic acid IPRS* in 1 ml of *ethanol* (*95 per cent*) and dilute to 100.0 ml with the dissolution medium.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 280 volumes of *acetonitrile* and 720 volumes of a 0.05 M phosphate buffer prepared by dissolving 6.81 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 6.5 with *2 M sodium hydroxide*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 100 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_{24}H_{40}O_4$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{24}H_{40}O_4$.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel*.

Mobile phase A. A mixture of 5 volumes of *acetic acid*, 5 volumes of *methanol* and 90 volumes of *chloroform*.

Mobile phase B. A mixture of 1.25 volumes of *acetic acid*, 50 volumes of *ethyl acetate* and 50 volumes of *2,2,4-trimethylpentane*.

Test solution. Disperse a quantity of the powdered tablets containing 0.1 g of Ursodeoxycholic Acid with 10 ml of *ethanol* (*95 per cent*) with the aid of ultrasound for 15 minutes, centrifuge.

Reference solution (a). A solution containing 0.01 per cent w/v of *lithocholic acid IPRS*, 0.05 per cent w/v of *cholic acid IPRS* and 0.15 per cent w/v of *chenodeoxycholic acid IPRS* in *methanol*. Dilute 1.0 ml of the solution to 10.0 ml with *acetone*.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with *ethanol* (*95 per cent*).

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 10 cm with mobile phase A, dry in air and then develop the plate to 8 cm in the same direction with mobile phase B. After removal of the plate, heat at 110° to 120° for 30 minutes. Spray immediately with a solution containing 5.0 per cent w/v of *phosphomolybdic acid* in a mixture of 5 ml of *sulphuric acid* and 95 ml of *glacial acetic acid*. Heat the plate for a further 5 to 10 minutes at 110° to 120° until dark blue spots appear on a white or pale blue background. In the chromatogram obtained with the test solution, the area of any spot corresponding to lithocholic acid is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.1 per cent), any spot corresponding to cholic acid is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.5 per cent). Any spot corresponding to chenodeoxycholic acid is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (1.5 per cent). Any other secondary spot is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Other tests. Comply with the tests stated under tablets.

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Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. A solution containing 535 ml of *water*, 6.0 g of *sodium dihydrogen orthophosphate dihydrate*, 2.0 g of *disodium hydrogen orthophosphate dihydrate*, 65 ml of *tetrabutylammonium hydroxide solution* and 400 ml of *acetonitrile*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.3 g of Ursodeoxycholic Acid in 20 ml of the solvent mixture with the aid of ultrasound for 15 minutes and filter.

Reference solution. A 1.5 per cent w/v solution of *ursodeoxycholic acid IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: a mixture of 500 volumes of *acetonitrile* and 500 volumes of a solution containing 1.2 per cent w/v of *sodium dihydrogen orthophosphate dihydrate*, 0.4 per cent w/v of *disodium hydrogen orthophosphate dihydrate* and 1.08 per cent w/v of *tetrabutylammonium hydrogen sulphate* in *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of $C_{24}H_{40}O_4$ in the tablets.

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V

Valacyclovir Hydrochloride 3907
Valacyclovir Tablets 3909
Valganciclovir Hydrochloride 3910
Valganciclovir Tablets 3913
Valproic Acid 3915
Valproic Acid Capsules 3916
Valproic Acid Oral Solution 3916
Valsartan 3917
Valsartan Tablets 3918
Valsartan and Hydrochlorothiazide Tablets 3919
Vancomycin Hydrochloride 3921
Vancomycin Capsules 3922
Vancomycin Intravenous Infusion 3923
Vancomycin Oral Solution 3924
Vanillin 3925
Vasopressin 3926
Vasopressin Injection 3927
Vecuronium Bromide 3928
Vecuronium Bromide Injection 3929
Venlafaxine Hydrochloride 3930
Venlafaxine Prolonged-release Capsules 3931
Venlafaxine Prolonged-release Tablets 3932
Venlafaxine Tablets 3933
Verapamil Hydrochloride 3935
Verapamil Injection 3936
Verapamil Hydrochloride Prolonged-release Tablets 3937
Verapamil Tablets 3938
Vildagliptin 3939
Vildagliptin Tablets 3941
Vildagliptin and Metformin Tablets 3942

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Vinblastine Sulphate 3945
Vinblastine Injection 3946
Vincristine Sulphate 3947
Vincristine Injection 3948
Vinorelbine Tartrate 3950
Vinorelbine Injection 3951
Voglibose 3952
Voglibose Dispersible Tablets 3953
Voglibose Tablets 3954
Voriconazole 3954
Voriconazole Injection 3956
Voriconazole Tablets 3958

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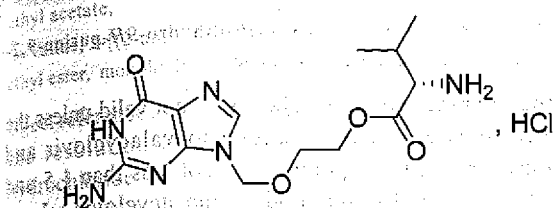
Valacyclovir Hydrochloride



$C_{13}H_{20}N_6O_4,HCl$ Mol. Wt. 360.8

Valacyclovir Hydrochloride is *L*-Valine, 2-[(2-amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl)methoxy] ethyl ester, monohydrochloride.

Valacyclovir Hydrochloride contains not less than 95.0 per cent and not more than 102.0 per cent of $C_{13}H_{20}N_6O_4.HCl$, calculated on the anhydrous basis.

Category. Antiviral

Description. A white to off-white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *valacyclovir hydrochloride IPRS* or with the reference spectrum of valacyclovir hydrochloride.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

C. A 5 per cent w/v solution gives the reaction (A) of chlorides (2.3.1).

Tests

Related substances.

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254* (prewash the plate with *methanol*).

Mobile phase. A mixture of 54 volumes of *methylene chloride*, 34 volumes of *methanol*, 12 volumes of *tetrahydrofuran* and 3 volumes of *ammonia solution*.

Test solution. Dissolve 0.25 g of the substance under examination in 2 ml of *water*, with the aid of ultrasound for 20 minutes and dilute to 5.0 ml with *ethanol* and filter.

Reference solution (a). Dissolve 5 mg, each of, *valacyclovir related compound D IPRS* and *valacyclovir related compound G IPRS*, 10 mg, each of, *valacyclovir related compound E IPRS* and *valacyclovir related compound F IPRS* in 2 ml of *water* and 6 ml of *ethanol*, with the aid of ultrasound for 20 minutes and dilute to 10.0 ml with *ethanol*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with *ethanol*.

Reference solution (c). Dilute 0.5 ml of reference solution (a) to 10.0 ml with *ethanol*.

Apply to the plate 4 µl of the test solution and reference solution (b) and (c). Allow the mobile phase to rise 10 cm. Dry the plate in air. Examine the plate under ultraviolet light at 254 nm and visually estimate the valacyclovir related compounds E and G in the sample using the appropriate reference solution spots. The chromatograms obtained with the reference solution (b) and (c) each show three clearly separated spots due to valacyclovir related compound D, E and G. Spray the plate with 0.01 per cent w/v of *fluorescamine* in *ethylene dichloride* and examine the plate under ultraviolet light at 365 nm to estimate the level of valacyclovir related compound F in the sample using the appropriate reference solution spot.

Name	Relative R_f value
Valacyclovir hydrochloride	1
Valacyclovir related compound D[1*]	1.1
Valacyclovir related compound E[2]	1.3
Valacyclovir related compound F[3]	1.8
Valacyclovir related compound G[4]	1.9

*This impurity is controlled in Method B of Related substances.

[1]2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-ethyl-*L*-valinate,

[2]2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-[(benzyloxy)carbonyl]-*L*-valinate,

[3]2-hydroxyethyl-*L*-valinate,

[4]*N*,*N*-dimethylpyridin-4-amine.

In the chromatogram obtained with the test solution, any spot corresponding to valacyclovir related compound E is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (0.2 per cent), any spot corresponding to valacyclovir related compound F is not more intense than the corresponding spot in the chromatogram obtained with reference solution (c) (0.1 per cent) and any spot corresponding to valacyclovir related compound G is not more intense than the corresponding spot in the chromatogram obtained with reference solution (c) (0.05 per cent).

B. Determine by liquid chromatography (2.4.14).

Solvent mixture. 20 volumes of *ethanol* and 80 volumes of *water*.

Test solution. Dissolve 40 mg of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

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Reference solution. A solution containing 0.04 per cent w/v of *valacyclovir hydrochloride IPRS*, 0.00008 per cent w/v of *valacyclovir related compound C IPRS* and 0.00016 per cent w/v of *acyclovir related compound A IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with phenyl group bonded to porous silica (5µm),
- column temperature: 15°,
- mobile phase: A. a 0.3 per cent w/w solution of *trifluoroacetic acid* in *water*,
  B. a 0.3 per cent w/w solution of *trifluoroacetic acid* in *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
5	90	10
35	60	40
35.1	90	10
45	90	10

Name	Relative retention time
Guanine[1,2]	0.31
Acyclovir[1,3]	0.42
Acyclovir alaninate[4]	0.54
Valacyclovir	1.00
Valacyclovir related compound C[5]	1.06
Acyclovir related compound A[1,6]	1.09
Valacyclovir related compound D[7]	1.17
Acyclovir isoleucinate[8]	1.30
N-formyl valacyclovir[9]	1.61
Guaninyl valacyclovir[10]	1.66
Bis valacyclovir[11]	2.00

[1]This impurity is controlled in Method C of Related substances,

[2]2-amino-1*H*-purin-6(9*H*)-one (guanine),

[3]9-[(2-hydroxyethoxy)methyl]guanine (acyclovir),

[4]9-[(2-hydroxyethoxy)methyl]guanine *L*-alaninate,

[5]2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-methyl-*L*-valinate,

[6]2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl acetate,

[7]2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-ethyl-*L*-valinate,

[8]9-[(2-hydroxyethoxy)methyl]guanine *L*-isoleucinate,

[9]9-[(2-hydroxyethoxy)methyl]guanine *N*-formyl-*L*-valinate,

[10][*N*²-(guanine-*N*²-yl)methyl]-9-[(2-hydroxyethoxy)methyl]guanine *L*-valinate,

[11]2,2'[Methylenebis[imino(6-oxo-1,6-dihydro-9*H*-purine-9,2-diyl)methylene-oxy]]diethyl di(*L*-valinate).

Inject the reference solution. The test is not valid unless the resolution between the peaks due to valacyclovir and valacyclovir related compound C is not less than 1.5 and valacyclovir related compound C and acyclovir related compound A is not less than 1.5 and the tailing factor is not more than 1.5 for valacyclovir peak.

Inject the test solution. The area of any peak corresponding to acyclovir alaninate, acyclovir isoleucinate and guaninyl valacyclovir, each of, is not more than 0.2 per cent, the area of any peak corresponding to valacyclovir related compound C, bis valacyclovir, each of is not more than 0.3 per cent, the area of any peak corresponding to valacyclovir related compound D is not more than 0.5 per cent, the area of any peak corresponding to N-formyl valacyclovir is not more than 0.8 per cent and the area of any other secondary peak is not more than 0.1 per cent, calculated by area normalization.

C. Determine by liquid chromatography (2.4.14),

Test solution. Dissolve 50 mg of the substance under examination in *0.05 M hydrochloric acid* and dilute to 100.0 ml with the *0.05 M hydrochloric acid*.

Reference solution (a). A 0.05 per cent w/v solution of *valacyclovir hydrochloride IPRS* in *0.05 M hydrochloric acid*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *0.05 M hydrochloric acid*.

NOTE — Valacyclovir hydrochloride IPRS contains a detectable quantity of D-valacyclovir.

Chromatographic system

- a stainless steel column 15 cm x 4.0 mm, a crown ether coated on silica gel substrate. The active site is (S)—18-crown-6-ether (5 µm) (Such as Crown Pak CR (+)),
- column temperature: 10°,
- mobile phase: a mixture 19 volumes of *water*, 1 volume of *methanol* and 0.1 volume of *perchloric acid*,
- flow rate: 0.75 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Name	Relative retention time	Correction factor
Guanine and acyclovir[1,2]	0.18	0.66
Acyclovir related compound A[3]	0.42	0.89
D-Valacyclovir[4]	0.55	—
Valacyclovir	1.0	—

[1]2-amino-1*H*-purin-6(9*H*)-one (guanine),

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9-[(2-hydroxyethoxy)methyl]guanine (acyclovir),
2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl acetate,
D-Valine, 2-[(2-amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl) methoxy]ethyl ester, monohydrochloride.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to valacyclovir hydrochloride and *D*-valacyclovir is not less than 2.0 in the chromatogram obtained with reference solution (a) and the relative standard deviation for replicate injections is not more than 5.0 per cent, in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to guanine and acyclovir is not more than twice the area of principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent), the area of any peak corresponding to acyclovir related compound A is not more than 0.2 times the area of principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), the area of any peak due to *D*-valacyclovir is not more than 3 times the area of the principal peak in the chromatogram obtained with the reference solution (b) (3.0 per cent).

The sum of all the impurities from related substance test A, B and C is not more than 5.0 per cent.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 2.0 per cent (for the anhydrous form) and 5.0 to 11.0 per cent (for the hydrous form).

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances C.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to valacyclovir hydrochloride and *D*-valacyclovir is not less than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{13}H_{20}N_6O_4$,HCl.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Valacyclovir Tablets

Valacyclovir Hydrochloride Tablets

Valacyclovir Tablets contain valacyclovir hydrochloride equivalent to not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of valacyclovir, $C_{13}H_{20}N_6O_4$.

Usual strengths. 500 mg; 1000 mg.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

B. Disperse a quantity of powdered tablets containing 0.1 g of Valacyclovir Hydrochloride in 10 ml of *water*. It gives reaction (A) of chlorides (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with 0.1 per cent v/v solution of *orthophosphoric acid*.

Reference solution. Dissolve a quantity of *valacyclovir hydrochloride IPRS* in 0.1 per cent v/v solution of *orthophosphoric acid* and dilute with the same medium to obtain a solution of known concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 5.0 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 95 volumes of 0.1 per cent v/v solution of *orthophosphoric acid* and 5 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

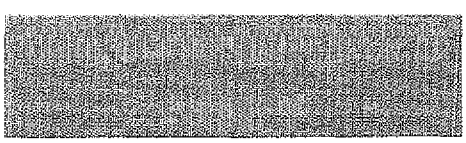
Calculate the content of $C_{13}H_{20}N_6O_4$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{13}H_{20}N_6O_4$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 0.1 per cent v/v solution of *orthophosphoric acid* in *water*.

Test solution. Disperse a quantity of powdered tablets containing 0.25 g of Valacyclovir Hydrochloride in 80 ml of *0.1 M hydrochloric acid*, with the aid of ultrasound with intermittent shaking and dilute to 100.0 ml with *0.1 M*



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hydrochloric acid. Dilute 1.0 ml of the solution to 25.0 ml with the solvent mixture and filter.

Reference solution (a). A 0.01 per cent w/v solution of *valacyclovir hydrochloride IPRS* in the solvent mixture.

NOTE — Valacyclovir hydrochloride IPRS contains a detectable amount of D-valacyclovir.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.0 mm, a crown ether coated silica gel substrate. The active site is (S)-=18-crown-6-ether,(5µm) (Such as Crownpak CR(+)),
- column temperature: 10°,
- mobile phase: a mixture of 5 volumes of *methanol* and 95 volumes of the solvent mixture,
- flow rate: 0.75 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Name	Relative retention time	Correction factor
Acyclovir[1]	0.56	0.71
D-Valacyclovir[2*]	0.82	—
Valacyclovir	1.00	—

*Process impurity included for identification only. To be controlled in drug substance.

[1]2-Amino-9-[(2-hydroxyethoxy)methyl]-1,9-dihydro-6*H*-purin-6-one.

[2]*D*-Valine, 2-[(2-amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl)methoxy] ethyl ester, monohydrochloride.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peak due to *D*-valacyclovir and valacyclovir is not less than 1.3, the tailing factor is not more than 2.0 for valacyclovir peak in the chromatogram obtained with reference solution (a), and the relative standard deviation for replicate injection is not more than 5.0 per cent in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to acyclovir is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14). as described under Related substances using following modifications.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to *D*-valacyclovir and valacyclovir is not less than 1.3, the tailing factor is not more than 2.0 for the valacyclovir peak and the relative standard deviation for replicate injection is not more than 2.0 per cent.

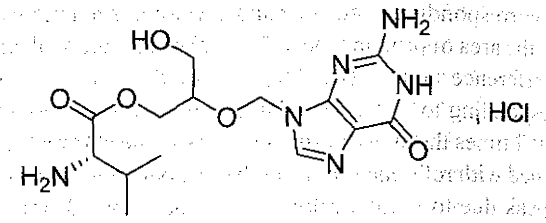
Inject reference solution (a) and the test solution.

Calculate the content of $C_{13}H_{20}N_6O_4$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of valacyclovir.

Valganciclovir Hydrochloride



$C_{14}H_{22}N_6O_5,HCl$ Mol. Wt. 390.8

Valganciclovir Hydrochloride is *L*-Valine, ester with 9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]guanine, monohydrochloride.

Valganciclovir Hydrochloride contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{14}H_{22}N_6O_5,HCl$, calculated on the anhydrous and solvent free basis.

Category. Antiviral

Description. A white to off-white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *valganciclovir hydrochloride IPRS* or with the reference spectrum of valganciclovir hydrochloride.

B. When examined in the range of 200 nm to 400 nm (2.4.7), a 0.001 per cent w/v solution in *methanol*, shows an absorption maxima at the same wavelength as obtained with the same concentration of the reference solution.

C. A 5.0 per cent w/v solution gives reaction (A) of chlorides (2.3.1).

Tests

Limit of isopropyl alcohol. Not more than 1.0 per cent.

Determine by gas chromatography (2.4.13).

Internal standard solution. A 0.1 per cent v/v solution of *1,4-dioxane* in *dimethylformamide*.

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Test solution. Dissolve 100 mg of the substance under examination in 2.0 ml of internal standard solution.

Reference solution (a). Dilute 1.0 ml of *isopropyl alcohol* and 0.1 ml of *toluene* to 100.0 ml with *dimethylformamide.*

Reference solution (b). Dilute 0.1 ml of reference solution (a) to 2.0 ml with the internal standard solution.

Chromatographic system

- a capillary column 30 m x 0.53 mm, coated with 6 per cent cyanopropylphenyl- 94 per cent dimethylpolysiloxane (film thickness 3.0 µm) (Such as DB-624),
- temperature:
  - column. 40° for 10 minutes, 40° to 240° @ 25° per minute and hold at 240° for 15 minutes,
  - inlet port at 250° and detector at 300°,
- flame ionization detector,
- split ratio :1:15,
- flow rate: 10.5 ml per minute, using nitrogen as the carrier gas.
- injection volume: 0.5 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to 1,4-dioxane and toluene is not less than 8.0, the column efficiency is not less than 6000 theoretical plates for 1,4-dioxane peak and the relative standard deviation of peak area ratios of isopropyl alcohol to the 1,4-dioxane for replicate injections is not more than 15.0 per cent.

Inject reference solution (b) and the test solution.

Calculate the content of isopropyl alcohol using ratio of peak area of isopropyl alcohol to that of peak area of 1,4-dioxane.

Related substances.

A. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in 100.0 ml of *0.001 M hydrochloric acid.*

Reference solution. A solution containing 0.02 per cent w/v of *valganciclovir hydrochloride IPRS* and 0.001 per cent w/v of *methoxymethylguanine IPRS* in *0.001 M hydrochloric acid.*

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm) (Such as zorbax SB C18),
- mobile phase: A. a buffer solution prepared by dissolving 11.5 g of *monobasic ammonium phosphate* in 900 ml of *water,* adjusted to pH 2.8 with *orthophosphoric acid* and dilute to 1000 ml with *water,*
  B. *methanol,*
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	92	8
5	92	8
15	80	20
30	30	70
30.1	92	8
45	92	8

Name	Relative retention time	Correction factor
Guanine*	0.28	0.53
Ganciclovir*	0.42	0.71
Methoxymethylguanine*	0.81	—
Valganciclovir	1.00	—
Isovalganciclovir*#	1.26	—
Monoacetoxyganciclovir*	1.36	0.77
Homologue of valganciclovir (H)	1.47	0.77
Homologue of valganciclovir (I)	1.52	0.71
Bis-valine ester of ganciclovir**	1.61	1.41
Homologue of valganciclovir(G)*#	1.66	—
Genciclovir monopropionate**	2.09	0.91
Valganciclovir dimer* (stereoisomer A)	2.49	—
Valganciclovir dimer* (stereisomer B)	2.52	—
Valganciclovir dimer* (stereisomer C)	2.54	—

*Specified impurity.

**Other identified impyrity.

*# Reported as the sum of diasteromers.

NOTE — The retention time for the second peak of valganciclovir is between 5 and 8.5 minutes.

Inject the reference solution. The test is not valid unless the resolution between the first peak of valganciclovir and methoxymethylguanine peak is not less than 1.0 and the resolution between the two peaks of valganciclovir (R and S esters of L-valine) is not less than 3.0, the column efficiency is not less than 8000 theoretical plates and the tailing factor is not more than 1.4 for the second peak of valganciclovir.

Inject the test solution. The area of any peak corresponding to bis-valine ester of ganciclovir, homologue of valganciclovir (H), homologue of valganciclovir (I), valganciclovir dimer (stereisomer A), valganciclovir dimer (stereisomer B) and valganciclovir dimer (stereisomer C), each of, is not more than 0.1 per cent, the area of any peak corresponding to

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monoacetoxyganciclovir and ganciclovir monopropionate, each of, is not more than 0.15 per cent, the area of any peak corresponding to guanine and homologue of valganciclovir (G), each of, is not more than 0.25 per cent, the area of any peak corresponding to methoxymethylguanine is not more than 0.3 per cent, the area of any peak corresponding to isovalganciclovir is not more than 0.5 per cent, the area of any peak corresponding to ganciclovir is not more than 1.5 per cent and the sum of the areas of peaks corresponding to bis valine ester of ganciclovir and ganciclovir mono propionate is not more than 0.25 per cent, the area of any other secondary peak is not more than 0.1 per cent and the sum of areas of all other secondary peaks is not more than 0.25 per cent, calculated by area normalization.

Diastereomer ratio. Using the chromatogram obtained with the test solution under Related substances A. Calculate the percentage of valganciclovir (*R* and *S* esters of *L*-valine) by the following expression.

$$100 \times \left(\frac{A_1}{A_1 + A_2} \right)$$

$$100 \times \left(\frac{A_2}{A_1 + A_2} \right)$$

where, A_1 and A_2 are the peak areas of the valganciclovir (*R* and *S* esters of *L*-valine). The diastereomer ratio is (45:55) to (55:45).

B. Determine by liquid chromatography (2.4.14), as described under Related substances test A with the following modifications.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with phenyl groups bonded to porous silica (3.5 µm) (Such as zorbax SB phenyl),
- mobile phase: A. a buffer solution prepared by diluting 2.5 ml of *triethylamine* to 1000 ml *water*, adjusted to pH 3.0 with *trifluoroacetic acid*,
  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	93	7
10	93	7
20	70	30
21	93	7
35	93	7

Name	Relative retention time
Valganciclovir	1.00
Ganciclovir mono-*N*-methyl valinate	1.2

NOTE — The retention time for the second peak of valganciclovir is between 6 and 9 minutes.

Inject the reference solution. The test is not valid unless the resolution between the two peaks of valganciclovir (*R* and *S* esters of *L*-valine) is not less than 1.3, the column efficiency is not less than 8000 theoretical plates and the tailing factor is not more than 1.2 for the second peak of valganciclovir.

Inject the test solution. The area of any peak corresponding to ganciclovir mono-*N*-methyl valinate (sum of distereomers) is not more than 0.3 per cent, the area of any other secondary peak is not more than 0.1 per cent and the sum of areas of all other secondary peaks is not more than 0.25 per cent, calculated by area normalization.

The sum of all the impurities calculated under method A and B is not more than 3.0 per cent.

Enantiomeric purity. Not less than 97.0 per cent.

Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 10 mg of the substance under examination in *0.001 M hydrochloric acid* and dilute to 50.0 ml with *0.001 M hydrochloric acid*.

Reference solution. A solution containing 0.02 per cent w/v of *valganciclovir hydrochloride IPRS* and 0.002 per cent w/v of *D-valganciclovir IPRS* in *0.001 M hydrochloric acid*.

Chromatographic system

- a stainless steel column 15 cm x 4.0 mm, packed with crown ether coated bonded to porous silica (5 µm) (Such as crownpack CR (+),
- mobile phase: a 1.62 per cent w/v solution of *perchloric acid* in *water*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to second peak of *D*-valine ester and first peak of valganciclovir is not less than 3.5, the column efficiency is not less than 1800 theoretical plates for the second peak of valganciclovir.

Inject the test solution and calculate the enantiomeric purity by the following expression.

$$100 \times \left(\frac{A}{A + B} \right)$$

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Where, A is the sum of the peak area of valganciclovir (*R* and *S* esters of L-valine) and B is the sum of the peak area of enantiomeric impurities (*R* and *S* esters of D-valine).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 8.0 per cent, determined on 0.1 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 20 mg of the substance under examination in *0.001 M hydrochloric acid* and dilute to 100.0 ml with *0.001 M hydrochloric acid*.

Reference solution (a). A 0.02 per cent w/v solution of *valganciclovir hydrochloride IPRS* in *0.001 M hydrochloric acid*.

Reference solution (b). A solution containing 0.02 per cent w/v of *valganciclovir hydrochloride IPRS* and 0.001 per cent w/v of *methoxymethylguanine IPRS* in *0.001 M hydrochloric acid*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm) (Such as Zorbax SB C-18),
- mobile phase: a mixture of 92 volumes of a buffer solution prepared by dissolving 11.5 g of *monobasic ammonium phosphate* in 900 ml of *water*, adjusted to pH 2.8 with *orthophosphoric acid* and dilute to 1000 ml with *water* and 8 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the first peak of valganciclovir and methoxymethylguanine peak is not less than 1.0 and the resolution between the two peaks of valganciclovir (*R* and *S* esters of L-valine) is not less than 3.0, the column efficiency is not less than 8000 theoretical plates for the second peak of valganciclovir and the tailing factor is not more than 1.4 for the second peak of valganciclovir in the chromatogram obtained with reference solution (b). The relative standard deviation of the correction factor (C) for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (a), the correction factor (C) calculate by the following expression.

$$C = \frac{\left(\frac{W_s}{R_s}\right)}{100}$$

where, W_s is the weight (in mg) of *valganciclovir IPRS* in reference solution (a) preparation and R_s is the sum of the areas of the two peaks of valganciclovir diastereomers in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{14}H_{22}N_6O_5$.HCl

Storage. Store protected from moisture, at temperature not exceeding 25°.

Valganciclovir Tablets

Valganciclovir Hydrochloride Tablets

Valganciclovir Tablets contain not less than 93.0 per cent and not more than 105.0 per cent of the stated amount of valganciclovir, $C_{14}H_{22}N_6O_5$.

Usual strength. 450 mg

Identification

A. When examined in the range of 200 nm to 350 nm (2.4.7), a 0.001 per cent w/v solution in *0.001 M hydrochloric acid*, shows an absorption maximum as obtained with *valganciclovir hydrochloride IPRS* of the same concentration.

B. In the Assay, the diastereomeric peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with reference solution (b).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter through 10 µm polyethylene filter, dilute if necessary, with the dissolution medium. Measure the absorbance of the filtrate, suitably diluted with the medium if necessary, at the maximum at about 254 nm (2.4.7) using 0.02 cm cuvette. Calculate the content of $C_{14}H_{22}N_6O_5$ in the medium from the absorbance obtained from a solution of known concentration of *valganciclovir hydrochloride IPRS* in dissolution medium.

Q. Not less than 80 per cent of the stated amount of $C_{14}H_{22}N_6O_5$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 450 mg of valganciclovir in 800 ml of *0.001 M*

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hydrochloric acid, with the aid of ultrasound and dilute to 1000.0 ml with *0.001 M hydrochloric acid*.

Reference solution (a). A solution containing 0.001 per cent w/v of *ganciclovir mono-N-methyl valinate IPRS (2-(RS)-[(Guanin-9-yl)methoxy]-3-hydroxypropyl N-methyl-L-valinate)* and 0.78 per cent w/v of *valganciclovir hydrochloride IPRS* in *0.001M hydrochloric acid*. Dilute 1.0 ml of the solution to 100.0 ml with *0.001 M hydrochloric acid*.

Reference solution (b). A 0.009 per cent w/v solution of *valganciclovir hydrochloride IPRS* in *0.001 M hydrochloric acid*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, phenyl groups bonded to porous silica (3.5 µm) (Such as Zorbax SB phenyl),
- mobile phase: a mixture of 93 volumes of 0.25 per cent v/v solution of *triethylamine* in *water*, adjusted to pH 3.0 with *trifluoroacetic acid* and 7 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 50 µl.

Name	Relative retention time	Correction factor
Guanine	0.51	0.53
Ganciclovir	0.66	0.71
Valganciclovir[1]	1.0	—
Valganciclovir[2]	1.07	—
Ganciclovir mono-*N*-methyl valinate 1*	1.21	—
Ganciclovir mono-*N*-methyl valinate 2*	1.30	—
Methoxymethylguanine*	1.45	—
Isovalganciclovir[1]*	1.55	—
Isovalganciclovir[2]*	1.61	—
Ganciclovir divalinate*	2.13	—
Monoacetoxyganciclovir*	2.31	—
Isomonochloroganciclovir*	2.52	—
Homologue[1]*	2.69	—
Homologue[2]*	2.77	—

*Process impurity include for identification only and not included in the calculation of total degradation products.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due second diastereomeric valganciclovir and first ganciclovir mono-*N*-methyl valinate is not less than 2.0 in the chromatogram obtained with reference solution (a), the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 3.0 for the second diastereomeric valganciclovir peak and the relative standard deviation for replicate injections is not more than 2.0 per cent for the sum of the areas of two valganciclovir peaks in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to guanine is not more than 0.05 times the sum of the areas of valganciclovir diastereomers in the chromatogram obtained with reference solution (b) (1.0 per cent), the area of any peak corresponding to ganciclovir is not more than 0.1 times the sum of the areas of valganciclovir diastereomers in the chromatogram obtained with reference solution (b) (2.0 per cent). The area of any other secondary peak is not more than 0.2 per cent and the sum of areas of all other secondary peaks is not more than 0.5 per cent, calculated by area normalization.

The sum of all the impurities is not more than 3.5 per cent.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modifications.

Test solution. Disperse 5 intact tablets in 300 ml of *0.001 M hydrochloric acid*, with the aid of mechanical shaker until the tablets are fully disintegrated and dilute to 500.0 ml with *0.001 M hydrochloric acid*. Dilute 2.0 ml of the solution to 100.0 ml with *0.001 M hydrochloric acid* and filter.

Reference solution (b). A 0.01 per cent w/v solution of *valganciclovir hydrochloride IPRS* in *0.001 M hydrochloric acid*.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due second diastereomeric valganciclovir and first ganciclovir mono-N-methyl valinate is not less than 2.0 in the chromatogram obtained with reference solution (a), the column efficiency is not less than 3000 theoretical plates, the tailing factor is not more than 3.0 for the second diastereomeric valganciclovir peak and the relative standard deviation for replicate injections is not more than 2.0 per cent for the sum of the areas of two valganciclovir peaks in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution.

Calculate the content of $C_{14}H_{22}N_5O_5$ in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

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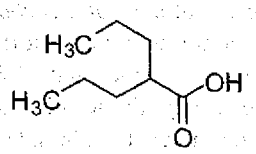
Valproic Acid



$C_8H_{16}O_2$ Mol. Wt. 144.2

Valproic acid is 2-propylpentanoic acid.

Valproic acid contains not less than 99.0 per cent and not more than 101.0 per cent of $C_8H_{16}O_2$.

Category. Anticonvulsant.

Description. A colourless or very slightly yellow liquid, slightly viscous.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *valproic acid IPRS* or with the reference spectrum of valproic acid.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 50 volumes of *ether* and 50 volumes of *dichloromethane*.

Test solution. A 1.0 per cent w/v solution of the substance under examination in *methanol*.

Reference solution. A 1.0 per cent w/v solution of *valproic acid IPRS* in *methanol*.

Apply 2 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air and spray with *bromocresol green solution*. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

C. To 1 ml add 3.0 ml of dilute *sodium hydroxide solution*. Add 3.0 ml of *water* and 1.0 ml of a 10 per cent w/v solution of *cobalt nitrate*. A violet precipitate is formed, filter it. The precipitate dissolves in *dichloromethane*.

Tests

Appearance of solution. A 20.0 per cent w/v solution of valproic acid in 8.5 per cent w/v sodium hydroxide solution is clear (2.4.1) and not more intensely colored than reference solution YS5 (2.4.1).

Refractive index (2.4.27). 1.422 to 1.425.

Related substances. Determine by gas chromatography (2.4.13).

Internal standard solution. A 0.005 per cent w/v solution of *butyric acid* in *heptane*.

Test solution. A 5.0 per cent w/v solution of the substance under examination in the internal standard solution. Dilute 1.0 ml of the solution to 10.0 ml with *heptane*.

Reference solution. Dissolve 20 mg of the substance under examination and 20 mg of *2-(1-methylethyl)pentanoic acid (valproic acid impurity C) IPRS* in 10.0 ml of *heptane*. Dilute 1.0 ml of the solution to 10.0 ml with *heptane*.

Chromatographic system
- a fused-silica capillary column 30 m x 0.53 mm, coated with wide-bore macrogol 20000 2-nitroterephthalate (0.5 µm),
- temperature.
  column 130° from 0-10 minutes and 130° - 190° from 10-30 minutes
- inlet port and detector 220°,
- a flame ionisation detector,
- flow rate: 8 ml per minute, using nitrogen as the carrier gas.

Inject 1 µl of the reference solution. The test is not valid unless the resolution between the peaks due to valproic acid impurity C and valproic acid is not less than 3.0.

Inject 1µl of the reference solution and the test solution. In the chromatogram obtained with the test solution the area of peak due to any impurity is not more than the area of the peak due to the internal standard (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 3 times the area of the peak due to the internal standard (0.3 per cent). Ignore any peak with an area less than 0.1 times the area of the peak due to the internal standard (0.01 per cent).

Heavy metals (2.3.13). Dissolve 2.0 g in 20 ml *ethanol (80 per cent)* and use 12 ml of the solution. The resulting solution complies with the limit test for heavy metals, Method D (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb)* obtained by diluting *lead standard solution (100 ppm Pb)* with *ethanol (80 per cent)*.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Dissolve 0.1g in 25 ml of *ethanol (95 per cent)*. Add 2 ml of *water*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01442 g of $C_8H_{16}O_2$.

Storage. Store protected from moisture.

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Valproic Acid Capsules

Valproic Acid Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of valproic acid, $C_8H_{16}O_2$.

Usual strength. 250 mg.

Identification

A. In the Assay, the retention time ratio of the principal peak to the internal standard peak in the chromatogram obtained with the test solution and reference solution, are not more than 2.0 per cent.

B. Disperse a quantity of the content of capsules containing about 250 mg of valproic acid, add 20 ml of *1 M sodium hydroxide*, shake and allow the layers to separate. Filter the aqueous layer, add 4 ml of *hydrochloric acid*, mix and extract with 40 ml of *n-heptane*. Filter the *n-heptane* layer through glass wool and evaporate the solvent completely on a steam bath with the aid of a current of air. Transfer 2 drops of the residue to a test tube containing 0.5 ml, each of, 2 per cent v/v of *potassiumm iodide solution* and 4 per cent v/v of *potassium iodate solution* and mix, a yellow colour is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of a 0.5 per cent w/v solution of *sodium lauryl sulphate* in simulated intestinal fluid prepared by dissolving 6.8 g of *monobasic sodium phosphate* in 250 ml *water*, mix and 77 ml of *0.2 M sodium hydroxide*, add 500 ml of *water*. Adjusted to pH 6.8 with *0.2 M sodium hydroxide* or *0.2 M hydrochloric acid* dilute to 1000 ml with *water*, adjusted to pH 7.5 with *5 M sodium hydroxide*,

Speed and time. 50 rpm and 60 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by gas chromatography (2.4.13) as described under Assay, using the following solutions.

Test solution. To 10 ml of the filtrate, add about 3.0 g of *sodium chloride*, and mix on a vortex mixer for 5 minutes. Add 1.0 ml of *6 M hydrochloric acid* and 5.0 ml of internal standard solution, shake and allow to separate, remove the *n-heptane* layer and filter. Discard the aqueous layer.

Reference solution. Weigh a quantity of *valproic acid IPRS* and dilute with the dissolution medium to obtain a solution having a concentration similar to that of the test solution. Take 10 ml of the solution and proceed as in test solution beginning with the words "add about 3.0 g.....".

Calculate the content of $C_8H_{16}O_2$ in the capsule.

Q. Not less than 85 per cent of the stated amount of $C_8H_{16}O_2$.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by gas chromatography (2.4.13).

Test solution. Take 20 capsules in a container and add 150 ml of *dichloromethane*, cool in a *solid carbon dioxide-acetone mixture* until the contents have solidified. If necessary, transfer the mixture of capsules and *dichloromethane* to a blender jar and blend until all the solids are reduced to fine particles. Transfer the mixture to a 500-ml flask, add *n-heptane* to volume, mix and allow the solids to settle. Transfer an accurately measured volume of the solution, containing about 250 mg of valproic acid, to a 100-ml flask, dilute with *n-heptane* to volume and mix. Transfer 5.0 ml to a container equipped with a closure. Add 2.0 ml of internal standard solution, close the container and mix.

Reference solution. A 0.25 per cent w/v solution of *valproic acid IPRS* in *n-heptane*. Transfer 5.0 ml to a container equipped with a closure. Add 2.0 ml of internal standard solution, close the container and mix.

Internal standard solution. A 0.5 per cent w/v solution of *biphenyl* in *n-heptane*.

Chromatographic system

- a glass column 1.8 m x 2 mm, packed with 10 per cent phase G34 on 80 to 100 mesh support S1A,
- temperature: column. 150°, injection port and detector. 250°,
- a flame ionization detector,
- flow rate: 40 ml per minute using nitrogen as the carrier gas.

The relative retention time with reference to valproic acid for biphenyl is about 2.0.

Inject 2 µl of the reference solution. The test is not valid unless the resolution between the peak due to valproic acid and biphenyl is not less than 3.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject 2 µl of the reference solution and the test solution.

Calculate the content of $C_8H_{16}O_2$ in the capsules.

Storage. Store protect from moisture, at a temperature not exceeding 30°.

Valproic Acid Oral Solution

Valproic Acid Oral Solution contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of valproic acid, $C_8H_{16}O_2$.

Usual strengths. 40 mg per ml; 50 mg per ml.

Identification

A. In the Assay, the retention time ratio of the principal peak to the internal standard peak in the chromatogram obtained

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with the test solution and reference solution is not more than 2.0 per cent.

B. Dissolve a volume of solution containing about 250 mg of valproic acid in 40 ml of *water* and 2.0 ml of *hydrochloric acid*. Mix and extract with 40 ml of *n-heptane*. Filter the *n-heptane* layer through glass wool into a beaker, and evaporate the solvent completely on a steam bath with the aid of a current of air. Transfer 2 drops of the residue to a test tube containing 0.5 ml each 2 per cent v/v of *potassium iodide solution* and 4 per cent v/v solution of *potassium iodate solution* and mix; a yellow colour is produced.

Tests

pH (2.4.24). 7.0 to 8.0.

Other tests. Complies with the tests stated under Oral Liquids.

Assay. Determine by gas chromatography (2.4.13).

Test solution. Dissolve a volume of solution containing about 250 mg of valproic acid in 40 ml of *water*, add 2.0 ml of *hydrochloric acid* and extract with 80 ml of *n-heptane* until the aqueous layer is clear. Filter the *n-heptane* layer through glass wool with small portions of *n-heptane*, add the rinsings to the flask, dilute to 100.0 ml with *n-heptane* and mix. Transfer 5.0 ml to a container equipped with a closure. Add 2.0 ml of the internal standard solution, close the container and mix.

Reference solution. A 0.25 per cent w/v solution of *valproic acid IPRS* in *n-heptane*. Transfer 5.0 ml to a container equipped with a closure. Add 2.0 ml of internal standard solution and mix.

Internal standard solution. A 0.5 per cent w/v solution of *biphenyl* in *n-heptane*.

Chromatographic system
- glass column 1.8 m x 2 mm, packed with 10 per cent phase G34 on 80 to 1010 mesh support S1A,
- temperature:
  column, 150°,
  injection port and detector at 250°,
- a flame ionization detector,
- flow rate: 40 ml per minute using nitrogen as the carrier gas.

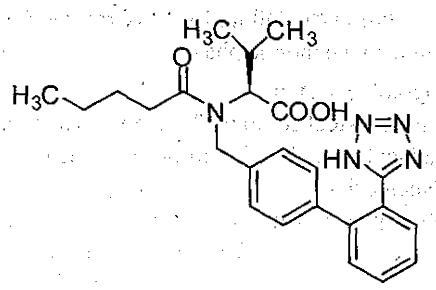
The relative retention time with reference to valproic acid for biphenyl is about 2.0.

Inject 2 µl of the reference solution. The test is not valid unless the resolution between valproic acid and biphenyl is not less than 3.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject 2 µl each of the reference solution and the test solution. Calculate the content of $C_8H_{16}O_2$ in oral solution.

Storage. Store protected from moisture.

Valsartan



$C_{24}H_{29}N_5O_3$ Mol. Wt. 435.5

Valsartan is *N*-pentanonyl-*N*-[2′-(1*H*-tetrazol-5-yl)biphenyl-4-ylmethyl]-L-valine.

Valsartan contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{24}H_{29}N_5O_3$, calculated on the anhydrous basis.

Category. Antihypertensive.

Production

As Nitrosamines are classified as probable human carcinogens, their presence in valsartan should be avoided or limited as much as possible. For this reason, manufacturers of valsartan for human use are expected to perform an assessment of the risk of *N*-nitrosamine formation and contamination during their manufacturing process; if this assessment identifies a potential risk, the manufacturing process should be modified to minimise contamination and a control strategy implemented to detect and control *N*-nitrosamine impurities in valsartan. The general chapter 5.11. Nitrosamine Impurities is available to assist manufacturers.

Description. A white to almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *valsartan IPRS* or with the reference spectrum of valsartan.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). −69.0° to −64.0°, determined in 1.0 per cent w/v solution in *methanol*, at 20°.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 100.0 ml of the mobile phase.

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Reference solution (a). A 0.05 per cent w/v solution of *valsartan IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 12.5 cm x 3.0 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Nucleosil),
- mobile phase: a mixture of 50 volumes of *water*, 50 volumes of *acetonitrile* and 0.1 volume of *glacial acetic acid*,
- flow rate: 0.4 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram three times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.2 times the area of the peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than 0.5 times the area of the peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 2.0 per cent, determined on 1 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in 50.0 ml of the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.005 per cent w/v solution of *valsartan IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 12.5 cm x 3.0 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Purospher 18e),
- mobile phase: a mixture of 50 volumes of *water*, 50 volumes of *acetonitrile* and 0.1 volume of *glacial acetic acid*,
- flow rate: 0.4 ml per minute,
- spectrophotometer set at 273 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{24}H_{29}N_5O_3$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Valsartan Tablets

Valsartan Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of valsartan, $C_{24}H_{29}N_5O_3$.

Usual strengths. 40 mg; 80 mg; 160 mg; 320 mg.

Identification

A. Shake a quantity of the powdered tablets containing 0.1 g of Valsartan with 40 ml of *methanol*, filter and evaporate the filtrate to dryness. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained from *valsartan IPRS* or with the reference spectrum of valsartan.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *phosphate buffer pH 6.8*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtered solution, suitably diluted with the medium if necessary, at the maximum at about 250 nm (2.4.7). Calculate the content of $C_{24}H_{29}N_5O_3$ in the medium from the absorbance obtained from a solution of known concentration of *valsartan IPRS* prepared by dissolving in minimum amount of *methanol* and diluted with the dissolution medium.

Q. Not less than 70 per cent of the stated amount of $C_{24}H_{29}N_5O_3$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets containing 25 mg of Valsartan with 15 ml of mobile phase and dilute to 25.0 ml with the mobile phase, filter.

Reference solution (a). A 0.1 per cent w/v solution of *valsartan IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system as described under Assay.

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Inject reference solution (a). The test is not valid unless the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 50 mg of Valsartan in 25 ml of the mobile phase and dilute to 100.0 ml with the mobile phase and filter. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.005 per cent w/v solution of *valsartan IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of *water*, 50 volumes of *acetonitrile* and 0.1 volume of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 273 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{24}H_{29}N_5O_3$ in the tablets.

Storage. Store protected from light and moisture.

Valsartan and Hydrochlorothiazide Tablets

Valsartan and Hydrochlorothiazide Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of valsartan, $C_{24}H_{29}N_5O_3$ and hydrochlorothiazide, $C_7H_8ClN_3O_4S_2$.

Usual strength. Valsartan, 80 mg and Hydrochlorothiazide, 12.5 mg; Valsartan 160 mg, and Hydrochlorothiazide, 12.5 mg.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 80 volumes of *ethyl acetate*, 20 volumes of *dehydrated alcohol* and 10 volumes of a 25 per cent w/v solution of *ammonium hydroxide*.

Test solution. Disperse a quantity of powdered tablets containing about 20 mg of valsartan in 100.0 ml of *acetone*, sonicate for 15 minutes and filter.

Reference solution. A solution equivalent to test concentrations of *valsartan IPRS* and *hydrochlorothiazide IPRS* in *acetone*.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 7 cm. Dry the plate in a current of warm air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. In the Assay, the retention time of the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 1000 ml of *phosphate buffer pH 6.8*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of the filtered solution, suitably diluted with the medium if necessary, at the maximum at about 250 nm for valsartan and 272 nm for hydrochlorothiazide (2.4.7). Calculate the content of $C_{24}H_{29}N_5O_3$ and $C_7H_8ClN_3O_4S_2$ in the medium from the absorbance obtained from a solution of known concentration of *valsartan IPRS* and *hydrochlorothiazide IPRS* in the same medium.

Calculate the content of valsartan dissolved by the formula:

$$\frac{(AT2 \times A1\ per\ cent\ H_{272\,nm}) - (AT1 \times A1\ per\ cent\ H_{250\,nm})}{(A1\ per\ cent\ V_{250\,nm} \times A1\ per\ cent\ H_{272\,nm}) - (A1\ per\ cent\ V_{272\,nm} \times A1\ per\ cent\ H_{250\,nm})} \times 12{,}500$$

Calculate the content of hydrochlorothiazide dissolved by the formula:

$$\frac{(AT1 \times A1\ per\ cent\ V_{250\,nm}) - (AT2 \times A1\ per\ cent\ V_{272\,nm})}{(A1\ per\ cent\ H_{272\,nm} \times A1\ per\ cent\ V_{250\,nm}) - (A1\ per\ cent\ H_{250\,nm} \times A1\ per\ cent\ V_{272\,nm})} \times 80{,}000$$

where, *AT1* = absorbance of the test solution at 272 nm,
AT2 = absorbance of the test solution at 250 nm,
$A1\ per\ cent\ V_{272\,nm}$ = absorptivity (1 per cent, 0.2 cm, 272 nm) of valsartan in medium,
$A1\ per\ cent\ V_{250\,nm}$ = absorptivity (1 per cent, 0.2 cm, 250 nm) of valsartan in medium,

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A1 per cent $H_{272\ nm}$ = absorptivity (1 per cent, 0.2 cm, 272 nm) of hydrochlorothiazide in medium,

A1 per cent $H_{250\ nm}$ = absorptivity (1 per cent, 0.2 cm, 250 nm) of hydrochlorothiazide in medium,

Q. Not less than 80 per cent of the stated amount of $C_{24}H_{29}N_5O_3$ and $C_7H_8ClN_3O_4S_2$.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay using the following solutions.

Solvent mixture. Equal volumes of *acetonitrile* and *water*.

Test solution. Disperse a quantity of powdered tablets containing about 62.5 mg of Hydrochlorothiazide with 5.0 ml of *water* and 100 ml of solvent mixture, sonicate for 15 minutes and shake for 30 minutes. Dilute to 250 ml with the solvent mixture, centrifuge and dilute 25.0 ml of the supernatant to 200.0 ml with the solvent mixture.

Reference solution (a). A solution containing 0.003 per cent w/v of *benzothiadiazine impurity A (4-amino-6-chloro-1,3-benzenedisulphonamide) IPRS*, 0.006 per cent w/v of *hydrochlorothiazide IPRS*, 0.008 per cent w/v of *valsartan IPRS* and 0.02 per cent w/v of *valsartan impurity B (N-butyryl-N-{[2'-(1H-tetrazole-5-yl)biphenyl-4-yl]methyl}-L-valine) IPRS* in the solvent mixture.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Reference solution (c). Dilute 10 ml of reference solution (b) to 100.0 ml with the solvent mixture.

Inject reference solutions (b) and (c). The test is not valid unless in the chromatogram obtained with reference solution (b), the resolution between valsartan impurity B and valsartan and between benzothiadiazine impurity A and hydrochlorothiazide is not less than 1.4. The relative standard deviation for replicate injections is not more than 10 per cent in the chromatogram obtained with reference solution (c).

Inject reference solution (a), (b) and (c) and the test solution. The area of the peak due to benzothiadiazine impurity A is not more than 1.0 per cent; the area of any secondary peak other than valsartan impurity A is not more than 0.2 per cent; and the sum of areas of all the secondary peaks other than valsartan impurity A is not more than 1.3 per cent. (Valsartan impurity A is the enantiomer of valsartan and coelutes with valsartan in this test.)

Uniformity of content. Complies with the tests stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay using the following solution as the test solution.

Solvent mixture. Equal volumes of *acetonitrile* and *water*.

Test solution. Disperse 1 tablet with 5.0 ml of *water* and about 100 ml of the solvent mixture, sonicate for 15 minutes and dilute to 200.0 ml with the solvent mixture. Centrifuge a portion of the solution and dilute a volume of the clear supernatant with the solvent mixture to obtain a 0.02 per cent w/v solution of valsartan.

Calculate the content of $C_{24}H_{29}N_5O_3$ and $C_7H_8ClN_3O_4S_2$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *acetonitrile* and *water*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 62.5 mg of Hydrochlorothiazide with 5.0 ml of *water* and 100 ml of solvent mixture, sonicate for 15 minutes and shake for 30 minutes. Dilute to 250 ml with the solvent mixture, centrifuge and dilute 25.0 ml of the supernatant to 200.0 ml with the solvent mixture. Dilute a suitable volume of the solution with the solvent mixture to obtain a 0.02 per cent w/v solution of Valsartan.

Reference solution. Transfer about 12.5 mg of *hydrochlorothiazide IPRS* to a 200 ml volumetric flask and add about 12.5 J mg of *valsartan IPRS*, J being the ratio of the stated amount, in mg, of valsartan to the stated amount, in mg, of hydrochlorothiazide per tablet. Add about 100 ml of solvent mixture, sonicate for 15 minutes, dilute to volume and mix. Transfer 25 ml of the solution to a 50 ml flask, dilute with solvent mixture to volume and mix. Dilute a suitable volume of the solution with the solvent mixture to obtain a 0.02 per cent w/v solution of *valsartan IPRS*.

Chromatographic system

- a stainless steel column 12.5 cm x 3.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 90 volumes of *water*, 10 volumes of *acetonitrile* and 0.1 volume of *trifluroacetic acid*,
  B. a mixture of 90 volumes of *acetonitrile*, 10 volumes of *water* and 0.1 volume of *trifluroacetic acid*,
- a gradient programme using the conditions given below,
- flow rate: 0.4 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
25	10	90
27	90	10
40	90	10

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

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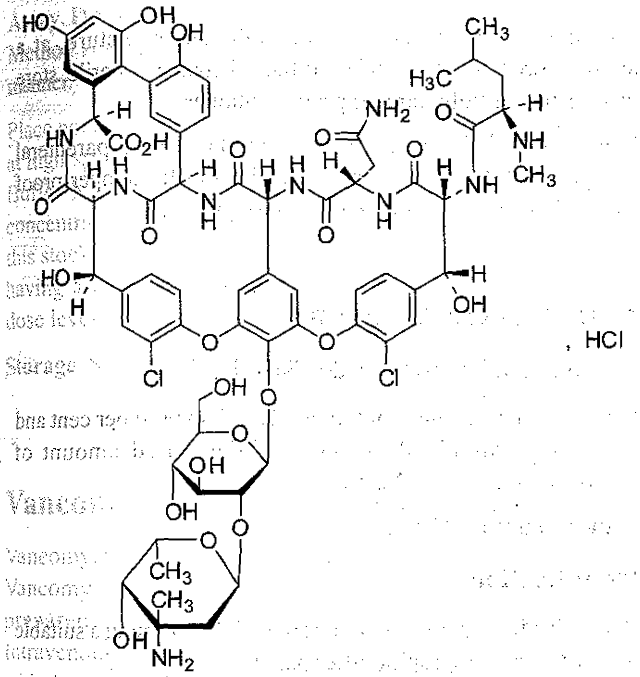
Inject the reference solution and the test solution.

Calculate the content of $C_{24}H_{29}N_5O_3$ and $C_7H_8ClN_3O_4S_2$ in the tablets.

Storage. Store protected from moisture.

Vancomycin Hydrochloride



$C_{66}H_{75}Cl_2N_9O_{24}$, HCl Mol.Wt. 1485.7

Vancomycin Hydrochloride is a mixture of related glycopeptides, consisting principally of the monohydrochloride of (3*S*,6*R*,7*R*,22*R*,23*S*,26*S*,30a*S*,36*R*,38a*R*)-3-(2-amino-2-oxoethyl)-44-[[2-*O*-(3-amino-2,3,6-trideoxy-3-*C*-methyl-β-L-*lyxo*-hexopyranosyl)-β-D-glucopyranosyl]oxy]-10,19-dichloro-7,22,28,30,32-pentahydroxy-6-[[(2*R*)-4-methyl-2-(methylamino)pentanoyl]amino]-2,5,24,38,39-pentaoxo-2,3,4,5,6,7,23,24,25,26,36,37,38,38a-tetradecahydro-22*H*-8,11:18,21-dietheno-23,36-(iminomethano)-13,16:31,35-dimetheno-1*H*,13*H*-[1,6,9]oxadiazacyclohexadecinol[4,5-*m*] [10,2,16]benzoxadiazacyclotetracosine-26-carboxylic acid (vancomycin B).

It is produced by certain strains of *Amycolatopsis orientalis* or obtained by any other means.

Vancomycin Hydrochloride contains not less than 1050 IU per mg of $C_{66}H_{75}Cl_2N_9O_{24}$, HCl, calculated on the anhydrous basis and not less than 93.0 per cent of vancomycin B.

Category. Antibacterial.

Description. A white or almost white hygroscopic powder.

Identification

A. In the test for Vancomycin B, the principal peak in the chromatogram obtained with test solution (a) corresponds to that in the chromatogram obtained with the reference solution.

B. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution in *water* is clear (2.4.1), absorbance of the solution at 450 nm (2.4.7) is not more than 0.1.

pH (2.4.24). 2.5 to 4.5, determined in a 5.0 per cent w/v solution in *carbondioxide-free water*.

Vancomycin B. Determine by liquid chromatography (2.4.14).

NOTE — Use freshly prepared solutions.

Test solution (a). Dissolve 10 mg of the substance under examination in mobile phase A and dilute to 5.0 ml with mobile phase A.

Test solution (b). Dilute 2.0 ml of test solution (a) to 50.0 ml with mobile phase A.

Test solution (c). Dilute 0.5 ml of test solution (b) to 20.0 ml with mobile phase A.

Reference solution. A 0.05 per cent w/v solution of *vancomycin hydrochloride IPRS* in *water.* Heat at 65° for 24 hours, allow to cool.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 1 volume of *tetrahydrofuran*, 7 volumes of *acetonitrile* and 92 volumes of buffer solution prepared by diluting 1 ml of *triethylamine* to 500 ml with *water*, adjusted to pH 3.2 with *orthophosphoric acid*,

  B. a mixture of 1 volume of *tetrahydrofuran*, 29 volumes of *acetonitrile* and 70 volumes of buffer solution,
- a gradient programme using conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
13	100	0
22	0	100
26	0	100
28	100	0

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Inject the reference solution and test solution (a), (b) and (c). The test is not valid unless the resolution between the two principal peaks in the chromatogram obtained with the reference solution is not less than 5.0, signal-to-noise ratio for the principal peak in the chromatogram obtained with test solution (c) is not less than 5.0 and the tailing factor for the peak due to vancomycin in the chromatogram obtained with the test solution (b) is not more than 1.6.

Calculate the percentage content of vancomycin B hydrochloride using the following expression:

$$\frac{A_b \times 100}{A_b + \left(\frac{A_t}{25}\right)}$$

where A_b = area of the peak due to vancomycin B in the chromatogram obtained with test solution (b);

A_t = sum of the areas of the peaks due to impurities in the chromatogram obtained with test solution (a).

Related substances. Determine by liquid chromatography (2.4.14), as described under Vancomycin B with following modifications.

Inject test solutions (a), (b) and (c). In the chromatogram obtained with test solution (a), the area of any secondary peak is not more than 4.0 per cent and the sum of areas of all the secondary peaks is not more than 7.0 per cent. Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with test solution (c) (0.1 per cent).

Calculate the percentage content of each impurity using the following expression:

$$\frac{\left(\frac{A_i}{25}\right) \times 100}{A_b + \left(\frac{A_t}{25}\right)}$$

A_i = area of the peak due to an impurity in the chromatogram obtained with test solution (a);

A_b = area of the peak due to vancomycin B in the chromatogram obtained with test solution (b);

A_t = sum of the areas of the peaks due to impurities in the chromatogram obtained with test solution (a).

Heavy metals (2.3.13). 0.67 g complies with the limit test for heavy metals, Method B (30 ppm).

Sulphated ash (2.3.18). Not more than 1.0 per cent.

Bacterial endotoxins (2.2.3). Not more than 0.25 Endotoxin Unit per mg.

Vancomycin Hydrochloride intended for use in the manufacture of parenteral preparations without a further appropriate sterilization procedure complies with the following additional requirement.

Sterility (2.2.11). Complies with the test for sterility.

Water (2.3.43). Not more than 5.0 per cent, determined on 0.5 g.

Assay. Determine by the microbiological assay of antibiotics, Method A or Method B (2.2.10) using *vancomycin hydrochloride IPRS*.

Storage. Store protected from light and moisture, at a temperature between 2° to 8°. If the substance is sterile, store in a sterile, air tight, tamper proof container.

If it is intended for use in the manufacture of parenteral preparations, the container should be sterile, tamper-proof and sealed so as to exclude micro-organisms.

Vancomycin Capsules

Vancomycin Hydrochloride Capsules

Vancomycin Capsules contain not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of vancomycin, $C_{66}H_{75}Cl_2N_9O_{24}$.

Usual strength. 125 mg.

Identification

Determine by paper chromatography (2.4.15), using a suitable sheet of chromatographic filter paper.

Mobile phase. A mixture of 6 volumes of *butyl alcohol*, 4 volumes of *water* and 3 volumes of *pyridine*.

Test solution. Disperse the content of capsules containing about 100 mg of vancomycin in 100.0 ml of water.

Reference solution. A 0.1 per cent w/v solution of *vancomycin hydrochloride IPRS* in water.

Apply to the plate 5 µl of each solution. Develop by descending chromatography for about 7 hours. After development, dry the paper and place it on an inoculated agar surface of sufficient area to accommodate the paper and prepared for vancomycin assay described in microbiological assay of antibiotics (2.2.10) except to use Medium B. Remove the paper from the agar surface after 30 minutes and incubate the agar medium at 37° for 18 hours; clear zones of inhibition are produced at corresponding positions on the two chromatograms.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

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Medium: 900 ml of *water*,

Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Carry out the method as described under Assay.

Q. Not less than 85 per cent of the stated amount of $C_{66}H_{75}Cl_2N_9O_{24}$.

Water (2.3.43). Not more than 8.0 per cent.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by the microbiological assay of antibiotics, Method A (2.2.10) on a solution prepared in the following manner.

Place not less than 5 capsules in a glass blender jar and blend at high speed for 3 to 5 minutes with a sufficient volume of Buffer No. 3 to yield a stock solution having a convenient concentration of vancomycin. Dilute a measured volume of this stock solution with Buffer No. 3 to obtain a test dilution having a concentration assumed to be equal to the median dose level of the standard.

Storage. Store protected from moisture.

Vancomycin Intravenous Infusion

Vancomycin Intravenous Infusion is a sterile solution of Vancomycin Hydrochloride in Water for Injections. It is prepared by dissolving Vancomycin Hydrochloride for Intravenous Infusion in Water for Injections and then diluting with the requisite volume of a suitable diluent in accordance with the manufacturer's instructions.

Vancomycin Intravenous Infusion contains not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of vancomycin, $C_{66}H_{75}Cl_2N_9O_{24}$.

The intravenous infusion complies with the tests stated under Parenteral Preparations.

Storage. Vancomycin Intravenous Infusion should be used immediately after preparation but, in any case, within the period recommended by the manufacturer when prepared and stored strictly in accordance with the manufacturer's instructions.

Usual strengths. 500 mg; 1 g.

Vancomycin Hydrochloride for Intravenous Infusion

Vancomycin Hydrochloride for Intravenous Infusion is a sterile material consisting of Vancomycin Hydrochloride with or without excipients. It is filled in a sealed container.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Vancomycin Hydrochloride for Intravenous Infusion contains not less than 88.0 per cent of Vancomycin B.

Identification

A. In the test for vancomycin B, the retention time of the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution of Vancomycin Hydrochloride is clear (2.4.1) and the absorbance of the solution at 450 nm (2.4.7) is not more than 0.1.

pH (2.4.24). 2.5 to 4.5, determined on a 5.0 per cent w/v solution of Vancomycin Hydrochloride.

Vancomycin B. Determine by liquid chromatography (2.4.14).

NOTE—Use the solutions within 4 hours of preparation.

Test solution (a). Dissolve a quantity of the substance under examination in sufficient mobile phase A to produce a solution containing 2,000 IU of vancomycin per ml.

Test solution (b). Dilute 1.0 ml of test solution (a) to 25.0 ml with mobile phase A.

Test solution (c). Dilute 1.0 ml of test solution (b) to 40.0 ml with mobile phase A.

Reference solution. A 0.05 per cent w/v solution of *vancomycin hydrochloride IPRS* in *water*. Heat at 65° for 24 hours and allow to cool.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- mobile phase: A. a mixture of 1 volumes of *tetrahydrofuran*, 7 volumes of *acetonitrile* and 92 volumes of buffer solution prepared by diluting 1 ml of *triethylamine* to 500 ml with *water*, adjusted to pH 3.2 with *orthophosphoric acid*,

  B. a mixture of 1 volume of *tetrahydrofuran*, 29 volumes of *acetonitrile* and 70 volumes of buffer solution,
- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
13	100	0
21	0	100
25	100	0
35	100	0

Inject the reference solution, test solution (b) and (c). The test is not valid unless in the chromatogram obtained with test solution (c), signal-to- noise ratio of the principal peak is not less than 5; the tailing factor of the vancomycin peak in the chromatogram obtained with test solution (b) is not more than 1.6 and the resolution between the two principal peaks in the chromatogram obtained with the reference solution is not less than 5.0.

Related substances. Determine by liquid chromatography (2.4.14), using test solutions (a), (b) and (c) as described under Vancomycin B.

Chromatographic system as described under Vancomycin B.

In the chromatogram obtained with test solution (a) calculate the content of each impurity.

The content of any impurity is not more than 4.0 per cent and the sum of the contents of any such impurities is not more than 12.0 per cent. Ignore any peak with an area less than that of the principal peak in the chromatogram obtained with test solution (c) (0.1 per cent).

Water (2.3.43). Not more than 5.0 per cent, determined on 0.5 g.

Bacterial endotoxins (2.2.3). Dissolve the contents of the sealed container in *tris-chloride buffer pH 7.4* prepared using *water BET* to give a solution containing 9000 Units of vancomycin per ml. Carry out the test on the resulting solution; the maximum allowable endotoxin concentration of the solution is 2.5 Units of endotoxins per ml. Carry out the test using the maximum valid dilution of the prepared solution calculated from the declared sensitivity of the lysate used in the test.

Assay. Determine the weight of the contents of ten containers as described under test for Uniformity of weight under Parenteral Preparations (Powders for Injection).

Determine on the mixed contents of ten containers by microbiological assay of antibiotics, Method A or Method B (2.2.10).

Labelling. The label states (1) the total number of Units of vancomycin in the container and (2) the number of Units of vancomycin per mg.

Vancomycin Oral Solution

Vancomycin Oral Solution is a solution of Vancomycin Hydrochloride in a suitable flavoured vehicle. It is prepared by dissolving Vancomycin Hydrochloride for Oral Solution in the requisite amount of a suitable diluent.

The oral solution complies with the tests stated under Oral Liquids.

Storage. Vancomycin Oral Solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer when prepared and stored strictly in accordance with the manufacturer's instructions.

Usual strength. 50 mg per ml.

Vancomycin Hydrochloride for Oral Solution

Vancomycin Hydrochloride for Oral Solution is a dry powder consisting of Vancomycin Hydrochloride with or without excipients. It is filled in a sealed container.

The contents of the sealed container comply with the requirements stated under Powders and Granules for Oral Solutions and Oral Suspensions stated under Oral Liquids and with the following requirements.

Vancomycin Hydrochloride for Oral Solution contains not less than 88.0 per cent of Vancomycin B.

Identification

A. In the test for Vancomycin B, the retention time of the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 10.0 per cent w/v solution is clear (2.4.1) and the absorbance at 450 nm (2.4.7) is not more than 0.1.

pH (2.4.24). 2.5 to 4.5, determined on a 5.0 per cent w/v solution of Vancomycin Hydrochloride.

Vancomycin B. Determine by liquid chromatography (2.4.14).

NOTE—Use the solutions within 4 hours of preparation.

Test solution (a). Dissolve a quantity of the powder in mobile phase A to produce a solution containing 2,000 IU of vancomycin per ml.

Test solution (b). Dilute 1.0 ml of test solution (a) to 25.0 ml with mobile phase A.

Test solution (c). Dilute 1.0 ml of test solution (b) to 40.0 ml with mobile phase A.

Reference solution. A 0.05 per cent w/v solution of *vancomycin hydrochloride IPRS* in *water*. Heat at 65° for 24 hours and allow to cool.

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Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with end-capped octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- mobile phase: A. a mixture of 4 ml of *triethylamine* diluted with 1996 ml of *water*, adjusted to pH 3.2 with *orthophosphoric acid* (solution A), 10 volumes of *tetrahydrofuran* and 70 volumes of *acetonitrile* to 920 volumes of solution A,
  B. a mixture of 10 volumes of *tetrahydrofuran*, 290 volumes of *acetonitrile* and 700 volumes of solution A,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
13	100	0
21	0	100
25	100	0
35	100	0

Inject each solution. The test is not valid unless in the chromatogram obtained with test solution (c) signal-to- noise ratio of the principal peak is not less than 5.0. The tailing factor of the principal peak in the chromatogram obtained with test solution (b) is not more than 1.6; and the resolution between the two principal peaks in the chromatogram obtained with the reference solution is not less than 5.0.

Related substances. Determine by liquid chromatography (2.4.14), using test solution (a), (b) and (c) as described under Vancomycin B.

Use chromatographic system as described under Vancomycin B.

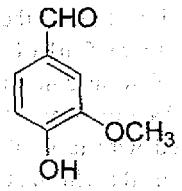
Inject the reference solution and test solution (c). The area of any secondary peak is not more than 4.0 per cent and the sum of the areas of all the secondary peaks is not more than 12.0 per cent. Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with the test solution (c) (0.1 per cent).

Water (2.3.43). Not more than 5.0 per cent, determined on 0.5 g.

Assay. Determine on the mixed contents of ten containers by the microbiological assay of antibiotics, Method A or Method B (2.2.10).

Labelling. The label states (1) the total number of Units of vancomycin in the container and (2) the number of Units of vancomycin per mg.

Vanillin



$C_8H_8O_3$ Mol. Wt. 152.2

Vanillin is 4-hydroxy-3-methoxybenzaldehyde.

Vanillin contains not less than 99.0 per cent and not more than 101.0 per cent of $C_8H_8O_3$, calculated on the dried basis.

Category. Pharmaceutical aid (flavouring agent).

Description. A white or slightly yellow powder or crystalline needles.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *vanillin IPRS* or with the reference spectrum of vanillin.

B. In the test for Related substances, the principal spot in the chromatogram obtained with test solution (b) corresponds to that in the chromatogram obtained with reference solution (b). Examine the chromatograms in daylight after spraying.

C. To 5 ml of a saturated solution add 0.2 ml of *ferric chloride solution*; a blue colour is produced. Heat to 80°; the solution becomes brown and a white precipitate is produced on cooling.

D. Melting range (2.4.21). 81° to 84°.

Tests

Appearance of solution. A 5.0 per cent w/v solution in *ethanol (95 per cent)* is clear (2.4.1) and not more intensely coloured than reference solution BS6 (2.4.1).

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 98.5 volumes of *dichloromethane*, 1 volume of *methanol* and 0.5 volume of *anhydrous acetic acid*.

Test solution (a). Dissolve 0.2 g of the substance under examination in 10.0 ml of *methanol*.

Test solution (b). Dissolve 0.2 g of the substance under examination in 100.0 ml of *methanol*.

Reference solution (a). Dissolve 10 mg of the substance under examination in 100.0 ml of *methanol*.

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Reference solution (b). A 0.2 per cent w/v solution of *vanillin IPRS* in *methanol*.

Use an unsaturated tank and allow the mobile phase to rise 10 cm. Apply to the plate 5 µl of each solution. After development, dry the plate in cold air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a). Spray with *dinitrophenylhydrazine-aceto-hydrochloric solution* and examine in daylight. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

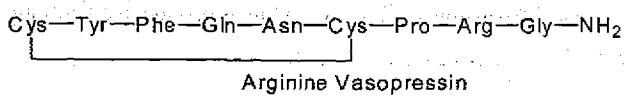
Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying over *phosphorus pentoxide* for 4 hours.

Assay. Weigh 0.12 g, dissolve in 20 ml of *ethanol (95 per cent)*, add 60 ml of *carbon dioxide-free water*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01521 g of $C_8H_8O_3$.

Storage. Store protected from light and moisture.

Vasopressin



Arginine Vasopressin

Vasopressin, 8-L-arginine

$C_{46}H_{65}N_{15}O_{12}S_2$ Mol. Wt. 1084.2

Vasopressin is a polypeptide hormone having the properties of causing the contraction of vascular and other smooth muscles and of antidiuresis. It is prepared by chemical synthesis or by animal origin.

Vasopressin contains not less than 95.0 per cent and not more than 102.0 per cent of $C_{46}H_{65}N_{15}O_{12}S_2$, calculated on anhydrous and acetic acid- free basis.

1 mg of anhydrous Vasopressin is equivalent to 410 Vasopressin Units.

Category. Antidiuretic.

Description. A white or almost white powder.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Acetic acid. Note more than 15.0 per cent.

Determine by liquid chromatography (2.4.14).

Solvent mixture. 95 volumes of a 0.07 per cent v/v solution of *orthophosphoric acid* in *water*, adjusted to pH 3.0 with 42 per cent w/v solution of *sodium hydroxide* and 5 volumes of *methanol*.

Test solution. Prepare the solution as described under reference solution. The amount of material used can be adopted depending on the amount of acetic acid expected.

Reference solution. A 0.01 per cent w/v solution of *glacial acetic acid IPRS* in the solvent mixture (The concentration can be adjusted depending on the amount of acetate or acetic acid expected to be present in the substance under examination).

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a 0.07 per cent v/v solution of *orthophosphoric acid* in *water*, adjusted to pH 3.0 with 42 per cent w/v solution of *sodium hydroxide*,
  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
5	95	5
10	50	50
20	50	50
22	95	5

The retention time of acetic acid peak is 3 to 4 minutes.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 5 per cent.

Inject the reference solution and the test solution.

Calculate the content of acetic acid.

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

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Inject the test solution. In the chromatogram obtained with the test solution, the sum of areas of all the secondary peaks is not more than 5 per cent of the area of the principal peak.

Microbial contamination (2.2.9). Total viable count not more than 200 cfu per g. For products of animal origin, 1 g is free from *Escherichia coli* and 10 g is free from *Salmonella* and *Shigella*.

Water (2.3.43). Not more than 8.0 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 10 mg of the substance under examination in 0.25 per cent v/v solution of *glacial acetic acid* and dilute to 25.0 ml with the *glacial acetic acid*.

Reference solution (a). Dissolve the entire contents of a vial of *vasopressin IPRS* in a known volume of 0.25 per cent v/v solution of *glacial acetic acid*.

Reference solution (b). A solution containing 0.0025 per cent w/v, each of, *lypressin IPRS* and *vasopressin IPRS* in 0.25 per cent v/v *glacial acetic acid*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 87 volumes of buffer solution prepared by dissolving 6.6 g of *dibasic ammonium phosphate* in about 950 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*, dilute to 1000 ml with *water* and 13 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Equilibrate the column at least for 1 hour.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to vasopressin and the lypressin is not less than 1.1 and the relative standard deviation for replicate injections is not more than 2.0 per cent for vasopressin.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{46}H_{65}N_{15}O_{12}S_2$.

Storage. Store protected from moisture, preferably of type I glass and at a temperature between 2° to 8°.

Vasopressin Injection

Vasopressin Injection is sterile solution of vasopressin in suitable diluent.

Each ml of Vasopressin Injection contains an activity of not less than 90.0 per cent and not more than 110.0 per cent of the stated on the label in Vasopressin Units. It may contain a suitable preservative.

Usual strength. 20 Units per ml.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 2.5 to 4.5.

Bacterial endotoxins (2.2.3). Less than 17.0 Endotoxin Units per Unit of Vasopressin.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 5.0 g of *chlorobutanol* in 5.0 ml of *glacial acetic acid*, add 1.1 g of *sodium acetate*, 5.0 g of *ethanol (95 per cent)*, and dilute to 1000 ml with *water*.

Test solution. Dilute 2.0 ml (20 Units per ml) of the injection to 25.0 ml with 0.25 per cent w/v of *glacial acetic acid*.

Reference solution (a). Dissolve the contents of one vial of *vasopressin IPRS* in a known volume of the solvent mixture to obtain the same concentration of test solution.

Reference solution (b). A solution containing 0.0025 per cent w/v, each of, *lypressin IPRS* and *vasopressin IPRS* in 0.25 per cent v/v *glacial acetic acid*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temeperature: 40°,
- mobile phase: a mixture of 87 volumes of buffer solution prepared by dissolving 6.6 g of *dibasic ammonium phosphate* in about 950 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*, dilute to 1000 ml with *water* and 13 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between vasopressin and lypressin is not less than 1.1 and relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{46}H_{65}N_{15}O_{12}S_2$ in the injection.

Storage. Store in single-dose or multiple-dose containers, preferably of type I glass. Do not freeze.

Labelling. The label states (1) the number of Units of the Vasopressin Units per ml; (2) indicate its origin (animal or synthetic).

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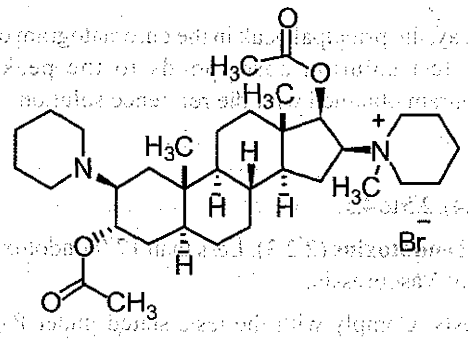
Vecuronium Bromide



$C_{34}H_{57}BrN_2O_4$ Mol. Wt. 638

Vecuronium Bromide is 1-(3α,17β-Diacetoxy-2β-piperidino-5α-androstan-16β-yl)-1-methylpiperidinium bromide.

Vecuronium Bromide contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{34}H_{57}BrN_2O_4$, calculated on the anhydrous basis.

Category. Non-depolarizing neuromuscular blocker.

Description. A white or almost white crystals or crystalline powder.

Identification

A. Complies with the test for Specific optical rotation.

B. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *vecuronium bromide IPRS* or with the reference spectrum of vecuronium bromide.

C. It gives reaction (A) of bromides (2.3.1).

Tests

Appearance of solution. A 1.0 per cent w/v solution in 0.5 per cent v/v of *hydrochloric acid* is clear (2.4.1) and not more intensely coloured than reference solution BYS7 (2.4.1).

Specific optical rotation (2.4.22). +30.5° to +35.0°, determined on 1.0 per cent w/v solution in *0.05 M hydrochloric acid*.

Impurity B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. Dissolve 1 g of *sodium bromide* in 5 ml of *water*. Add 85 ml of *2- propanol*, then 10 ml of *acetonitrile*.

Test solution. Dissolve 0.1 g of the substance under examination in *dichloromethane* and dilute to 5.0 ml with *dichloromethane*.

Reference solution (a). A solution containing 0.1 per cent w/v, each of, the substance under examination and *vecuronium bromide impurity B IPRS (pancuronium bromide IPRS)* in *dichloromethane*.

Reference solution (b). A 0.005 per cent w/v solution of *vecuronium bromide impurity B IPRS* in *dichloromethane*.

Apply to the plate 1 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air for 30 minutes and spray with a 0.25 per cent w/v solution of *iodine* in a mixture of equal volumes of *methanol* and *dichloromethane*. Any spot due to vecuronium bromide impurity B is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent). The chromatogram obtained with reference solution (a) shows 2 clearly separated spots.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Use freshly prepared solutions.

Test solution. Dissolve 40 mg of the substance under examination in a 0.02 per cent w/v solution of *hydrochloric acid* in *methanol* and dilute to 20.0 ml with *methanol*.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with a 0.02 per cent w/v solution of *hydrochloric acid* in *methanol*.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100.0 ml with a 0.02 per cent w/v solution of *hydrochloric acid* in *methanol*.

Reference solution (c). Dilute 10.0 ml of reference solution (b) to 50.0 ml with a 0.02 per cent w/v solution of *hydrochloric acid* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 50 volumes of a 1.8 per cent w/v solution of *tetramethylammonium hydroxide*, adjusted to pH 6.5 with *orthophosphoric acid*, 250 volumes of *methanol* and 700 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Vecuronium impurity C[1]	0.8	1.4
Vecuronium impurity D[2]	0.9	—
Vecuronium (Retention time: about 5 minutes)	1.0	—
Vecuronium impurity E[3]	1.2	—
Vecuronium impurity A[4]	1.3	0.6

[1]1-[17β-(acetyloxy)-3α-hydroxy-2β-(piperidin-1-yl)-5α-androstan-16β-yl]-1-methylpiperidinium,

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1-[3α,17β-dihydroxy-2β-(piperidin-1-yl)-5α-androstan-16β-yl]-1-methylpiperidinium,

1-[3α-(acetyloxy)-17β-hydroxy-2β-(piperidin-1-yl)-5α-androstan-16β-yl]-1-methylpiperidinium,

2β,16β -bis(piperidin-1-yl)-5α -androstane-3α,17β -diyl diacetate.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b), (c) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to vecuronium bromide impurities A, C, D, E is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent), the area of any other secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 2.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent). Ignore any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 4.0 per cent, determined on 0.3 g.

Assay. Dissolve 0.45 g in 50 ml of *glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.0638 g of $C_{34}H_{57}BrN_2O_4$.

Vecuronium Bromide intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 10.0 Endotoxin Units per mg of vecuronium bromide.

Storage. Store protected from light and moisture.

Vecuronium Bromide Injection

Vecuronium Bromide Injection is a sterile material consisting of Vecuronium Bromide with or without auxiliary agents. It is supplied in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile Water for Injections, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Vecuronium Bromide Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of vecuronium bromide, $C_{34}H_{57}BrN_2O_4$.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Description. A white to off white lyophilized mass.

Identification

In the Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a).

Tests

Solution A. Reconstitute the content of a vial with Water for Injections.

pH (2.4.24). 3.5 to 4.5, determined in solution A.

Light absorption (2.4.7). Not more than 0.1 at 420 nm, determined on solution A.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture A. Dilute 8.5 ml of *concentrated hydrochloric acid* to 100 ml with *water*. Dilute 10 ml of the solution to 1000 ml with *acetonitrile*.

Solvent mixture B. Dissolve 1 g of *citric acid monohydrate* and 730 mg of *disodium hydrogen phosphate* in *water* and dilute to 100 ml with *water*.

Test solution. Reconstitute 10 vials with Water for Injections and dilute the solution containing 20 mg of vecuronium bromide to 50.0 ml with solvent mixture A, sonicate for 30 to 40 minutes, filter. Dilute 10.0 ml of the solution to 25.0 ml with solvent mixture A.

Reference solution (a). Dissolve 20 mg of *vecuronium bromide IPRS* in 5.0 ml of solvent mixture B and dilute to 50.0 ml with solvent mixture A, sonicate for 20 minutes, filter. Dilute 10.0 ml of the solution to 25.0 ml with solvent mixture A.

Reference solution (b). Transfer 25 mg of *vecuronium bromide IPRS* and 10 mg of *potassium carbonate* (previously dried at 105° for 30 minutes) in 25-ml volumetric flask and make up the volume with *methanol*. Shake vigorously, decant the solution and inject immediately.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with porous silica (5 µm) (Such as Hypersil silica),
- column temperature: 40°,
- mobile phase: a mixture of 60 volumes of a solution prepared by dissolving 8.0 g of *sodium perchlorate* in

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6 ml of *water* an *dilute* to 1000 ml with *acetonitrile* and 40 volumes of a solution prepared by dissolving 160 mg of *ammonium chloride* in 100 ml of 25 per cent w/v *ammonium hydroxide solution* and dilute 8 ml of the solution to 1000 ml with *methanol*,

- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 100 µl.

Name	Relative retention time
Vecuronium bromide (Retention time: about 5 minutes)	1.0
Vecuronium bromide mono impurity	1.15
Vecuronium bromide diol impurity	1.65

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to vecuronium bromide and vecuronium bromide mono impurity is not less than 1.5.

Inject the test solution. The area of any peaks due to vecuronium bromide mono impurity and diol impurity is not more than 1.0 per cent. The sum of the areas of all the secondary peaks is not more than 2.0 per cent, calculated by area normalization method.

Water (2.3.43). Not more than 3.0 per cent, determined on 0.1 g.

Bacterial Endotoxins (2.2.3). Not more than 50 Endotoxin Units per mg of Vecuronium Bromide.

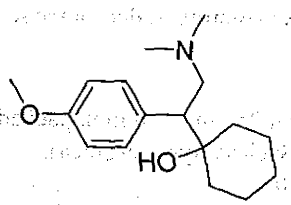
Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modifications.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to vecuronium bromide and vecuronium bromide mono impurity is not less than 1.5, the column efficiency is not less than 3500 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{34}H_{57}BrN_2O_4$ in the injection.

Venlafaxine Hydrochloride



and enantiomer, HCl

$C_{17}H_{28}ClNO_2$ Mol. Wt. 313.9

Venlafaxine Hydrochloride is 1-[(1*RS*)-2-(dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexanol hydrochloride.

Venlafaxine Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{17}H_{28}ClNO_2$, calculated on the dried basis.

Category. Antidepressant.

Description. A white or almost white powder. It shows polymorphism (2.5.11).

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *venlafaxine hydrochloride IPRS* or with the reference spectrum of venlafaxine hydrochloride.

NOTE —If the spectra obtained in the solid state show differences, dissolve the substance under examination and reference substance in *2-propanol*, evaporate to dryness and record new spectra using the residues.

B. It gives reaction (A) of chlorides (2.3.1).

Tests

Acidity or alkalinity. Dissolve in 0.2 g in 10 ml of *carbon dioxide free water*. Add 0.05 ml of *methyl red solution* and 0.1 ml of 0.01M *hydrochloric acid*. The solution is pink. Not more than 0.2 ml of *0.01 M sodium hydroxide* is required to change the colour of the indicator to yellow.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution(a). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution(b). Dissolve the contents of a vial of *venlafaxine* for system suitability *IPRS* (containing impurities D and F) in 1.0 ml of the mobile phase.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 149 volumes of a buffer solution prepared by dissolving 1.7 g of *ammonium dihydrogen orthophosphate* in 149 ml of *water*, adjusted to pH 4.4 with *orthophosphoric acid* and 51 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

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Name	Relative retention time
Venlafaxine impurity D[1]	0.9
Venlafaxine (Retention time is about 9 minutes)	1.0
Venlafaxine impurity F[2]	3.4

[1](1-[(1*RS*)-1-(4-methoxyphenyl)-2-(methylamino)ethyl] cyclohexanol,
[2]((2*RS*)-2-(cyclohex-1-enyl)-2-(4-methoxyphenyl)-*N*,*N*-dimethyl-ethanamine.

Run the chromatogram 10 times the retention time of venlafaxine.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to venlafaxine and venlafaxine impurity D is not less than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of the peak due to venlafaxine impurity F is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent), the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Ignore any peak with an area less than 0.5 times of the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in a vacuum at 80° for 3 hours.

Assay. Weigh accurately about 0.25 g dissolve in a mixture of 5 ml of 0.01 M *hydrochloric acid* and 50 ml of *ethanol (95 per cent)*. Titrate with *0.1 M sodium hydroxide*. Determining the end-point potentiometrically (2.4.25). Read the volume added between the 2 points of inflexion.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.03139 g of $C_{17}H_{28}ClNO_2$.

Venlafaxine Prolonged-release Capsules

Venlafaxine Hydrochloride Prolonged-release Capsules

Venlafaxine Prolonged-release Capsules manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be same.

Venlafaxine Prolonged-release Capsules contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of venlafaxine, $C_{17}H_{27}NO_2$.

Usual strengths. 37.5 mg; 75 mg; 150 mg.

Identification

Solvent mixture. 30 volumes of *cyclohexane* and 70 volumes of *dichloromethane*.

Dissolve a quantity of the powdered tablets containing about 350 mg of venlafaxine in a 100 ml of the solvent mixture for 30 minutes, filter the extract through *anhydrous sodium sulphate* and evaporate the filtrate to dryness. Wash the residue with the solvent mixture, filter and dry the residue. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *venlafaxine hydrochloride IPRS* treated in the same manner or with the reference spectrum of venlafaxine hydrochloride.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered capsules containing 200 mg of venlafaxine in 80 ml of a 2.4 per cent v/v solution of *orthophosphoric acid* with the aid of ultrasound for 30 minutes, cool and dilute to 100.0 ml with *water* and centrifuge.

Reference solution (a). Dilute 1.0 ml of test solution to 500.0 ml with mobile phase A.

Reference solution (b). A 0.2 per cent w/v solution of *venlafaxine impurity IPRS* (containing impurity D and F) in mobile phase A.

Reference solution (c). Dilute 25.0 ml of reference solution (a) to 100.0 ml with mobile phase A.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as Partisil ODS 3),
- mobile phase: A. a mixture of 1 volume of *triethylamine*, 20 volumes of *acetonitrile* and 80 volumes of *water*, adjusted to pH 3.5 with *orthophosphoric acid*,
  B: a mixture of 1 volume of *triethylamine*, 50 volumes of *acetonitrile* and 50 volumes of *water*, adjusted to pH 3.5 with *orthophosphoric acid*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 226 nm,
- injection volume: 20 µl.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
20	100	0
30	0	100
45	0	100
48	100	0
60	100	0

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to venlafaxine and venlafaxine impurity D is not less than 1.5.

Inject reference solution (a), (c) and the test solution. In the chromatogram obtained with the test solution, the area of any peak due to venlafaxine impurity D (1-[(1*RS*)-1-(4-methoxyphenyl)-2-(methylamino) ethyl] cyclohexanol) or venlafaxine impurity F ((2*RS*)-2-(cyclohex-1-enyl)-2-(4-methoxyphenyl)-*N*,*N*-dimethylethanamine) is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the sum of areas of all the secondary peaks is not more than 2.5 times of the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the mixed contents of 20 capsules containing 175 mg of Venlafaxine in 100.0 ml of *methanol*, ultrasound for 30 minutes and filter. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (a). A 0.01 per cent w/v of *venlafaxine hydrochloride IPRS* in the mobile phase.

Reference solution (b). A 0.01 per cent w/v solution of *venlafaxine impurity IPRS* (containing impurity D) in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as Zorbax Rx C18),
- mobile phase: a mixture of 20 volumes of *acetonitrile* and 80 volumes of a 1 per cent v/v solution of *triethylamine* previously adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 226 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to impurity D and venlafaxine is not less than 1.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{17}H_{27}NO_2$ in the Capsules.

1 mg of $C_{17}H_{27}NO_2$, HCl is equivalent to 0.884 mg of $C_{17}H_{27}NO_2$.

Labelling. The label states the strength in terms of the equivalent amount of venlafaxine.

Venlafaxine Prolonged-release Tablets

Venlafaxine Hydrochloride Prolonged-release Tablets

Venlafaxine Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be same.

Venlafaxine Prolonged-release Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of venlafaxine, $C_{17}H_{27}NO_2$.

Usual strengths. 25 mg; 37.5 mg; 75 mg.

Identification

Solvent mixture. 30 volumes of *cyclohexane* and 70 volumes of *dichloromethane*.

Dissolve a quantity of the powdered tablets containing about 350 mg of venlafaxine in a 100 ml of the solvent mixture and shake for 30 minutes, filter the extract through *anhydrous sodium sulphate* and evaporate the filtrate to dryness. Wash the residue with the solvent mixture, filter and dry the residue. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *venlafaxine hydrochloride IPRS* treated in the same manner or with the reference spectrum of venlafaxine hydrochloride.

Tests

Dissolution (2.5.2). Complies with the test stated under Tablets.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing 200 mg of venlafaxine in 80 ml of a 2.4 per cent v/v solution of *orthophosphoric acid* with the aid of ultrasound for 30 minutes, cool and dilute to 100.0 ml with *water* and filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A and further Dilute 1.0 ml of the solution to 5.0 ml with mobile phase A.

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Reference solution (b). A 0.2 per cent w/v solution of *venlafaxine impurity IPRS* (containing impurity D and F) in mobile phase A.

Reference solution (c). Dilute 25.0 ml of reference solution (a) to 100.0 ml with mobile phase A.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as Partisil ODS 3),
- mobile phase: A. a mixture of 1 volume of *triethylamine*, 20 volumes of *acetonitrile* and 80 volumes of *water* adjusted to pH 3.5 with *orthophosphoric acid*.
  B: a mixture of 1 volume of *triethylamine*, 50 volumes of *acetonitrile* and 50 volumes of *water* adjusted to pH 3.5 with *orthophosphoric acid*.
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 226 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
20	100	0
30	0	100
45	0	100
48	100	0
60	100	0

Name	Relative retention time
Venlafaxine impurity D[1]	0.8
Venlafaxine (Retention time is about 13 minutes)	1.0
Venlafaxine impurity F[2]	1.7

[1](1-[(1*RS*)-1-(4-methoxyphenyl)-2-(methylamino) ethyl] cyclohexanol,

[2]((2*RS*)-2-(cyclohex-1-enyl)-2-(4-methoxyphenyl)-*N*,*N*-dimethylethanamine.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to venlafaxine and venlafaxine impurity D is not less than 1.5.

Inject reference solution (a), (c) and the test solution. In the chromatogram obtained with the test solution, the area of any peak due to venlafaxine impurity D or venlafaxine impurity F is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the sum of areas of all the secondary peaks is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 85 mg of Venlafaxine in 50.0 ml of *methanol* with the aid of ultrasound for 30 minutes, mix and filter. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (a). A 0.01 per cent w/v solution of *venlafaxine IPRS* in the mobile phase.

Reference solution (b). A 0.01 per cent w/v solution of *venlafaxine impurity IPRS* (containing impurity D) in the mobile phase.

Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as Zorbax Rx C18),
- mobile phase: a mixture of 20 volumes of *acetonitrile* and 80 volumes of a 1 per cent v/v solution of *triethylamine* adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 226 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to impurity D and venlafaxine is not less than 1.0.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{17}H_{27}NO_2$ in the tablets.

1 mg of $C_{17}H_{27}NO_2$, HCl is equivalent to 0.884 mg of $C_{17}H_{27}NO_2$.

Labelling. The label states the strength in terms of the equivalent amount of venlafaxine.

Venlafaxine Tablets

Venlafaxine Hydrochloride Tablets

Venlafaxine Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of venlafaxine, $C_{17}H_{27}NO_2$.

Usual strengths. 25 mg; 37.5 mg; 75 mg.

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Identification

Solvent mixture. 30 volumes of *cyclohexane* and 70 volumes of *dichloromethane.*

Dissolve a quantity of the powdered tablets containing about 350 mg of venlafaxine in a 100 ml of the solvent mixture with aid of ultrasound for 30 minutes, filter the extract through *anhydrous sodium sulphate* and evaporate the filtrate to dryness. Wash the residue with the solvent mixture, filter and dry the residue. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *venlafaxine hydrochloride IPRS* treated in the same manner or with the reference spectrum of venlafaxine hydrochloride.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Reject first few ml of the filtrate and dilute a suitable volume of the filtrate with dissolution medium to obtain a 0.0025 per cent w/v solution of venlafaxine. Measure the absorbance of the filtrate, at the maximum at about 274 nm (2.4.7). Calculate the content of $C_{17}H_{27}NO_2$ in the medium from the absorbance obtained from a solution having a known concentration of *venlafaxine hydrochloride IPRS* similar to the concentration of the test solution.

1 mg of $C_{17}H_{27}NO_2$,HCl is equivalent to 0.884 mg of $C_{17}H_{27}NO_2$.

Q. Not less than 75 per cent of the stated amount of $C_{17}H_{27}NO_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of powdered tablets containing 200 mg of venlafaxine in 80 ml of a 2.4 per cent v/v solution of *orthophosphoric acid* with the aid of ultrasound for 30 minutes, cool and dilute to 100.0 ml with *water* and centrifuge.

Reference solution (a). Dilute 1.0 ml of the test solution to 500.0 ml with mobile phase A.

Reference solution (b). A 0.2 per cent w/v solution of *venlafaxine impurity IPRS* (containing impurity D and F) in mobile phase A.

Reference solution (c). Dilute 25.0 ml of reference solution (a) to 100.0 ml with mobile phase A.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as Partisil ODS 3);
- mobile phase: A. a mixture of 1 volume of *triethylamine*, 20 volumes of *acetonitrile* and 80 volumes of *water*, adjusted to pH 3.5 with *orthophosphoric acid*;
  B: a mixture of 1 volume of *triethylamine*, 50 volumes of *acetonitrile* and 50 volumes of *water*, adjusted to pH 3.5 with *orthophosphoric acid*,
- a gradient programme using the conditions given below;
- flow rate: 1 ml per minute,
- spectrophotometer set at 226 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
20	100	0
30	0	100
45	0	100
48	100	0
60	100	0

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to venlafaxine and venlafaxine impurity D is not less than 1.5.

Inject reference solutions (a), (c) and the test solution. In the chromatogram obtained with the test solution, the area of any peak due to venlafaxine impurity D ((1-[(1*RS*)-1-(4-methoxyphenyl)-2-(methylamino) ethyl] cyclohexanol) or venlafaxine impurity F ((2*RS*)-2-(cyclohex-1-enyl)-2-(4-methoxyphenyl)-*N*,*N*-dimethylethanamine) is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent of each), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the sum of areas of all the secondary peaks is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of Venlafaxine in 250.0 ml of a 0.2 per cent v/v solution of *orthophosphoric acid* with the aid of ultrasound for 30 minutes, cool, mix and centrifuge. Dilute 4.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (a). A 0.009 per cent w/v solution of *venlafaxine hydrochloride IPRS* in the mobile phase.

Reference solution (b). A 0.01 per cent w/v solution of *venlafaxine impurity IPRS* in the mobile phase.

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Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm), (Such as Zorbax C8),
- mobile phase: a mixture of 25 volumes of *acetonitrile* and 75 volumes of a 1 per cent v/v solution of *triethylamine* previously adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 226 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to impurity D and venlafaxine is not less than 1.0.

Inject reference solution (a) and the test solution.

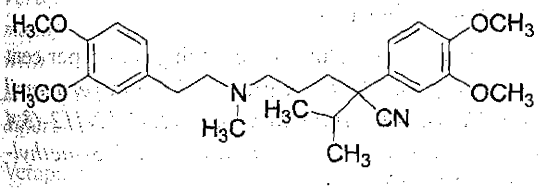
Calculate the content of $C_{17}H_{27}NO_2$ in the tablets.

1 mg of $C_{17}H_{27}NO_2$, HCl is equivalent to 0.884 mg of $C_{17}H_{27}NO_2$.

Labelling. The label states the strength in terms of the equivalent amount of venlafaxine.

Verapamil Hydrochloride

Verapamil Chloride; Iproveratril Hydrochloride



, HCl

$C_{27}H_{38}N_2O_4$, HCl, Mol. Wt. 491.1

Verapamil Hydrochloride is (*RS*)-2-(3,4-dimethoxyphenyl)-5-[[2-(3,4-dimethoxyphenyl)ethyl](methyl)amino]-2-(1-methylethyl)pentanenitrile hydrochloride.

Verapamil Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{27}H_{38}N_2O_4$, HCl, calculated on the dried basis.

Category. Calcium channel blocker.

Description. A white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B and C may be omitted if tests A and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *verapamil hydrochloride IPRS* or with the reference spectrum of verapamil hydrochloride.

B. When examined in the range 220 nm to 360 nm (2.4.7), a 0.002 per cent w/v solution in *0.01 M hydrochloric acid* shows absorption maxima at about 229 nm and 278 nm and there may be a shoulder at about 282 nm. The ratio of the absorbance at the maximum at about 278 nm to that at the maximum at about 229 nm is 0.35 to 0.39.

C. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to verapamil in the chromatogram obtained with reference solution (a).

D. It gives reaction (B) of chlorides (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution in *carbon dioxide-free water* prepared with the aid of gentle heat is clear (2.4.1), and colourless (2.4.1).

pH (2.4.24). 4.5 to 6.0, determined in a 5.0 per cent w/v solution prepared with the aid of gentle heat.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 63 volumes of mobile phase A and 37 volumes of mobile phase B.

Test solution. Dissolve 25 mg of the substance under examination in 10.0 ml of the solvent mixture.

Reference solution (a). Dissolve 5 mg, each of, of *verapamil hydrochloride IPRS* and *(2RS)-2-(3,4-dimethoxyphenyl)-2-[2-[[2-(3,4-dimethoxy-phenyl)ethyl](methyl)amino]ethyl]-3-methylbutanenitrile IPRS* (*verapamil impurity A IPRS*) in 20.0 ml of the solvent mixture. Dilute 1 ml of the solution to 10 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with palmitamidopropylsilane bonded to porous silica (5 µm),
- mobile phase: A. a 0.7 per cent w/v solution of *dipotassium hydrogen phosphate* adjusted to pH 7.2 with *orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 278 nm,
- injection volume: 10 µl.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	63	37
22	63	37
27	35	65
35	35	65
36	63	37
50	63	37

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to verapamil and verapamil impurity A is not less than 5.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.01 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh 0.4 g, dissolve in 40 ml of *anhydrous glacial acetic acid*, add 6 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.04911 g of $C_{27}H_{38}N_2O_4$, HCl.

Storage. Store protected from light and moisture.

Verapamil Injection

Verapamil Hydrochloride Injection; Verapamil Chloride Injection; Iproveratril Hydrochloride Injection

Verapamil Injection is a sterile solution of Verapamil Hydrochloride in Water for Injections.

Verapamil Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of verapamil hydrochloride, $C_{27}H_{38}N_2O_4$, HCl.

Usual strength. 2.5 mg per ml.

Identification

A. Dilute a volume containing 10 mg of Verapamil Hydrochloride to 5 ml with *0.1 M hydrochloric acid*, extract with 5 ml of *ether*, discard the ether extract and make the aqueous layer just alkaline with *2 M potassium carbonate*. Extract with 5 ml of *ether*, filter the ether layer through *anhydrous sodium sulphate* and evaporate to dryness. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *verapamil hydrochloride IPRS* treated in the same manner or with the reference spectrum of verapamil.

B. To a volume containing 5 mg of Verapamil Hydrochloride add 0.2 ml of a 5 per cent w/v solution of *mercuric chloride*; a white precipitate is produced.

C. To a volume containing 5 mg of Verapamil Hydrochloride add 0.5 ml of *3 M sulphuric acid* and 0.2 ml of *dilute potassium permanganate solution*; a violet precipitate is produced which quickly dissolves to produce a very pale yellow solution.

Tests

pH (2.4.24). 4.5 to 6.0.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dilute the injection with the mobile phase to obtain a solution containing 0.125 per cent w/v of Verapamil Hydrochloride.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Further dilute 1.0 ml the solution to 10.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.005 per cent w/v, each of, *verapamil hydrochloride IPRS* and *verapamil impurity I ((2RS)-2-(3,4-dimethoxyphenyl)-2-[2-[[2-(3,4-dimethoxyphenyl)ethyl](methyl)amino]ethyl]-3-methylbutanenitrile) IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 12.5 cm x 4 mm, packed with octadecylsilane bonded to porous silica (3 µm) (Such as Hypersil ODS),
- mobile phase: a mixture of 1 volume of *n-heptylamine*, 4.7 volumes of *glacial acetic acid*, 58 volumes of *acetonitrile* and 137 volumes of *0.01 M sodium acetate*,
- flow rate: 0.85 ml per minute,
- spectrophotometer set at 278 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to verapamil and verapamil impurity I is not less than 2.0.

Inject reference solution (a) and the test solution. Run the chromatogram 4 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal

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peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than three times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). Ignore any peak with an area less than 0.5 times that of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Bacterial endotoxins (2.2.3). Not more than 16.7 Endotoxin Units per mg of verapamil hydrochloride.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Dilute a measured volume containing 5 mg of Verapamil Hydrochloride to 100.0 ml with *0.01 M hydrochloric acid* and measure the absorbance of the resulting solution at the maximum at about 278 nm (2.4.7). Calculate the content of $C_{27}H_{38}N_2O_4,HCl$ taking 118 as the specific absorbance at 278 nm.

Storage. Store protected from light.

Verapamil Hydrochloride Prolonged-release Tablets

Verapamil Hydrochloride Sustained-release Tablets; Verapamil Hydrochloride Extended-release Tablets

Verapamil Hydrochloride Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Verapamil Hydrochloride Prolonged-release Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of verapamil hydrochloride $C_{27}H_{38}N_2O_4,HCl$.

Usual strengths. 120 mg; 240 mg.

Identification

A. Shake a quantity of the powdered tablets containing 0.1 g of Verapamil Hydrochloride with 25 ml of *0.1 M hydrochloric acid* for 10 minutes, filter, extract the filtrate with 25 ml of *ether*, discard the ether extract and make the aqueous layer just alkaline with *2 M potassium carbonate sesquihydrate*. Extract with 25 ml of *ether*, filter the ether layer through *anhydrous sodium sulphate* and evaporate to dryness. The residue complies with the following tests.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *verapamil hydrochloride IPRS* treated in the same manner or with the reference spectrum of verapamil.

B. Chlorides (2.3.12). Dissolve a quantity of powdered tablets containing 0.24 g of *verapamil hydrochloride* with 6 ml of *methanol* for a few minutes, allow to stand for 5 minutes and diluting 0.7 ml of the supernatant liquid to 2 ml with *water*. The resulting solution yields complies with the limit test for chlorides.

Tests

Dissolution (2.5.2). Complies with the test stated under Tablets.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. A equal volumes of *0.01 M hydrochloric acid* and *methanol*.

Test solution. Disperse a quantity of the powdered tablets containing 0.24 g of verapamil hydrochloride with 95.0 ml of the solvent mixture with the aid of ultrasound for 5 minutes shaking occasionally, dilute to 100.0 ml with solvent mixture and mix. Centrifuge 50 ml of the resulting solution, dilute the supernatant liquid with the solvent mixture to obtain 0.072 per cent w/v solution of verapamil hydrochloride and filter through 0.45 µm filter.

Reference solution(a). Dilute 1 volume of test solution to 200 volumes with solvent mixture.

Reference solution (b). Dilute 1 volume of reference solution(a) to 5 volumes with solvent mixture.

Chromatographic system
- a stainless steel column 12.5 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature 40°,
- mobile phase: a mixture of 700 volumes of 0.082 per cent w/v solution of *anhydrous sodium acetate* and 3.3 per cent w/v solution of *anhydrous acetic acid*, 300 volume of *acetonitrile* and 3 volume of *2-heptylamine*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 278 nm,
- injection volume: 20 µl.

Inject test solution (a). The area of any secondary peak is not more than 0.4 times the area of the principal peak in chromatogram obtained with the reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.12 g of verapamil hydrochloride in

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methanol, mix with the aid of ultrasound for 30 minutes and occasionally shaking for 10 minutes. Add sufficient *methanol* to obtain 0.048 per cent w/v solution of verapamil hydrochloride mix and filter.

Reference solution. A 0.048 per cent w/v solution of *verapamil hydrochloride IPRS* in the *methanol*.

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica or ceramic microparticles (10 µm),
- mobile phase: a mixture of 3 volumes of 0.164 per cent w/v solution of *anhydrous sodium acetate* previously adjusted to pH 7.0 with a 5 per cent v/v solution of *glacial acetic acid* and 7 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 25 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{38}N_2O_4$,HCl in the tablets.

Verapamil Tablets

Verapamil Hydrochloride Tablets; Verapamil Chloride Tablets; Iproveratril Hydrochloride Tablets

Verapamil Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of verapamil hydrochloride, $C_{27}H_{38}N_2O_4$, HCl.

Usual strengths. 40 mg; 80 mg; 120 mg; 160 mg.

Identification

A. Shake a quantity of the powdered tablets containing 0.1 g of Verapamil Hydrochloride with 25 ml of *0.1 M hydrochloric acid*, filter, extract the filtrate with 25 ml of *ether*, discard the ether extract and make the aqueous layer just alkaline with *2 M potassium carbonate*. Extract with 25 ml of *ether*, filter the ether layer through *anhydrous sodium sulphate* and evaporate to dryness. The residue complies with the following tests.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *verapamil hydrochloride IPRS* treated in the same manner or with the reference spectrum of verapamil.

B. Shake a quantity of the powdered tablets containing 0.1 g of Verapamil Hydrochloride with 10 ml of *dichloromethane*, filter, evaporate the filtrate to dryness and dissolve the residue in 10 ml of *water* (solution A). To 2 ml of the resulting solution add 0.2 ml of a 5 per cent w/v solution of *mercuric chloride*; a white precipitate is produced.

C. To 2 ml of solution A obtained in test B add 0.5 ml of *3 M sulphuric acid* and 0.2 ml of *dilute potassium permanganate solution*; a violet precipitate is produced which quickly dissolves to produce a very pale yellow solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium: 900 ml of *0.01 M hydrochloric acid*,
Speed and time: 50 rpm for 30 minutes.

Withdraw a suitable volume of the medium and filter, rejecting the first few ml of filtrate. Dilute a suitable volume of the filtrate with the medium, if necessary. Measure the absorbance of the resulting solution at the maximum at about 278 nm and 300 nm (2.4.7). Calculate the content of verapamil hydrochloride, $C_{27}H_{38}N_2O_4S$,HCl in the medium from the difference in absorbance at 278 nm and 300 nm, obtained from a solution of known concentration of *verapamil hydrochloride IPRS* in the dissolution medium.

Q. Not less than 75 per cent of the stated amount of $C_{27}H_{38}N_2O_4S$,HCl.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Shake a quantity of the powdered tablets containing about 0.24 g of Verapamil Hydrochloride with 100.0 ml of the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.005 per cent w/v, each of, *verapamil hydrochloride IPRS* and *(2RS)-2-(3,4-dimethoxyphenyl)-2-[2-[[2-(3,4-dimethoxyphenyl)ethyl]-(methyl)amino]ethyl]-3-methylbutanenitrile (verapamil impurity A) IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 12.5 cm x 4 mm, packed with octadecylsilane bonded to porous silica (3 µm) (Such as Hypersil ODS),
- mobile phase: a mixture of 1 volume of n-*heptylamine*, 4.7 volumes of *glacial acetic acid*, 58 volumes of *acetonitrile* and 137 volumes of *0.01 M sodium acetate*,
- flow rate: 0.85 ml per minute,
- spectrophotometer set at 278 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to verapamil and verapamil impurity A is not less than 2.0.

Inject reference solution (a) and the test solution. Run the chromatogram 4 times the retention time of the principal peak.

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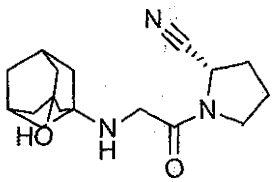
In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than three times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.1 g of Verapamil Hydrochloride, shake with 150 ml of *0.1 M hydrochloric acid* for 10 minutes, add sufficient *0.1 M hydrochloric acid* to produce 200.0 ml and filter. Dilute 10.0 ml of the filtrate to 100.0 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 278 nm (2.4.7). Calculate the content of $C_{27}H_{38}N_2O_4$, HCl taking 118 as the specific absorbance at 278 nm.

Vildagliptin



$C_{17}H_{25}N_3O_2$ Mol. Wt. 303.4

Vildagliptin is (S)-1-[2-(3-Hydroxyadamant-1-ylamino)acetyl] pyrrolidine-2-carbonitrile.

Vildagliptin contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{17}H_{25}N_3O_2$, calculated on the dried basis.

Category. Antidiabetic.

Description. A white to off white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *vildagliptin IPRS* or with the reference spectrum of vildagliptin.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Appearance of solution. A 5.0 per cent w/v solution is clear (2.4.1) and not more intensely colored than reference solution BYS5 and GYS5 (2.4.1).

Enantiomeric purity. Not more than 0.5 per cent.

Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *n-hexane* and *ethanol.*

Test solution. Dissolve 100 mg of the substance under examination in 5 ml of *ethanol*, with the aid of ultrasound and dilute to 10.0 ml with *n-hexane.*

Reference solution (a). A 0.01 per cent w/v solution of *vildagliptin IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 20.0 ml with the solvent mixture.

Reference solution (c). Dissolve 100 mg of *vildagliptin IPRS* and 1 mg of R-enantiomer of vildagliptin (1-[(3-Hydroxyadamant-1-ylamino)-acetyl]-pyrrolidine-(2R)-carbonitrile) in 5 ml of *ethanol*, with the aid of ultrasound and dilute to 10.0 ml with *n-hexane.*

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with amylase tris-3,5-dimethyl phenylcarbamate (10 µm) (Such as chiralpak AD),
- column temperature: 40°,
- mobile phase: a mixture of 90 volumes of *n-hexane*, 5 volumes of *ethanol*, 5 volumes of *methanol* and 0.1 volume of *trifluoroacetic acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 10 µl.

The relative retention time with reference to vildagliptin for R-enantiomer of vildagliptin is about 0.8.

Inject reference solution (a) and (c). The test is not valid unless the resolution between the peaks due to vildagliptin and R-enantiomer of vildagliptin is not less than 1.5 in the chromatogram obtained with reference solution (c) and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with test solution, the area of any peak corresponding to R-enantiomer of vildagliptin is not more than 0.5 times the area of principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than the area of principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

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Diazabicycloundecene Impurity. Not more than 0.1 per cent.

Determine by liquid chromatography (2.4.14).

Solvent mixture. 10 volumes of *acetonitrile* and 90 volumes of *water*.

Test solution. Dissolve 150 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution. A 0.0015 per cent w/v solution of *diazabicycloundecene IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm × 3 mm, packed with octadecylsilane bonded to porous silica (3.5 µm), (Such as XTerra MS),
- column temperature: 60°,
- mobile phase: A. a buffer solution prepared by dissolving 2.72 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 5.5 with *0.1M sodium hydroxide*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 217 nm,
- injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
3.5	95	5
3.6	20	80
9.5	20	80
9.6	95	5
15	95	5

The retention time of diazabicycloundecene impurity (*1,8-Diazabicyclo-[5.4.0]undec-7-en*) peck is about 2.5 minutes.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with test solution, the area of any peak corresponding to diazabicycloundecene impurity is not more than the area of principal peak in the chromatogram obtained with the reference solution (0.1 per cent).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 95 volumes of *water*, 5 volumes of *acetonitrile* and 0.1 volume of *orthophosphoric acid*.

Test solution. Dissolve 40 mg of the substance under examination in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). A 0.08 per cent w/v solution of *vildagliptin IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Reference solution (c). A 0.0004 per cent w/v solution of *vildagliptin impurity A, B, C* and *D IPRS* in reference solution (a).

Chromatographic system

- a stainless steel column 15 cm × 3.0 mm, packed with octadecylsilane bonded to porous silica (3.5 µm), (Such as XTerra MS),
- column temperature: 32°,
- mobile phase: A. a buffer solution prepared by dissolving 1.74 g of *dipotassium hydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.5 with *orthophosphoric acid*,
  B. a mixture of 75 volumes of *acetonitrile* and 25 volumes of mobile phase A,
- a gradient programme using the conditions given below,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
10	34	66
12.5	34	66
12.6	95	5
15	95	5

Name	Relative retention time	Correction factor
Vildagliptin impurity A[1]	0.7	0.6
Vildagliptin impurity B[2]	0.75	0.9
Vildagliptin	1.0	—
Vildagliptin impurity C[3]	1.14	0.7
Vildagliptin impurity D[4]	1.50	0.6

[1]2-(3-hydroxy-adamantan-1-yl)-1-imino-hexahydro-pyrrolo[1,2-a]pyrazin-4-one (Cyclic amidine),

[2]1-[(3-Hydroxyadamant-1-ylamino)-acetyl]-(2S)-prolinamide (Amide),

[3]2-(3-Hydroxy adamant-1-yl)-hexahydro-pyrrolo[1,2-a]pyrazine-1,4-dione (Diketopiperazine),

[4]1;1'-[(3-Hydroxyadamant-1-ylamino)-acetyl]-bis-pyrrolidine-(2S)-carbonitrile (Dialkylated byproduct).

Inject reference solution (b) and (c). The test is not valid unless the resolution between the peaks due to vildagliptin and vildagliptin impurity C is not less than 2.0 in the chromatogram obtained with reference solution (c) and the relative standard

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deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to vildagliptin impurity A, C and D, each of, is not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent), the area of any peak corresponding to vildagliptin impurity B is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent), the area of any other secondary peak is not more 0.1 times than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.3.13): 2.0 g complies with limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{17}H_{25}N_3O_2$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Vildagliptin Tablets

Vildagliptin Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of vildagliptin, $C_{17}H_{25}N_3O_2$.

Usual strengths. 50 mg; 100 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 1000 ml of *0.01 M hydrochloric acid*,

Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A 0.005 per cent w/v solution of *vildagliptin IPRS* in the dissolution medium.

Chromatographic system

- a stainless steel column 5 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm), (Such as Waters XTerra MS 120 Å),
- column temperature: 35°,
- mobile phase: a mixture of 90 volumes of a buffer solution prepared by dissolving 1.3 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.5 with 10 per cent w/v solution of *dipotassium hydrogen phosphate* and 10 volumes of *acetonitrile*,
- flow rate: 2.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{25}N_3O_2$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{17}H_{25}N_3O_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 90 volumes of *0.02 M hydrochloric acid* and 10 volumes of *acetonitrile*.

Buffer solution. Dissolve 1.3 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.5 with 10 per cent w/v solution of *dipotassium hydrogen phosphate*.

Test solution. Disperse a quantity of powdered tablets containing 50 mg of vildagliptin in the solvent mixture, with the aid of ultrasound and dilute to 100.0 ml with the solvent mixture.

Reference solution (a). A 0.05 per cent w/v solution of *vildagliptin IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Reference solution (c). Dilute 5.0 ml of reference solution (b) to 50.0 ml with the solvent mixture.

Reference solution (d). A solution containing 0.05 per cent w/v of *vildagliptin IPRS* and 0.0005 per cent w/v, each of, vildagliptin impurity A, B and C in the solvent mixture.

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Chromatographic system

- a stainless steel column 5 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (3.5 µm), (Such as Waters, XTerra MS 120 Å),
- column temperature: 35°,
- mobile phase: A. a mixture of 40 volumes of the buffer solution, 60 volumes of *water*, 1.5 volumes of *methanol* and 1.5 volumes of *acetonitrile*,

  B. a mixture of 40 volumes of the buffer solution, 45 volumes of *acetonitrile* and 15 volumes of *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
1	100	0
3	90	10
8	30	70
8.1	100	0
11	100	0

Name	Relative retention time	Correction factor
Vildagliptin impurity A[1]	0.61	0.57
Vildagliptin impurity B[2]	0.75	0.81
Vildagliptin	1.0	--
Vildagliptin impurity C[3]	1.1	0.68

[1]2-(3-hydroxy-adamantan-1-yl)-1-imino-hexahydro-pyrrolo[1,2-a]pyrazin-4-one. (Cyclic amidine),

[2](S)1-[(3-Hydroxyadamant-1-ylamino)-acetyl]-2-prolinamide. (Amide),

[3]2-(3-Hydroxy adamantan-1-yl)-hexahydro-pyrrolo[1,2-a]pyrazine-1,4-dione. (Diketopiperazine).

Inject reference solution (b), (c) and (d). The test is not valid unless the resolution between the peaks due to vildagliptin impurity A and vildagliptin impurity B is not less than 2.5, tailing factor of vildagliptin peak is not more than 1.8 in the chromatogram obtained with reference solution (d), the relative standard deviation for replicate injections is not more than 5.0 per cent in the chromatogram obtained with reference solution (b) and the signal-to-noise ratio is not less than 10 for the principal peak in the chromatogram obtained with reference solution (c).

Inject reference solution (b), (c) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to vildagliptin impurity B is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent), the area of any peak corresponding to vildagliptin impurity A is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent), the area of any peak corresponding to vildagliptin impurity C is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any other secondary peak is not more 0.2 times than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 2.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.2 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modifications.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{17}H_{25}N_3O_2$ in the tablets.

Storage. Store protected at a temperature not exceeding 30°.

Vildagliptin and Metformin Tablets

Vildagliptin and Metformin Hydrochloride Tablets

Vildagliptin and Metformin Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amounts of vildagliptin, $C_{17}H_{25}N_3O_2$ and metformin hydrochloride, $C_4H_{11}N_5,HCl$.

Usual strengths. Vildagliptin, 50 mg and Metformin hydrochloride, 500 mg; Vildagliptin, 50 mg and Metformin hydrochloride, 850 mg; Vildagliptin, 50 mg and Metformin hydrochloride, 1000 mg.

Identification

In the Assay, the principal peaks in the chromatogram obtained with test solution (a) and test solution (b) correspond to the principal peaks in the chromatogram obtained with reference solution (a).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium. 900 ml of *0.01 M hydrochloric acid*,

Speed and time. 100 rpm and 30 minutes.

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Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Buffer solution. A solution prepared by dissolving 1.7 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*. Add 8.0 g of *ammonium hexafluorophosphate* and mix.

Test solution (a). Use the filtrate.

Test solution (b). Use the filtrate, dilute if necessary, with the dissolution medium to obtain a solution of known concentration similar to the expected concentration of the reference solution.

Reference solution. A solution containing 0.00556 per cent w/v, each of, *vildagliptin IPRS* and *metformin hydrochloride IPRS* in the dissolution medium.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to spherical silica (3.0 µm), (Such as Atlantis d C18 100 Å),
- column temperature: 40°,
- mobile phase: a mixture of 40 volumes of the buffer solution, 33 volumes of *water* and 27 volumes of *acetonitrile*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 218 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 for both the peaks.

Inject the reference solution and test solution (a) for vildagliptin and test solution (b) for metformin hydrochloride.

Calculate the content of $C_{17}H_{25}N_3O_2$ and $C_4H_{11}N_5$, HCl in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{17}H_{25}N_3O_2$ and $C_4H_{11}N_5$,HCl .

Benzyltrimethylammonium hydroxide. Not more than 0.4 per cent.

Determine by gas chromatography (2.4.13).

NOTE — Use freshly prepared solutions.

Internal standard solution. A 0.5 per cent v/v solution of *benzyl alcohol* in *acetone*. Dilute 1.0 ml of the solution to 10.0 ml with *acetone*. Dilute 2.0 ml of the solution to 200.0 ml with *acetone*.

Test solution. Disperse a quantity of the powdered tablets containing 62.5 mg of Vildagliptin in the internal standard and vortex for 2 minutes. Then stir the solution for 45 minutes, with the aid of magnetic stirrer, dilute to 50.0 ml with the internal standard and centrifuge at 4000 rpm for 30 minutes. Use supernatant liquid.

Reference solution (a). A solution containing 0.000625 per cent w/v of *benzyltrimethylammonium hydroxide IPRS (1-amino-adamantan-3-ol)* in the internal standard solution.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 10.0 ml with the internal standard solution.

Reference solution (c). A 0.125 per cent w/v solution of *vildagliptin IPRS* in reference solution (a).

Chromatographic system

- a fused-silica capillary column, 15 m x 0.25 mm coated with crossbond 5 per cent diphenyl and 95 per cent dimethylpolysiloxane with film thickness of 1.0 µm (Such as Rtx-5 amine),
- temperature:
  column 100° for 4 minutes, 100° to 290° @ 35° per minutes and hold at 290°, for 14 minutes,
- inlet port at 250° and detector at 300°,
- flame ionisation detector,
- split ratio of 5:1,
- flow rate: 1.0 ml per minute using nitrogen as the carrier gas,
- injection volume: 1 µl.

Inject reference solution (a), (b) and (c). The test is not valid unless the resolution between the peaks due to benzyltrimethyl ammonium hydroxide and benzyl alcohol is not less than 2.5 in the chromatogram obtained with reference solution (c), the relative standard deviation of the peak area ratio due to benzyltrimethylammonium hydroxide and internal standard for replicate injections is not more than 10.0 per cent in the chromatogram obtained with reference solution (a) and the signal-to-noise ratio is not less than 10 in the chromatogram obtained with reference solution (b).

Inject reference solution (a) and the test solution.

Calculate the content of benzyltrimethyl ammonium hydroxide, using ratio of the peak area of benzyltrimethyl ammonium hydroxide to that of peak area of the internal standard.

Related substances. Determine by liquid chromatography (2.4.14).

Buffer solution. Dissolve 1.7 g of *potassium dihydrogen phosphate* in 1000 ml of *water* and adjusted to pH 3.0 with *ortho phosphoric acid*, add 8 g of *ammonium hexafluorophosphate* and mix.

Solvent mixture. 90 volumes of *water*, 10 volumes of *acetonitrile* and 0.1 volume of *orthophosphoric acid*.

Solution A. 40 volumes of the buffer solution, 60 volumes of *water* and 2.5 volumes of *acetonitrile*.

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Test solution (a). Disperse 10 intact tablets in the solvent mixture, with the aid of magnetic stirrer for 45 minutes, dilute to 500.0 ml with the solvent mixture. Centrifuge to get a clear supernatant, filter. Dilute 10.0 ml of the filtrate to 20.0 ml with the solvent mixture.

Test solution (b). Dilute a suitable volume of test solution (a) with solution A to obtain a solution having 0.02 per cent w/v of Metformin Hydrochloride.

Reference solution (a). A solution containing 0.05 per cent w/v of *vildagliptin IPRS* and 0.02 per cent w/v of *metformin hydrochloride IPRS* in the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture.

Reference solution (c). A 0.005 per cent w/v solution of *dicyandiamide IPRS* in the solvent mixture.

Reference solution (d). Dilute 5.0 ml of reference solution (b) and 0.2 ml of reference solution (c) to 50.0 ml with the solvent mixture.

Reference solution (e). A solution containing 0.0125 per cent w/v, each of, *vildagliptin impurities A, B, C IPRS* and *dicyandiamide IPRS* in the solvent mixture. Dilute 2.0 ml of the solution to 50.0 ml with reference solution (a).

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to spherical silica (3.0 µm) (Such as Waters, Atlantis d C18 100 Å),
- column temperature: 35°,
- mobile phase A: a mixture of 40 volumes of the buffer solution and 60 volumes of *water,*
  B: a mixture of 40 volumes of the buffer solution and 60 volumes of *acetonitrile,*
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm (for vildagliptin) and 218 nm (for metformin hydrochloride),
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	97	3
1	97	3
2	85	15
5	75	25
12	25	75
12.1	97	3
18	97	3

Name	Relative retention time	Correction factor
Dicyandiamide[1]	0.21	0.36
Metformin hydrochloride	0.41	—
Vildagliptin impurity A[2]	0.87	0.58
Vildagliptin impurity B[3]	0.83	0.77
Vildagliptin impurity C[4]	0.95	0.68
Vildagliptin	1.0	—

[1]1-cyanoguanidine,

[2]2-(3-hydroxy-adamantan-1-yl)-1-imino-hexahydro-pyrrolo[1,2-a]pyrazin-4-one. (Cyclic amidine),

[3](*S*)1-[(3-Hydroxyadamant-1-ylamino)-acetyl]-2-prolinamide. (Amide),

[4]2-(3-Hydroxy adamantan-1-yl)-hexahydro-pyrrolo[1,2-a]pyrazine-1,4-dione. (Diketopiperazine).

Inject reference solution (b), (d) and (e) at 210 nm. The test is not valid unless the resolution between the peaks due to vildagliptin impurity B and vildagliptin impurity A is not less than 1.8 in the chromatogram obtained with reference solution (e), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent for both the peaks in the chromatogram obtained with reference solution (b). The signal-to-noise ratio is not less than 10 in the chromatogram obtained with reference solution (d) for all the three peaks.

Inject reference solution (b) and test solution (a) at 210 nm. In the chromatogram obtained with test solution (a), the area of any peak corresponding to vildagliptin impurity A and vildagliptin impurity C, each of, is not more than the area of vildagliptin peak in the chromatogram obtained with reference solution (b) (1.0 per cent), the area of any peak corresponding to vildagliptin impurity B is not more than twice the area of vildagliptin peak in the chromatogram obtained with reference solution (b) (2.0 per cent), the area of any other secondary peak is not more than 0.2 times the area of the vildagliptin peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 3.5 times the area of vildagliptin peak in the chromatogram obtained with reference solution (b) (3.5 per cent). Ignore the peaks due to metformin hydrochloride and any peak with an area less than 0.05 times the area of the vildagliptin peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Inject reference solution (b) and test solution (b) at 218 nm. In the chromatogram obtained with the test solution (b), the area of any peak corresponding to dicyandiamide is not more than 0.02 times the area of principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent), the area of any other secondary peak is not more than 0.1 the area of the metformin hydrochloride peak in the chromatogram obtained with reference solution (b) (0.1 per cent), and the sum of the

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areas of all the secondary peaks is not more than 0.6 times the area of metformin hydrochloride peak in the chromatogram obtained with reference solution (b) (0.6 per cent). Ignore the peak due to vildagliptin.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4. 14), as described under Dissolution, using the following modifications.

Solvent mixture. 90 volumes of *water*, 10 volumes of *acetonitrile* and 0.1 volume of *orthophosphoric acid*.

Test solution. Disperse one intact tablet in the solvent mixture, with the aid of ultrasound and dilute to 200.0 ml with the solvent mixture, filter.

Reference solution. A 0.025 per cent w/v solution of *vildagliptin IPRS* in the solvent mixture.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{25}N_3O_2$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances.

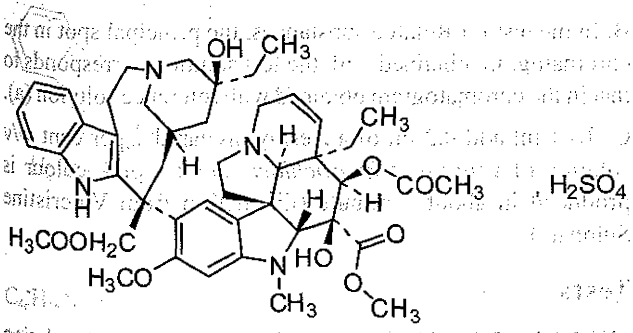
Inject reference solution (a). The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent for both the peaks.

Inject reference solution (a) and test solution (a) and (b).

Calculate the content of $C_{17}H_{25}N_3O_2$ in test solution (a) and $C_4H_{11}N_5$, HCl in test solution (b) in the tablets.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Vinblastine Sulphate



$C_{46}H_{58}N_4O_9,H_2SO_4$ Mol. Wt. 909.1

Vinblastine Sulphate is methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-acetoxy-3a-ethyl-9-[(5*S*,7*R*,9*S*)-5-ethyl-5-hydroxy-9-methoxycarbonyl-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[(5,4-*b*)indol-9-yl]-5-hydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate sulphate.

Vinblastine Sulphate contains not less than 95.0 per cent and not more than 104.0 per cent of $C_{46}H_{58}N_4O_9$, H_2SO_4, calculated on the dried basis.

Category. Anticancer.

Description. A white or yellowish, amorphous or crystalline powder; very hygroscopic.

CAUTION— Handle Vinblastine Sulphate with great care since it is a potent cytotoxic agent. Avoid contact with eyes; irritant to tissues.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *vinblastine sulphate IPRS* or with the reference spectrum of vinblastine sulphate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (b).

C. A 10 per cent w/v solution gives the reaction of sulphates (2.3.1).

Tests

Appearance of solution. A 0.5 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution YS5 (2.4.1).

pH (2.4.24). 3.5 to 5.0, determined in a 0.15 per cent w/v solution.

Related substances. In the Assay, the area of any secondary peak in the chromatogram obtained with the test solution is not greater than that of the principal peak in the chromatogram obtained with reference solution (c) and the sum of the areas of any such peaks is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c). Ignore any peak with an area less than that of the peak in the chromatogram obtained with reference solution (d).

Loss on drying (2.4.19). Not more than 15.0 per cent, determined by Method B, on an appropriately calibrated instrument using about 3.0 mg, weighed. Heat the substance under examination at the rate of 5° per minute between ambient temperature and 200° in a current of nitrogen for chromatography with a flow rate of 40 ml per minute. From the thermogram, determine the accumulated loss in weight between

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ambient temperature and a point on the plateau before decomposition is indicated (at about 160°).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in 100 ml of *water*.

Reference solution (a). A solution containing 0.1 per cent w/v each of *vinblastine sulphate IPRS* and *vincristine sulphate IPRS* in *water*.

Reference solution (b). A 0.1 per cent w/v solution of *vinblastine sulphate IPRS* in *water*.

Reference solution (c). A 0.002 per cent w/v solution of *vinblastine sulphate IPRS* in *water*.

Reference solution (d). A 0.0001 per cent w/v solution of *vinblastine sulphate IPRS* in *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm), (b) a guard column packed with a suitable silica gel placed between the pump and the injection device,
- mobile phase: a mixture of 50 volumes of *methanol*, 38 volumes of a 1.5 per cent v/v solution of *diethylamine* adjusted to pH 7.5 with *phosphoric acid* and 12 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 262 nm,
- injection volume: 10 µl.

NOTE — Store all solutions in ice before use.

Inject each solution and record the chromatograms for 3 times the retention time of the peak due to vinblastine.

The assay is not valid unless the resolution between the peaks due to vincristine and vinblastine in the chromatogram obtained with reference solution (a) is at least 4 and the signal-to-noise ratio of the peak in the chromatogram obtained with reference solution (d) is at least 5.

Calculate the percentage content of $C_{46}H_{58}N_4O_9,H_2SO_4$.

Vinblastine Sulphate intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 10.0 Endotoxin Units per mg of vinblastine sulphate.

Vinblastine Sulphate intended for use in the manufacture of parenteral preparations without a further appropriate sterilisation procedure complies with the following additional requirement.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store protected from light and moisture in a deep freezer (Below -18°). If the material is intended for use in the manufacture of parenteral preparations, it should be stored in sterile, tamper-evident glass containers and sealed so as to exclude microorganisms.

Labelling. The label states whether or not the contents are suitable for use in the manufacture of parenteral preparations.

Vinblastine Injection

Vinblastine Sulphate Injection

Vinblastine Injection is a sterile material consisting of Vinblastine Sulphate with or without auxiliary substances. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile 0.9 per cent w/v solution of Sodium Chloride, immediately before use.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Vinblastine Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of vinblastine sulphate, $C_{46}H_{58}N_4O_9,H_2SO_4$.

Usual strength. 10 mg.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Identification

CAUTION — Handle Vinblastine Injection with great care since it is a potent cytotoxic agent. Avoid contact with eyes; irritant to tissues.

A. When examined in the range 230 nm to 360 nm (2.4.7), the solution obtained in the Assay shows an absorption maximum at about 267 nm.

B. In the test for Related substances, the principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a).

C. To 1 ml add 0.2 ml of a freshly prepared 1 per cent w/v solution of *vanillin* in *hydrochloric acid*; a pink colour is produced in about 1 minute (distinction from Vincristine Sulphate).

Tests

pH (2.4.24). 3.5 to 5.0, determined in a 0.15 per cent w/v solution of the dried contents.

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Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 80 volumes of *toluene*, 40 volumes of *chloroform* and 6 volumes of *diethylamine*.

Test solution. Dissolve a quantity of the contents of a container in sufficient *methanol* to produce a solution containing the equivalent of 1 per cent w/v of dried vinblastine sulphate.

Reference solution (a). A 1 per cent w/v solution of *vinblastine sulphate IPRS* in *methanol*.

Reference solution (b). A 0.02 per cent w/v solution of *vincristine sulphate IPRS* in *methanol*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (b).

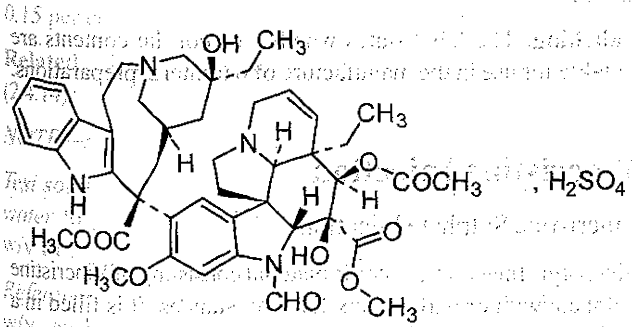
Bacterial endotoxins (2.2.3). Not more than 10.0 Endotoxin Units per mg of the dried contents.

Assay. Weigh the contents of 20 sealed containers. Weigh a quantity of the mixed contents containing about 20 mg of dried vinblastine sulphate and dissolve it in 100.0 ml of *methanol*. Dilute 10.0 ml to 100.0 ml with *methanol* and measure the absorbance of the resulting solution at the maximum at about 267 nm (2.4.7). Calculate the content of $C_{46}H_{58}N_4O_9,H_2SO_4$ taking 185 as the specific absorbance at 267 nm.

Storage. Store in sealed containers at a temperature of 2° to 8°.

Labelling. The label states (1) the strength in terms of the weight of dried vinblastine sulphate contained in it; (2) the names of auxiliary substances, if any; (3) that the contents are to be used by intravenous injection only; (4) the storage conditions.

Vincristine Sulphate



$C_{46}H_{56}N_4O_{10},H_2SO_4$ Mol. Wt. 923.1

Vincristine Sulphate is methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*) 4-acetoxy-3a-ethyl-9-[(5*S*,7*R*,9*S*)-5-ethyl-5-hydroxy-9- methoxycarbonyl-1,4,5,6,7,8,9,10-octahydro-2*H*-3, 7-methanoazacycloundecino[(5,4-*b*)indol-9-yl]-6-formyl-5-hydroxy- 8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-*1H*-indolizino[8,1-*cd*]carbazole-5-carboxylate sulphate.

Vincristine Sulphate contains not less than 95.0 per cent and not more than 104.0 per cent of $C_{46}H_{56}N_4O_{10},H_2SO_4$, calculated on the dried basis.

Category. Anticancer.

Description. A white to slightly yellowish, amorphous or crystalline powder; very hygroscopic.

CAUTION— Handle Vincristine Sulphate with great care since it is a potent cytotoxic agent. Avoid contact with eyes; irritant to tissues.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *vincristine sulphate IPRS* or with the reference spectrum of vincristine sulphate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to vincristine sulphate in the chromatogram obtained with reference solution (b).

C. A 10 per cent w/v solution gives the reaction for sulphates (2.3.1).

Tests

Appearance of solution. A 0.5 per cent w/v in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution YS7 (2.4.1).

pH (2.4.24). 3.5 to 4.5. Determined in a 0.1 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE— Keep the solutions in iced water before use.

Test solution. Dissolve about 50.0 mg of the substance under examination in 10.0 ml of *carbon dioxide-free water*. Dilute 1.0 ml of the solution to 5.0 ml with *water*.

Reference solution (a). A 0.1 per cent w/v solution of *vincristine sulphate IPRS* in *water*.

Reference solution (b). A 0.1 per cent w/v solution of *vinblastine sulphate IPRS* in reference solution (a).

Reference solution (c). Dilute 1.0 ml of the test solution to 50.0 ml with *water*.

Reference solution (d). Dilute 1.0 ml of reference solution (c) to 20.0 ml with *water*.

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Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: A. a 1.5 per cent v/v solution of *diethylamine*, adjusted to pH 7.5 with *orthophosphoric acid*,

B. *methanol*,
- a gradient programme using the condition given below,
- flow rate: 2 ml per minute,
- spectrophotometer set at 297 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	38	62
12	38	62
27	8	92
29	38	62
34	38	62

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to vincristine and vinblastine is not less than 4.

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent) and the sum of areas of all the secondary peaks is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (5.0 per cent). Ignore any peak with an area less than 0.1 per cent the area of the principal peak in the chromatogram obtained with reference solution (d).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined by Method B, on an appropriately calibrated instrument using about 3.0 mg, weighed. Heat the substance under examination at the rate of 5° per minute between ambient temperature and 200° in current of nitrogen for chromatography with a flow rate of 40 ml per minute.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in 100 ml of *water*.

Reference solution (a). A solution containing 0.1 per cent w/v, each of, *vinblastine sulphate IPRS* and *vincristine sulphate IPRS* in *water*.

Reference solution (b). A 0.1 per cent w/v solution of *vincristine sulphate IPRS* in *water*.

Reference solution (c). A 0.002 per cent w/v solution of *vincristine sulphate IPRS* in *water*.

Reference solution (d). A 0.0001 per cent w/v solution of *vincristine sulphate IPRS* in *water*.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm), (b) a guard column packed with a suitable silica gel placed between the pump and the injection device,
- mobile phase: a mixture of 70 volumes of *methanol* and 30 volumes of a 1.5 per cent v/v solution of *diethylamine* adjusted to pH 7.5 with *phosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 297 nm,
- injection volume: 10 µl.

NOTE — Store all solutions in ice before use.

Inject each solution and record the chromatograms for 3 times the retention time of the peak due to vincristine.

The assay is not valid unless the resolution between the peaks due to vincristine and vinblastine in the chromatogram obtained with reference solution (a) is at least 4 and the signal-to-noise ratio in the peak in the chromatogram obtained with reference solution (d) is at least 5.

Calculate the content of $C_{46}H_{56}N_4O_{10},H_2SO_4$.

Vincristine Sulphate intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 62.5 Endotoxin Units per mg.

Vincristine Sulphate intended for use in the manufacture of parenteral preparations without a further appropriate sterilisation procedure complies with the following additional requirement.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store protected from light in a deep freezer (Below -18°). If the material is intended for use in the manufacture of parenteral preparations, it should be stored in sterile, tamper-evident glass containers and sealed so as to exclude micro-organisms.

Labelling. The label states whether or not the contents are suitable for use in the manufacture of parenteral preparations.

Vincristine Injection

Vincristine Sulphate Injection

Vincristine Injection is a sterile material consisting of Vincristine Sulphate with or without auxiliary substances. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile 0.9 per cent w/v solution of Sodium Chloride, immediately before use.

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The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Vincristine Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of vincristine sulphate, $C_{46}H_{56}N_4O_{10},H_2SO_4$.

Usual strengths. The equivalent of 1 mg, 2 mg and 5 mg of dried vincristine sulphate.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Identification

CAUTION — Handle Vincristine Injection with great care since it is a potent cytotoxic agent. Avoid contact with eyes; irritant to tissues.

A. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (b).

B. Shake a quantity containing 1 mg of dried vincristine sulphate with 3 ml of *chloroform*, filter and wash the filter with 2 ml of *chloroform*. Evaporate the combined chloroform solutions to dryness at 40°. Add 0.2 ml of a freshly prepared 1 per cent w/v solution of *vanillin* in *hydrochloric acid* to the residue; an orange colour is produced in about 1 minute (distinction from vinblastine sulphate).

Tests

Appearance of solution. A solution prepared by dissolving the contents of a sealed container in 10 ml of *carbon dioxide-free water* is clear (2.4.1).

pH (2.4.24). 3.5 to 5.0, determined in a solution containing 0.15 per cent w/v solution of the dried contents.

Related substances. Deteremine by liquid chromatography (2.4.14).

NOTE—Keep the solutions in ice before use.

Test solution. Dissolve the contents of a sealed container in *water* to produce a solution containing about 0.1 per cent w/v of anhydrous vincristine sulphate.

Reference solution (a). A solution containing 0.1 per cent w/v, each of, *vincristine sulphate IPRS* and *vinblastine sulphate IPRS* in *water*.

Reference solution (b). A 0.1 per cent w/v solution of *vincristine sulphate IPRS* in *water*.

Reference solution (c). A 0.002 per cent w/v solution of *vincristine sulphate IPRS* in *water*.

Reference solution (d). A 0.0001 per cent w/v solution of *vincristine sulphate IPRS* in *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with endcapped octylsilane bonded to porous silica (5 µm), (Such as Zorbax C8),
- mobile phase: a mixture of 30 volumes of a 1.5 per cent v/v solution of *diethylamine* adjusted to pH 7.5 with *orthophosphoric acid* and 70 volumes of *methanol*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 297 nm,
- injection volume: 10 µl.

Inject reference solutions (a) and (d). The test is not valid unless the resolution between the peaks due to vincristine and vinblastine in the chromatogram obtained with reference solution (a) is not less than 4.0 and signal-to-noise ratio in the principal peak in the chromatogram obtained with reference solution (d) is not less than 5.0.

Inject reference solutions (c), (d) and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent) and the sum of the areas of all the secondary peaks is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (5.0 per cent). Ignore any peak with an area less than the of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

Uniformity of content. The content of anhydrous vincristine sulphate in each of 10 individual containers as determined in the Assay is not less than 90.0 per cent and not more than 110.0 per cent of the average except that in one container the content may be not less than 80.0 per cent and not more than 120.0 per cent of the average.

Bacterial endotoxins (2.2.3). Not more than 62.5 Endotoxin Units per mg of dried vincristine sulphate.

Assay. Dissolve the contents of a sealed container in a suitable volume of methanol to produce a solution containing about 0.005 per cent w/v of anhydrous vincristine sulphate. Measure the absorbance of the resulting solution at the maximum at 297 nm (2.4.7). Calculate the content of $C_{46}H_{56}N_4O_{10},H_2SO_4$ taking 177 as the specific absorbance at 297 nm.

Repeat the procedure with a further nine containers.

Calculate the average content of $C_{46}H_{56}N_4O_{10},H_2SO_4$ in the sealed container.

Storage. Store in sealed containers at a temperature of 2° to 8°.

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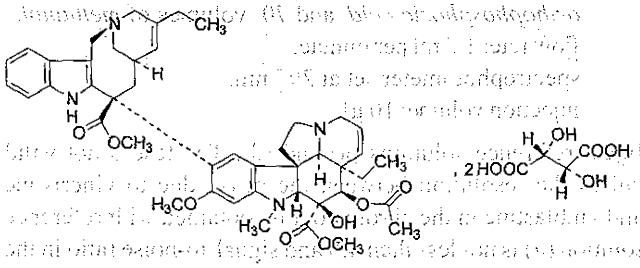
Labelling. The label states (1) the strength in terms of the weight of dried vincristine sulphate contained in it; (2) the names of auxilary substances, if any; (3) that the contents are to be used by intravenous injection only; (4) the storage conditions.

Vinorelbine Tartrate



$C_{45}H_{54}N_4O_8, (C_4H_6O_6)_2$ Mol Wt. 1079.1

Vinorelbine Tartrate is 3′,4′-didehydro-4′-deoxy-*C*′-norvincaleukoblastine ditartrate.

Vinorelbine Tartrate is the ditartrate salt of vinorelbine, a semisynthetic *Vinca* alkaloid; structurally relate to vinblastine.

Vinorelbine Tartrate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{45}H_{54}N_4O_8.2C_4H_6O_6$, calculated on the anhydrous basis.

Category. Anticancer.

Description. A white to yellow or light brown amorphous powder.

CAUTION — Vinorelbine Tartrate is cytotoxic; extra care required to prevent inhaling particles and exposing the skin to it.

Identification

A. Dissolve 10 mg in 5 ml of *water*, add 0.5 ml of 5 *M sodium hydroxide*, and extract with 5 ml of *methylene chloride*. Filter the organic layer through *anhydrous sodium sulphate*, and evaporate to dryness.

Determine by Infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *vinorelbine tartrate IPRS* treated in the same manner or with the reference spectrum of vinorelbine tartrate.

B. In the test for Related substances, the principal peak in the chromatogram of the test solution corresponds to peak in the chromatogram obtained with the refrence solution (a).

C. It gives the reactions for tartrate (2.3.1).

Tests

Appearance of solution. A 1.0 per cent w/v solution is clear (2.4.1).

Light absorption (2.4.7). The absorbance of 1.0 per cent w/v solution, at about 420 nm is not more than 0.03.

pH (2.4.24). 3.3 to 3.8, determined on a 1.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 35 mg of the substance under examination in 25 ml of the mobile phase.

Reference solution (a). A 0.14 per cent w/v solution of *vinorelbine tartrate IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system as described under Assay.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram 3 times the retention time of principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.3 times the area of the peak in the chromatogram obtained with reference solution (b) (0.3 per cent) and the sum of areas of all the secondary peaks is not more than the area of the peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore any secondary peak having area less than 0.1 per cent.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 4.0 per cent, determined on 0.1 g.

Bacterial endotoxins (2.2.3). Not more than 1.5 Endotoxin Unit per mg of vinorelbine base.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 10^2 CFU per g and total fungal count is not more than 50 CFU per g. 1 g is free from *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacteria* and *Clostridia*. 10 g is free from *Salmonella* and *Shigella*.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 35 mg of the substance under examination in 25.0 ml of mobile phase.

Reference solution. A 0.14 per cent w/v solution of *vinorelbine tartrate IPRS* in mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature 40°,

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– mobile phase: 62 volumes of *methanol* containing 1.22 g of *sodium 1-decane sulphonate* and 38 volumes of *phosphate buffer solution*, prepared by dissolving 6.9 g of *monobasic sodium phosphate* in 900 ml of *water*, adjusted to pH 4.2 with *orthophosphoric acid* and diluted to 1000 ml with *water*;
– flow rate: 1 ml per minute,
– spectrophotometer set at 267 nm,
– injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{45}H_{54}N_4O_8.2C_4H_6O_6$.

Storage. Store protected from light, in a deep freezer (below -18°).

Vinorelbine Injection

Vinorelbine Tartrate Injection

Vinorelbine Injection is a sterile solution of vinorelbine tartrate in Water for Injection.

Vinorelbine Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of vinorelbine, $C_{45}H_{54}N_4O_8$.

Usual strength. 10 mg per ml.

Description. A clear, colourless to pale yellow solution.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. When examined in the range 220 nm to 380 nm (2.4.7), a solution containing 0.01 per cent w/v of Vinorelbine Tartrate, exhibits the maxima at about 267 nm.

Tests

pH (2.4.24). 3.0 to 3.8.

Light absorption. The absorbance of the injection at about 420 nm (2.4.7), is not more than 0.06.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of injection containing 10 mg of Vinorelbine to 10 ml with the mobile phase.

Reference solution (a). A 0.14 per cent w/v solution of *vinorelbine tartrate IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). A solution containing 0.14 per cent w/v of *vinorelbine tartrate IPRS* and 0.001 per cent w/v of *vinorelbine impurity A IPRS* in *water*. Expose a portion of the solution in a suitable xenon lamp for about 1 hour, in order to generate an additional degradation product.

Chromatographic system as described under Assay.

The relative retention time with reference to vinorelbine for 3',4',7,8-tetradehydro-3,4'-dideoxy-3,6-epoxy-6,7-dihydro-C'-norvincaleukoblastine (photodegradation product) is about 0.8, for vinorelbine impurity A (4-*O*-deacetylvinorelbine) is about 1.2.

Inject reference solutions (a) and (c). The test is not valid unless the relative retention between vinorelbine tartrate and vinorelbine impurity A is not less than 1.1 in the chromatogram obtained with reference solution (c) and relative standard deviation for replicate injection is not more than 2.0 in the chromatogram obtained with reference solution (a).

Inject reference solution (b) and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak corresponding to vinorelbine impurity A is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent); the area of any peak corresponding to photodegradation product is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), the area of any other secondary peak is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

Other tests. Comply with the tests stated under Parenteral Preparation (Injections).

Bacterial endotoxins (2.2.3). Not more than 3.0 Endotoxin Units per mg of vinorelbine.

Sterility (2.2.11). Complies with the test for sterility.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Accurately measured volume of injection containing 10 mg of Vinorelbine, diluted to 10.0 ml with *water*.

Reference solution. A 0.14 per cent w/v solution of *vinorelbine tartrate IPRS* in *water*.

Chromatographic system
– a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
– column temperature: 40°,

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Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{21}NO_7$ in the tablets.

Storage. Store protected from moisture at a temperature not exceeding 30°.

Labelling. The label states that the tablets should be dispersed in water immediately before use.

Voglibose Tablets

Voglibose Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of voglibose, $C_{10}H_{21}NO_7$.

Usual strengths. 0.2 mg; 0.3 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Disperse 1 intact tablet in the mobile phase with the aid of ultrasound and dilute with the mobile phase to obtain a solution containing 0.002 per cent w/v of Voglibose.

Calculate the content of $C_{10}H_{21}NO_7$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Disperse 10 intact tablets in the mobile phase, with the aid of ultrasound and dilute with the mobile phase to obtain a solution containing 0.002 per cent w/v of Voglibose.

Reference solution. A 0.002 per cent w/v solution of *voglibose IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with polyamine chemically bonded to cross linked polyvinyl alcohol polymer (5 µm) (Such as Shodex Asahipak Amine P-50-4D),
- column temperature: 25°,
- mobile phase. a mixture of 1 volume of phosphate buffer pH 6.5 prepared by dissolving 1.56 g of *sodium dihydrogen phosphate dihydrate* in *water* to make 500 ml (solution A). Dissolve 3.58 g of *disodium hydrogen phosphate dodecahydrate* in *water* to make 500 ml (solution B). To a volume of solution A, add solution B until the mixture is adjusted to pH 6.5 and 2 volumes of *acetonitrile*,
- flow rate: 0.4 ml per minute,
- detector. spectrofluorometer,
- excitation wavelength. 350 nm and emission wavelength. 430 nm,
- injection volume: 100 µl.
- reaction coil temperature. 100°, (use a teflon tube 10 m in length and 0.5 mm in side diameters reaction coil).
- cooling coil temperature: 15°, (use a stainless steel tube 7 m in length and 0.25 mm inside diameter as cooling coil).
- flow rate of fluorescence reagent. 0.4 ml per minute, (prepared by dissolving about 6.25 g of taurine and 2.56 g of sodium periodate in 1000 ml of *water*).

The retention time of the principal peak is about 20 minutes. (Adjust the flow rate of mobile phase and fluorescent reagent to achieve the retention time of 20 minutes).

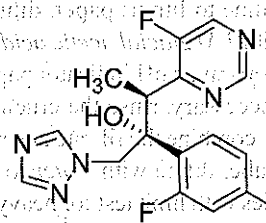
Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor for is not more than 1.5 and the relative standard deviation for replicate injections of is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{21}NO_7$ in tablets.

Storage. Store protected from moisture at a temperature not exceeding 30°.

Voriconazole



$C_{16}H_{14}F_3N_5O$ Mol. wt. 349.3

Voriconazole is (2*R*,3*S*)-2-(2,4-difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1-(1*H*-1,2,4-triazol-1-yl)butan-2-ol.

Voriconazole contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{16}H_{14}F_3N_5O$, calculated on the anhydrous basis.

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Category. Antifungal.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *voriconazole IPRS* or with the reference spectrum of voriconazole.

B. Enantiomeric purity (see Tests).

Tests

Appearance of solution. A 2.5 per cent w/v solution in 10.3 per cent w/v of *hydrochloric acid* is clear (2.4.1) and colourless (2.4.1).

Related substances. Determined by liquid chromatography (2.4.14).

Test solution (a). Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Test solution (b). Dilute 5.0 ml of test solution (a) to 100.0 ml with the mobile phase.

Reference solution (a). A 0.0025 per cent w/v solution of *voriconazole IPRS* in the mobile phase.

Reference solution (b). Dissolve 100 mg of the substance under examination in 10.0 ml of a 4 per cent w/v solution of *sodium hydroxide* and dilute to 20.0 ml with the mobile phase. Allow to stand for 30 minutes. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase (*in situ* degradation to obtain voriconazole impurities A and C).

Reference solution (c). Dilute 1.0 ml of test solution (a) to 100.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase and mix well.

Reference solution (d). A 0.0001 per cent w/v solution of *voriconazole impurity B IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to prorous silica (4µm) (Such as Nova-Pak C18),
- column temperature: 35°,
- mobile phase: a mixture of 15 volumes of *acetonitrile*, 30 volumes of *methanol* and 55 volumes of a 0.19 per cent w/v solution of *ammonium formate*, adjusted to pH 4.0 with *anhydrous formic acid* while stirring continuously,
- flow rate: 1 ml per minute,
- spectrophotometer at 256 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Voriconazole impurity A[1]	0.25	0.7
Voriconazole impurity C[2]	0.3	0.7
Voriconazole impurity B[3]	0.6	2.1
Voriconazole (Retention time: about 8 minutes)	1.0	—

[1]1-(2,4-difluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethanone,

[2]4-ethyl-5-fluoropyrimidine,

[3](2*RS*,3*SR*)-2-(2,4-difluorophenyl)-3-pyrimidin-4-yl-1-(1*H*-1,2,4-triazol-1- yl)butan-2-ol.

Use the chromatogram obtained with reference solution (b) to identify the peaks due to voriconazole impurities A and C and use the chromatogram obtained with reference solution (d) to identify the peak due to voriconazole impurity B.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to voriconazole impurity A and voriconazole impurity C is not less than 1.8.

Inject reference solution (c) and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak due to voriconazole impurities A, B and C is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.15 per cent), the area of any other secondary peak not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Enantiomeric purity. Determined by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in 2 ml of *acetonitrile* and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of *voriconazole impurity D IPRS ((2S,3R)-2-(2,4-difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1-(1H-1,2,4-triazol-1- yl)butan-2-ol IPRS)* in 2 ml of *acetonitrile* and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 25 mg of the substance under examination in 2 ml of *acetonitrile*, add 1.0 ml of reference solution (a) and dilute to 50.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

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Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with silica for chiral separation (5 µm),
- mobile phase: a mixture of 18 volumes of *acetonitrile* and 82 volumes of a 0.077 per cent w/v solution of *ammonium acetate*, adjusted to pH 5.0 with *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer at 256 nm,
- injection volume: 20 µl.

The relative retention time with reference to voriconazole (retention time: about 7 minutes) for voriconazole impurity D is about 1.5.

Inject reference solution (b).The test is not valid unless the resolution between the peaks corresponding to voriconazole and voricoazole impurity D is not less than 4.0.

Inject reference solution (c) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak due to voriconazole impurity D is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent).

Impurity E. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 5.0 ml of *methanol* and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 25 mg of *voriconazole impurity E IPRS ([(1R,4S)-7,7-dimethyl-2-oxobicyclo[2.2.1] hept-1-yl]methanesulfonic acid ((±)- 10-camphorsulfonic acid) IPRS)* in 50 ml of *methanol* and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 17 mg of *sodium chloride* in *water* and dilute to 200.0 ml with *water*. To 1.0 ml of the solution, add 1.0 ml of reference solution (a) and 25 ml of *methanol* and dilute to 50.0 ml with the mobile phase.

Reference solution (c). To 1.0 ml of reference solution (a), add 25 ml of *methanol* and dilute to 50.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.0 mm, packed with strongly basic anion exchange resin (8.5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 1500 ml of *water* and 500 ml of *methanol*. Add 175 µl of a 47 per cent w/v solution of *sodium hydroxide*,
- flow rate: 1 ml per minute,
- conductivity detector, use a self-regenerating anion suppressor,
- injection volume: 20 µl.

The relative retention time with reference to voriconazole impurity E (retention time: about 4 minutes) for chloride is about 1.5.

Inject reference solutions (b) and (c). The test is not valid unless in the chromatogram obtained with reference solution (b) the resolution between the peaks due to voriconazole impurity E and chloride is not less than 3.5 and in the chromatogram obtained with reference solution (c) the tailing factor for the peak due to voriconazole impurity E is not more than 1.7.

Inject reference solution (c) and the test solution. Run the chromatogram twice the retention time of the peak due to voriconazole impurity E. The area of any peak due to voriconazole impurity E is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Heavy metals (2.3.13). Dissolve 2.0 g in 20 ml of a mixture of 30 volumes of *water* and 70 volumes of *acetone*. 12 ml of the solution complies with the limit test for heavy metals, Method D (10 ppm) using 1.0 ml of *lead standard solution (10 ppm pb)*.

Water (2.3.43). Not more than 0.4 per cent, determined on 1.0 g.

Sulphated ash (2.3.18). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances with the following modifications.

Inject reference solution (a) and test solution (b).

Calculate the content of $C_{16}H_{14}F_3N_5O$.

If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Less than 0.2 Endotoxin Unit per mg of voriconazole.

Storage. If intended for use in the manufacture of parenteral preparations, the container should be sterile, airtight, tamper-proof.

Voriconazole Injection

Voriconazole Injection is a sterile material consisting of Voriconazole with or without buffering agents and other excipients. It is filled in a sealed container.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of the liquid stated on the label, immediately before use.

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The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Voriconazole Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of voriconazole, $C_{16}H_{14}F_3N_5O$.

Usual strength. 200 mg per vial.

Description. A white powder.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powder for Injections) and with the following requirements.

CAUTION — Voriconazole is anti fungal agent, care is required to prevent inhaling and exposing the skin to it.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 5.0 to 7.0, determined in a 1.0 per cent w/v solution in *water*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Mix the content of 10 containers. Dissolve a quantity of powder to obtain a solution containing 0.4 per cent w/v of voriconazole in mobile phase. Further dilute 5.0 ml of the solution to 20.0 ml with mobile phase.

Reference solution (a). Dissolve 50.0 mg of *voriconazole IPRS* in 5.0 ml *1 M sodium hydroxide*, dilute to 10.0 ml with the mobile phase. Sonicate if necessary. Allow to stand for 30 minutes. Further dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). A solution containing 0.0005 per cent w/v of *voriconazole impurity B IPRS* in the mobile phase.

Reference solution (c). A solution containing 0.0005 per cent w/v of *voriconazole IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 15cm x 4.6 mm, packed with octadecysilane bonded to porous silica (3.0 µm),
- column temperature: 35°,
- mobile phase: a mixture of 530 volumes of a buffer solution prepared by dissolving 1.9 g of *ammonium formate* in 1000 ml of *water*, adjusted to pH 4.0 with *formic acid*, 157 volumes of *acetonitrile* and 313 volumes of *methanol*,
- flow rate: 1.4 ml per minute,
- spectrophotometer set at 256 nm,
- injection volume: 20µl.

Name	Relative retention time	Correction factor
Impurity A[1]	0.25	0.7
Impurity B[2]	0.6	2.1
Impurity C[3]	0.3	0.7
Voriconazole	1.0	—

[1]1-(2,4-Difluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethanone,

[2](2RS,3SR)-2-(2,4-Difluorophenyl)-3-pyrimidin-4-yl-1-(1H-1,2,4-triazol-1-yl)butan-2-ol,

[3]4-Ethyl-5-fluoropyrimidine.

Inject reference solution (a). The test is not valid unless the resolution between impurity A and impurity C is not less than 1.8. Inject reference solution (b) to identify impurity B.

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent) and sum of areas of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent).

Bacterial endotoxins (2.2.3). Not more than 0.1 Endotoxins Unit per mg of voriconazole (for intravenous bolus injection) and not more than 1.5 Endotoxins Units per mg of voriconazole (for intravenous infusion).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Mix the content of 10 containers. Dissolve a quantity of the powder to obtain a solution containing 0.2 per cent w/v of Voriconazole in the mobile phase. Further dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.004 per cent w/v solution of *voriconazole IPRS* in the mobile phase.

Chromatographic systems

- a stainless column 25 cm x 4.6 mm, packed with octadecysilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 520 volumes of a buffer solution prepared by dissolving 11.62 g *tetramethylethyldiamine* in 1000 ml *water*, adjusted to pH 7.0 with *orthophosphoric acid* and 480 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2500 theoretical plates, and the tailing factor is not more than 2.0.

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Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{14}F_3N_5O$ in the injection.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states (1) direction for constituting the solution; (2) the name of any added buffering agent or pharmaceutical aids; (3) strength in terms of the equivalent amount of Voriconazole.

Voriconazole Tablets

Voriconazole Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of voriconazole, $C_{16}H_{14}F_3N_5O$.

Usual strengths. 50 mg; 200 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 75 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate and dilute, if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *voriconazole IPRS* in *methanol* and dilute with the dissolution medium to obtain a solution of the same concentration as that of the test solution.

Use chromatographic system as described under Assay.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{14}F_3N_5O$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{16}H_{14}F_3N_5O$.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Use freshly prepared solutions.

Solvent mixture. 50 volumes of *water* and 50 volumes of *acetonitrile*.

Test solution. Weigh and powder 20 tablets. Dissolve a quantity of the powder containing 50 mg of Voriconazole in 15 ml of the solvent mixture with the aid of ultrasound for 10 minutes and dilute to 100.0 ml with the solvent mixture and filter.

Reference solution. A 0.00025 per cent w/v solution of *voriconazole IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octydecasilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 70 volumes of *0.04 M ammonium dihydrogen phosphate*, adjusted to pH 6.0 with *ammonia* and 30 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 255 nm,
- injection volume. 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *water* and *acetonitrile*.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 50 mg of Voriconazole in 75 ml of the solvent mixture with the aid of ultrasound for 10 minutes and dilute to 100.0 ml with the solvent mixture and filter. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.005 per cent w/v solution of *voriconazole IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of *0.04 M ammonium dihydrogen phosphate*, adjusted to pH 6.0 with *ammonia* and 50 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 255 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{14}F_3N_5O$ in the tablets.

Storage. Store at a temperature not exceeding 30°.

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W

Warfarin Sodium 3961
Warfarin Sodium Clathrate 3962
Warfarin Tablets 3963
Purified Water 3964
Water for Injections 3965
Water for Injections in Bulk 3965
Sterile Water for Inhalation 3966
Sterile Water for Injections 3966
Wool Fat 3967
Hydrous Wool Fat 3968

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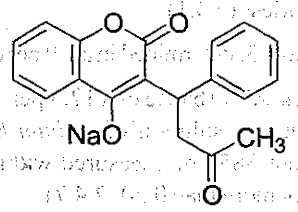
Warfarin Sodium



$C_{19}H_{15}NaO_4$ Mol. Wt. 330.3

Warfarin Sodium is sodium 2-oxo-3-[(1*RS*)-3-oxo-1-phenylbutyl]-2*H*-1-benzopyran-4-olate.

Warfarin Sodium contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{19}H_{15}NaO_4$, calculated on the anhydrous basis.

Category. Anticoagulant.

Description. A white powder; hygroscopic.

Identification

Test A may be omitted if tests B, C, D and E are carried out. Tests B and D may be omitted if tests A, C and E are carried out.

A. Dissolve 1 g in 25 ml of *water*, add 2 ml of *2 M hydrochloric acid* and filter. Wash the residue with *water* and dry over *phosphorus pentoxide*.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *warfarin sodium IPRS* treated in the same manner or with the reference spectrum of warfarin.

B. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (c).

C. Dissolve 1 g in 10 ml of *water*, add 5 ml of *nitric acid* and filter. To the filtrate add 2 ml of *potassium dichromate solution*, shake for 5 minutes and allow to stand for 20 minutes; the solution is not greenish blue when compared with a blank.

D. The residue obtained in test A, after washing with *water* and drying at 105°, melts at 159° to 163° (2.4.21).

E. The filtrate obtained in test A gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution is clear (2.4.1), and colourless (2.4.1).

pH (2.4.24). 7.6 to 8.6, determined in a 1.0 per cent w/v solution.

Phenolic ketones. Absorbance of a 12.5 per cent w/v solution in a 5.0 per cent w/v solution of *sodium hydroxide* at the maximum at about 385 nm, measured within 15 minutes of preparation, is not more than 0.20 (2.4.7).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 25 volumes of *methanol* and 75 volumes of *water*.

Test solution. Dissolve 40 mg of the substance under examination in 50.0 ml of the solvent mixture.

Reference solution (a). Dissolve 2 mg, each of, of *4-hydroxycoumarin* (warfarin impurity B) and *benzalacetone* (warfarin impurity C) in 25 ml of *methanol* and dilute to 100 ml with *water*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution (c). A 0.08 per cent w/v solution of *warfarin sodium IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with spherical nitrile silica gel (5 µm),
- mobile phase: a mixture of 1 volume of *glacial acetic acid*, 25 volumes of *acetonitrile* and 75 volumes of *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 20 µl.

The relative retention time with reference to warfarin for warfarin impurity B is about 0.4 and for warfarin impurity C is about 0.6.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to warfarin impurity B and warfarin impurity C is not less than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to warfarin impurity B and warfarin impurity C is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

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Water (2.3.43). Not more than 4.0 per cent, determined on 0.75 g.

Assay. Dissolve 0.1 g in sufficient *0.01 M sodium hydroxide* to produce 100.0 ml. Dilute 10.0 ml to 100.0 ml with *0.01 M sodium hydroxide* and further dilute 5.0 ml to 50.0 ml with *0.01 M sodium hydroxide*. Measure the absorbance of the resulting solution at the maximum at about 308 nm (2.4.7). Calculate the content of $C_{19}H_{15}NaO_4$ taking 431 as the specific absorbance at 308 nm.

Storage. Store protected from light and moisture.

Warfarin Sodium Clathrate

Warfarin Sodium Clathrate is a clathrate form of Warfarin Sodium consisting principally of Warfarin Sodium and Isopropyl Alcohol in a 2:1 molecular ratio.

Warfarin Sodium Clathrate contains not less than 97.0 per cent and more than 102.0 per cent of $C_{19}H_{15}NaO_4$ calculated on the anhydrous, isopropyl alcohol-free basis and not less than 8.0 per cent and not more than 8.5 per cent of isopropy alcohol.

Category. Anticoagulant.

Description. A white crystalline powder; hygroscopic.

Identification

Test A may be omitted if test B, C, D and E are carried out. Tests B and D may be omitted if tests A, C and E are carried out.

A. Dissolve about 1 g in 25 ml of *water*, add 2 ml of *2 M hydrochloric acid* and filter. Wash the precipitate 5 to 6 times with *water*. Dry the residue over *phosphorus pentoxide*.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *warfarin sodium IPRS* treated in the same manner or with the reference spectrum of warfarin.

B. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (c).

C. Dissolve 1 g in 10 ml of *water*, add 5 ml of *nitric acid* and filter. To the filtrate, add 2 ml of *potassium dichromate solution*, shake for 5 minutes and allow to stand for 20 minutes; the solution is not greenish-blue when compared with a blank.

D. The residue obtained in test A, after washing with *water* and drying at 105° melts at 159° to 163°.

E. The filtrate obtained in test A gives the reactions of sodium salts (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution is clear (2.4.1), and colourless (2.4.1).

pH (2.4.24). 7.2 to 8.3, determined in a 1.0 per cent w/v solution.

Phenolic ketones. Absorbance of a 12.5 per cent w/v solution in a 5.0 per cent w/v solution of *sodium hydroxide* at the maximum at about 385 nm, measured within 15 minutes of preparation, is not more than 0.20 (2.4.7).

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 25 volumes of *methanol* and 75 volumes of *water*.

Test solution. Dissolve 40 mg of the substance under examination in 50.0 ml of the solvent mixture.

Reference solution (a). Dissolve 2 mg, each of, *4-hydroxycoumarin* (warfarin impurity B) and *benzalacetone* (warfarin impurity C) in 25 ml of *methanol* and dilute to 100 ml with *water*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution (c). A 0.08 per cent w/v solution of *warfarin sodium IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with spherical nitrile silica gel (5 µm),
- mobile phase: a mixture of 1 volume of *glacial acetic acid*, 25 volumes of *acetonitrile* and 75 volumes of *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 260 nm,
- injection volume: 20 µl.

The relative retention time with reference to warfarin for warfarin impurity B is about 0.4 and for warfarin sodium impurity C is about 0.6.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to warfarin impurity B and warfarin impurity C is not less than 2.0.

Inject reference solution (b) and the test solution. Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution the area of the peak due to warfarin impurities B and C, each of, is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference

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solution (b) (0.3 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Isopropyl alcohol. Determine by gas chromatography (2.4.13).

Test solution. Dissolve 0.5 g of the substance under examination in sufficient *water* to produce 10 ml.

Reference solution (a). A solution containing 5.0 per cent w/v of the substance under examination and 0.5 per cent v/v of *propan-1-ol* (internal standard).

Reference solution (b). A solution containing 0.5 per cent v/v, each of, *propan-2-ol* and the internal standard.

Chromatographic system:
- a glass column 1.5 m × 4 mm, packed with porous polymer beads (125 to 150 mm) (Such as Porapak Q),
- temperature:
  column 150°,
  inlet port at 180° and detector at 200°,
- flow rate: 40 ml per minute, using nitrogen as the carrier gas.

Inject reference (b). The column temperature may be varied so that the resolution between propan-1-ol and propanol-2-ol is not less than 1.5, the tailing factor for the propan-2-ol is not more than 2.0, the tailing factor for the propan-1-ol is not more than 2.0 and the relative standard deviation of the ratio of the area due to the peak of propanol-2-ol to that due to propan-1-ol for five replicate injections of reference solution (b) is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of isopropyl alcohol w/w, assuming the weight per ml at 20° to be 0.785 g.

Water (2.3.43). Not more than 0.3 per cent, determined on 2.5 g.

Assay. Dissolve 0.1 g in sufficient *0.01 M sodium hydroxide* to produce 100.0 ml. Dilute 10.0 ml to 100.0 ml with *0.01 M sodium hydroxide* and further dilute 5.0 ml to 50.0 ml with *0.01 M sodium hydroxide*. Measure the absorbance of the resulting solution at the maximum at about 308 nm (2.4.7). Calculate the content of $C_{19}H_{15}NaO_4$ taking 431 as the specific absorbance at 308 nm.

Storage. Store protected from light and moisture.

Warfarin Tablets

Warfarin Sodium Tablets

Warfarin Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of warfarin sodium, $C_{19}H_{15}NaO_4$.

Usual strengths. 1 mg; 3 mg; 5 mg; 10 mg.

Identification

A. Extract a quantity of the powdered tablets containing 0.1 g of Warfarin Sodium with 30 ml of *water*, add 0.1 ml of *2 M hydrochloric acid*, filter, wash the precipitate with water and dry. Warm the residue gently with 3 ml of *ethanol (95 per cent)*, filter and add the filtrate to 25 ml of *water* containing 0.1 ml of *2 M hydrochloric acid*. Filter, wash the precipitate with water and dry it at 105°.

On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *warfarin sodium IPRS* treated in the same manner or with the reference spectrum of warfarin.

B. The final residue obtained in test A melts at about 159° (2.4.21).

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium: 900 ml of a 0.68 per cent w/v solution of *potassium dihydrogen phosphate* with the pH adjusted to 6.8 by the addition of *1 M sodium hydroxide*,

Speed and time: 100 rpm and 45 minutes.

For tablets containing 2 mg or less of warfarin sodium, use three tablets for each test; for tablets containing more than 2 mg of warfarin sodium, use a single tablet for each test.

Withdraw a suitable volume of the medium and filter. Measure the absorbance of a layer of suitable thickness of the filtrate, suitably diluted if necessary, at the maxima at about 307 nm and 360 nm (2.4.7), and calculate the difference between the two absorbances (DA). Calculate the total content of warfarin sodium, $C_{19}H_{15}NaO_4$, in the medium taking 428 as the specific absorbance value of DA.

Q. Not less than 70 per cent of the stated amount of $C_{19}H_{15}NaO_4$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture: 75 volumes of *water* and 25 volumes of *methanol*.

Test solution. Disperse a quantity of the powdered tablets containing 10 mg of Warfarin Sodium in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution (b). A solution containing 0.002 per cent w/v, each of, *4-hydroxycoumarin* and (E)-4-phenylbut-3-en-2-one in the solvent mixture.

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Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with cyano group bonded to porous silica (5 µm) (Such as Waters spherisorb CN),
- mobile phase: a mixture of 1 volume of *glacial acetic acid*, 15 volumes of *acetonitrile* and 85 volumes *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 283 nm,
- injection volume: 50 µl.

Name	Relative retention time	Correction factor
4-hydroxycoumarin	0.4	0.5
(E)-4-phenylbut-3-en-2-one	0.6	0.4
Warfarin	1.0	---

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to 4-hydroxycoumarin and (E)-4-phenylbut-3-en-2-one is not less than 2.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to 4-hydroxycoumarin and (E)-4-phenylbut-3-en-2-one, each of, is not more than 1.5 times the area of principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent), the area of any other secondary peak is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

Uniformity of content. Comply with the test stated under Tablets.

Determine by liquid chromatography (2.4.14).

Test solution. Shake one tablet with 10 ml of *0.01 M sodium hydroxide* for 15 minutes, add 10 ml of a 2 per cent v/v solution of *glacial acetic acid* in *acetonitrile*, centrifuge for 10 minutes and use the clear supernatant liquid.

Reference solution. Dissolve a quantity of *warfarin sodium IPRS* in equal mixture of *0.01M sodium hydroxide* and 2 per cent v/v solution of *glacial acetic acid* in *acetonitrile* and dilute to obtain a solution having a known concentration similar to the test solution.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 55 volumes of *acetonitrile*, 45 volumes of *water* and 1 volume of *glacial acetic acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 283 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution.

Determine the content of $C_{19}H_{15}NaO_4$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Shake a quantity of the powder containing 5 mg of Warfarin Sodium in 50 ml of *0.01 M sodium hydroxide* for 15 minutes and dilute to 100.0 ml with 2 per cent v/v solution of *glacial acetic acid* in *acetonitrile*, centrifuge for 10 minutes and use the clear supernatant liquid.

Reference solution. Dilute 5.0 ml of 0.01 per cent w/v solution of *warfarin IPRS* in *0.01 M sodium hydroxide* to 10.0 ml with the mobile phase.

Use chromatographic system as described under Uniformity of content.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0, and the relative standard deviation for replicate injection is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{15}NaO_4$ in the tablets.

Storage. Store protected from light.

Labelling. When the active ingredient is warfarin sodium clathrate the quantity is stated in the terms of equivalent amount of warfarin sodium.

Purified Water

H_2O Wt. 18.0

Purified Water is prepared by means of ion-exchange, reverse-osmosis, distillation, or by any other appropriate means from potable water that complies with all relevant specifications.

During its production, subsequent storage and distribution, adequate measures are taken to ensure that the microbial quality is controlled and counts do not proliferate. To detect any adverse trend, appropriate alert and action limits are set and followed. The adequacy of purification measures is indicated through conductivity displayed on-line or conducted off line (2.4.9). It contains no added substances.

Microbial contamination (2.2.9). (For monitoring purpose). Total viable count not more than 100 cfu per ml. Specified pathogens should be absent.

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Category. Pharmaceutical aid (solvent).

Description. A clear, colourless and odourless liquid.

Tests

Acidity or alkalinity. To 10 ml, freshly boiled and cooled in a borosilicate glass flask, add 0.05 ml of *methyl red solution*; the resulting solution is not red. To 10 ml add 0.1 ml of *bromothymol blue solution*; the resulting solution is not blue.

Heavy metals (2.3.13). Evaporate 150 ml to 15 ml; 12 ml of the solution complies with the limit test for heavy metals, Method D (0.1 ppm). Use *lead standard solution (1 ppm Pb)* to prepare the standard. Alternatively procedure as per ICP/IC/AAS may be used.

NOTE — If purified water complies with the requirements for conductivity prescribed for water for Injection in bulk, it is not necessary to carry out the test for heavy metals.

Nitrates. To 5 ml in a test-tube immersed in ice add 0.4 ml of a 10 per cent w/v solution of *potassium chloride*, 0.1 ml of *diphenylamine solution* and, dropwise with shaking, 5 ml of *nitrogen free sulphuric acid*. Transfer the tube to a water-bath at 50° and allow to stand for 15 minutes. Any blue colour in the solution is not more intense than that in a solution prepared at the same time and in the same manner using a mixture of 4.5 ml of *nitrate-free water* and 0.5 ml of *nitrate standard solution (2 ppm NO_3)* (0.2 ppm).

Total Organic Carbon or Oxidisable Substances. Carry out the test for total organic carbon (2.4.30) with a limit of 0.5 mg per litre (500 ppb) or alternatively the following test for oxidisable substances, to 100 ml add 10 ml *1 M sulphuric acid* and 0.1 ml of *0.02 M potassium permanganate* and boil for 5 minutes; the solution remains faintly pink.

Conductivity (2.4.9). Meets the requirements of the test.

Purified Water intended for use in the manufacture of dialysis solutions complies with the following additional requirements.

Aluminium (2.3.8). Not more than 10 ppb, determined using the following solutions.

Test solution. To 400 ml of the water under examination, add 10 ml of *acetate buffer solution pH 6.0* and 100 ml of *distilled water*.

Reference solution. Mix 2 ml of *aluminium standard solution (2 ppm Al)*, 10 ml of *acetate buffer solution pH 6.0* and 98 ml of *distilled water*.

Blank solution. Mix 10 ml of *acetate buffer solution pH 6.0* and 100 ml of *distilled water*. Tentative AAS/ICP/IC may be used.

Bacterial endotoxins (2.2.3). Not more than 0.25 Endotoxin Unit per ml.

Water for Injections

H_2O Mol. Wt. 18.0

Category. Pharmaceutical aid.

Water for Injections is water intended for use in the preparations of medicines for parenteral administration when water is used as a vehicle (Water for Injections in bulk) and for dissolving or diluting substances or preparations for injectable preparations (Sterile Water for Injections).

Water for Injections in Bulk

Production

Water for Injections is prepared by using purified water. This water is intended for use in the preparations of medicines for parenteral administration or for ophthalmic preparations. It is used as a vehicle for dissolving or diluting substances or for preparation of Sterile Water for Injections.

It is prepared from Purified Water through distillation (phase separation) process or any other process that is equivalent or superior to distillation in the removal of chemicals and microorganisms such as reverse osmosis, coupled with appropriate techniques. Distillation stills are constructed of neutral glass, quartz or suitable metal & must be suitably maintained to ensure the production of water free from bacterial endotoxin. It is collected and stored in conditions designed to prevent the growth of micro-organisms and to avoid any other re-contamination such as bacterial endotoxin.

During production and subsequent storage, adequate measures are taken to ensure that the microbial quality is controlled and monitored. The total viable count (2.2.9) is 10 CFU per 100 ml, determined by membrane filtration using at least 200 ml of this water is to be maintained. Appropriate action limits are set so as to detect any adverse notice.

Adequacy of water purification measures is demonstrated by following tests.

Microbial contamination (2.2.9). Total viable count not more than 10 CFU per 100 ml.

Total organic carbon (2.4.30). Not more than 0.5 mg per litre (500 ppb).

Conductivity (2.4.9). Meets the requirements of the test.

Description. A clear, colourless and odourless liquid.

Tests

Acidity or alkanity. To 10 ml, freshly boiled and cooled in a borosilicate glass flask, add 0.05 ml of *methyl red solution*;

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the resulting solution is not red. To 10 ml add 0.1 ml of *bromothymol blue solution*; the resulting solution is not blue.

Nitrates. To 5 ml in a test-tube immersed in ice add 0.4 ml of a 10 per cent w/v solution of *potassium chloride*, 0.1 ml of *diphenylamine solution* and, dropwise with shaking, 5 ml of *sulphuric acid*. Transfer the tube to a water-bath at 50° and allow to stand for 15 minutes. Any blue colour in the solution is not more intense than that in a solution prepared at the same time and in the same manner using a mixture of 4.5 ml of *nitrate-free water* and 0.5 ml of *nitrate standard solution (2 ppm NO_3)* (0.2 ppm).

Aluminium (2.3.8) *For water for injections intended for use in the manufacture of dialysis solutions.*

Not more than 10 ppb, determined using the following solutions.

Test solution. To 400 ml of the water under examination add 10 ml of *acetate buffer solution pH 6.0* and 100 ml of *distilled water*.

Reference solution. Mix 2 ml of *aluminium standard solution (2 ppm Al)*, 10 ml of *acetate buffer solution pH 6.0* and 98 ml of *distilled water*.

Blank solution. Mix 10 ml of *acetate buffer solution pH 6.0* and 100 ml of *distilled water*.

Bacterial endotoxins (2.2.3). Not more than 0.25 Endotoxin Unit per ml.

Storage. Store in containers designed to prevent the growth of microorganisms.

Sterile Water for Inhalation

Sterile Water for Inhalation is Water for Injection in Bulk that is distributed in suitable containers of glass or any other material, sealed and sterilized by heat under conditions that ensure that the water complies with the test for bacterial endotoxins. It is free from any added substances. Each container contains a sufficient quantity of Water for Inhalation to permit the withdrawal of nominal volume.

Sterile Water for Inhalation should not be used for parenteral administration or for other sterile dosage forms.

Category. Pharmaceutical aid

Description. A clear, colourless and odourless liquid

Tests

Oxidizable substances. To 100 ml add 10 ml of *1 M sulphuric acid*, and heat to boiling. For Sterile Water for Inhalation in containers having a fill volume of less than 50 ml, add 0.4 ml of *0.02 M potassium permanganate*, and boil for 5 minutes; where the fill volume is 50 ml or more, add 0.2 ml of *0.02 M potassium permanganate*, and boil for 5 minutes; the solution remains faintly pink.

Alternatively perform the test for Total organic carbon (2.4.30).

Total organic carbon (2.4.30). Meets the requirement of the test.

Alternately perform the test for Oxidizable substances.

Conductivity (2.4.9). Meets the requirements of the test.

Sterility (2.2.11). Complies with the test for sterility.

Bacterial Endotoxins (2.2.3). Less than 0.5 Endotoxin Units per ml.

Sterile Water for Injections

Sterile Water for Injections is Water for Injections in bulk that has been distributed in suitable containers of glass or any other material, sealed and sterilised by heat under conditions that ensure that the water complies with the test for bacterial endotoxins. It is free from any added substances. Each container contains a sufficient quantity of Water for Injections to permit the withdrawl of the nominal volume.

Category. Pharmaceutical aid.

Description. A clear, colourless liquid; odourless.

Tests

Appearance of solution. When examined in suitable conditions of visibility, it is clear (2.4.1) colourless (2.4.1) and practically free from suspended particles.

Acidity or alkalinity. To 20 ml, add 0.05 ml of *phenol red solution*. If the solution is yellow, it becomes red on the addition of 0.1 ml of *0.01 M sodium hydroxide*; if red, it becomes yellow on the addition of 0.15 ml of *0.01 M hydrochloric acid*.

Ammonium. For containers with a nominal volume less than 50 ml: maximum 0.6 ppm; for containers with a nominal volume equal to or greater than 50 ml: maximum 0.2 ppm.

Containers with a nominal volume less than 50 ml. To 20 ml, add 1 ml of *alkaline potassium tetraiodomercurate solution* and allow to stand for 5 minutes. When viewed vertically the solution is not more intensely coloured than a solution prepared at the same time by adding 1 ml of *alkaline potassium tetraiodomercurate solution* to a mixture of 4.0 ml of *ammonium standard solution (3 ppm NH_4)* and 16.0 ml of *ammonia-free water* (0.6 ppm).

Containers with a nominal volume equal to or greater than 50 ml. To 20 ml, add 1 ml of *alkaline potassium tetraiodomercurate solution* and allow to stand for 5 minutes. When

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viewed vertically the solution is not more intensely coloured than a solution prepared at the same time by adding 1 ml of *alkaline potassium tetraiodomercurate solution* to a mixture of 4.0 ml of *ammonium standard solution (1 ppm NH_4)* and 16.0 ml of *ammonia-free water* (0.2 ppm).

Calcium and magnesium. To 100 ml, add 2 ml of *ammonia buffer pH 10.0*, 50 mg of *mordant black II mixture* and 0.5 ml of *0.01 M disodium edetate*; a pure blue colour is produced.

Conductivity (2.4.9). Meets the requirements of the test.

Chlorides. To 10 ml, add 1 ml of *2 M nitric acid* and 0.2 ml of *0.1 M silver nitrate*; the appearance of the solution does not change for at least 15 minutes.

For containers with a nominal volume of 100 ml or less, 15 ml complies with the limit test for chlorides (2.3.12) (0.5 ppm), using a standard solution prepared by mixing 1.5 ml of *chloride standard solution (5 ppm Cl)* and 13.5 ml of *water*. For containers with a nominal volume greater than 100 ml, use the following test: to 10 ml add 1 ml of *dilute nitric acid* and 0.2 ml of *silver nitrate solution*. The solution shows no change in appearance for at least 15 minutes.

Nitrates. To 5 ml in a test-tube immersed in ice add 0.4 ml of a 10 per cent w/v solution of *potassium chloride*, 0.1 ml of *diphenylamine solution* and, dropwise with shaking, 5 ml of *sulphuric acid*. Transfer the tube to a water-bath at 50° and allow to stand for 15 minutes. Any blue colour in the solution is not more intense than that in a solution prepared at the same time and in the same manner using a mixture of 4.5 ml of *nitrate-free water* and 0.5 ml of *nitrate standard solution (2 ppm NO_3)* (0.2 ppm).

Sulphates. To 10 ml, add 0.1 ml of *2 M hydrochloric acid* and 0.1 ml of *barium chloride solution*. The appearance of the solution does not change for at least 1 hour.

Aluminium. Maximum 10 ppb, if intended for use in the manufacture of dialysis solutions.

Prescribed solution to 400 ml of the *water* to be examined add 10 ml of acetate buffer solution pH 6.0 and 100 ml of *distilled water*.

Reference solution. Mix 2 ml of *aluminium standard solution (2 ppm Al)*, 10 ml of *acetate buffer solution pH 6.0* and 98 ml of *distilled water*.

Blank solution. Mix 10 ml of *acetate buffer solution pH 6.0* and 100 ml of *distilled water*.

Oxidisable substances. Boil 100 ml with 10 ml of *1 M sulphuric acid*, add 0.4 ml of *0.02 M potassium permanganate* (for Sterile Water for Injections in containers with fill volume of less than 50 ml) or 0.2 ml of *0.02 M potassium permanganate* (for Sterile Water for Injections in containers with fill volume of 50 ml or more) and boil for 5 minutes. If a precipitate forms, cool in an ice-bath to room temperature and filter through a sintered glass filter (porosity No.3). The pink colour of the solution does not disappear completely.

Residue on evaporation. Evaporate 100 ml to dryness on a water-bath and dry the residue to constant weight at 105°. For containers with a nominal volume of 10 ml or less, the residue weighs not more than 4 mg (0.004 per cent) and for containers with a nominal volume greater than 10 ml, the residue weighs not more than 3 mg (0.003 per cent).

Particulate contamination (2.5.9). Complies with the requirements of Method 1 or Method 2.

Bacterial endotoxins (2.2.3). Not more than 0.25 Endotoxin Unit per ml.

Sterility (2.2.11). Complies with the test for sterility.

Storage. Store in single dose containers of not larger than one litre size.

Wool Fat

Anhydrous Lanolin

Wool Fat is purified, anhydrous, waxy material obtained from the wool of sheep. It may contain Butylated Hydroxytoluene as an antioxidant.

Category. Pharmaceutical aid (absorbent ointment base).

Description. A pale yellow, unctuous substance.

Identification

A. To a solution of 0.5 g in 5 ml of *chloroform* add 1 ml of *acetic anhydride* and 0.1 ml of *sulphuric acid*; a green colour develops.

B. To a solution of 50 mg in 5 ml of *chloroform* add 5 ml of *sulphuric acid* and shake; a red colour is produced and a strong fluorescence appears in the lower layer.

Tests

Melting range (2.4.21). 34° to 44°, determined by Method IV. To fill the metal cup, melt the substance under examination on a water-bath, cool to 50°, pour into the cup and allow to stand at 15° to 20° for 24 hours.

Acid value (2.3.23). Not more than 1.0, determined on 5.0 g dissolved in 25 ml of the prescribed mixture of solvents.

Peroxide value (2.3.35). Not more than 20.

Saponification value. (2.3.37). 90 to 105. Heat for 4 hours.

Water-absorption capacity. Weigh 10.0 g into a mortar. Add *water* in quantities of 0.2 to 0.5 ml from a burette and stir vigorously, incorporating all the *water* before proceeding to

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the next addition. The end-point is reached when visible droplets remain that cannot be incorporated; not less than 20 ml of *water* is absorbed.

Water-soluble acidic or alkaline substances. Shake vigorously 5.0 g, previously melted on a water-bath, for 2 minutes with 75 ml of *water* previously heated to 90° to 95°. Allow to cool and filter through filter paper previously washed with *water*. To 60 ml of the filtrate, which may not be clear, add 0.25 ml of *bromothymol blue solution*. Not more than 0.2 ml of *0.02 M hydrochloric acid* or 0.15 ml of *0.02 M sodium hydroxide* is required to change the colour of the solution.

Water-soluble oxidisable substances. To 10 ml of the filtrate obtained in the test for Water-soluble acidic or alkaline substances add 1 ml of *1 M sulphuric acid* and 0.1 ml of *0.02 M potassium permanganate*; the solution is not completely decolorised within 10 minutes.

Ammonia. To 10 ml of the filtrate obtained in the test for Water-soluble acidic or alkaline substances, add 1 ml of *1 M sodium hydroxide* and boil; the vapours do not turn red *litmus paper* blue.

Chlorides. Boil 1.0 g with 20 ml of *ethanol (90 per cent)* under a reflux condenser for 5 minutes, cool, add 40 ml of *water* and 0.5 ml of *nitric acid* and filter. To the filtrate add 0.15 ml of a 1 per cent w/v solution of *silver nitrate* in *ethanol (90 per cent)*. After 5 minutes, protected from light, any opalescence produced is not more intense than that obtained by adding 0.15 ml of a 1 per cent w/v solution of *silver nitrate* in *ethanol (90 per cent)* to a mixture of 0.2 ml of *0.02 M hydrochloric acid*, 20 ml of *ethanol (90 per cent)*, 40 ml of *water* and 0.5 ml of *nitric acid (150 ppm)*.

Paraffins. Prepare an alumina column 23 cm x 2 cm by adding a slurry of *anhydrous aluminium oxide* and *light petroleum (40° to 60°)* to a glass tube fitted with a tap and containing the light petroleum; the tap and absorbent cotton plugs should be free from grease. Allow to settle and reduce the depth of the solvent above the column to about 4 cm. Dissolve 3.0 g of the substance under examination in 50 ml of warm *light petroleum (40° to 60°)*, cool, pass the solution through the column at a rate of 3 ml per minute and wash with 250 ml of the light petroleum. Distill the combined eluate and washings to low bulk, evaporate to dryness on a water-bath and heat the residue at 105° for periods of 10 minutes until the difference between two successive weighings is not greater than 1 mg; the residue weighs not more than 30 mg.

Butylated hydroxytoluene *(if present)*. Not more than 200 ppm, determined by gas chromatography (2.4.13).

Test solution (a). A 10 per cent w/v solution of the substance under examination in *carbon disulphide*.

Test solution (b). A solution containing 10 per cent w/v of the substance under examination and 0.002 per cent w/v of *methyl n-decanoate* (internal standard) in *carbon disulphide*.

Reference solution. A solution containing 0.002 per cent w/v each of, *butylated hydroxytoluene* and the internal standard.

Chromatographic system

- a glass column 1.5 m x 4 mm, packed with silanised diatomaceous support (80 to 100 mesh) (such as Diatomite) impregnated with 10 per cent w/w of silicone gum rubber (methyl) (such as SE-30),
- temperature:
  column 150°,
  inlet port at 180° and detector at 300°,
- flow rate: 40 ml per minute, using nitrogen as the carrier gas.

Calculate the content of butylated hydroxytoluene in the substance under examination from the heights or areas of the peaks due to butylated hydroxytoluene and the internal standard in the chromatograms obtained with test solution (b) and the reference solution.

Sulphated ash (2.3.18). Not more than 0.15 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 1 hour.

Storage. Store protected from moisture.

Labelling. The label states the proportion of any butylated hydroxytoluene present.

Hydrous Wool Fat

Hydrous Wool Fat is a mixture of 75 per cent w/w of Wool Fat and 25 per cent w/w of Purified Water.

Category. Pharmaceutical aid (water-in-oil emulsion ointment base).

Description. A pale yellow, unctuous substance. On heating, it separates at first into two layers; with continued heating with stirring, water is driven off and the residue which is transparent while warm, cools to form a yellowish, tenacious, soft mass.

Identification

A. To a solution of 0.5 g in 5 ml of *chloroform* add 1 ml of *acetic anhydride* and 0.1 ml of *sulphuric acid*; a green colour develops.

B. To a solution of 50 mg in 5 ml of *chloroform* add 5 ml of *sulphuric acid* and shake; a red colour is produced and a strong fluorescence appears in the lower layer.

Tests

Melting range (2.4.21). 34° to 44°, determined by Method IV. To fill the metal cup, melt the substance under examination on

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a water-bath, cool to about 50°, pour into the cup and allow to stand at 15° to 20° for 24 hours.

Acid value (2.3.23). Not more than 1.0, determined on 5.0 g dissolved in 25 ml of the prescribed mixture of solvents.

Paraffins. Prepare an alumina column 23 cm x 2 cm by adding a slurry of *anhydrous aluminium oxide* and *light petroleum (40° to 60°)* to a glass tube fitted with a tap and containing the light petroleum; the tap and absorbent cotton plugs should be free from grease. Allow to settle and reduce the depth of the solvent above the column to about 4 cm. Dissolve 3 g of the substance under examination in 50 ml of warm *light petroleum (40° to 60°)*, cool, pass the solution through the column at a rate of 3 ml per minute and wash with 250 ml of the light petroleum. Distill the combined eluate and washings to low bulk, evaporate to dryness on a water-bath and heat the residue at 105° for periods of 10 minutes until the difference between two successive weighings is not more than 1 mg; the residue weighs not more than 30 mg.

Peroxide value (2.3.35). Not more than 15.

Saponification value (2.3.37). 67 to 79. Heat for 4 hours.

Water-absorption capacity. Weigh 10.0 g of the residue obtained in the test for Wool fat content into a mortar. Add *water* in quantities of 0.2 to 0.5 ml from a burette and stir vigorously, incorporating all the *water* before proceeding to the next addition. The end-point is reached when visible droplets remain that cannot be incorporated; not less than 20 ml of water is absorbed.

Water-soluble acidic or alkaline substances. Shake vigorously 6.7 g, previously melted on a water-bath, for 2 minutes with 75 ml of *water* previously heated to 90° to 95°. Allow to cool and filter through filter paper previously washed with *water*. To 60 ml of the filtrate, which may not be clear, add 0.25 ml of *bromothymol blue solution*. Not more than 0.2 ml of *0.02 M hydrochloric acid* or 0.15 ml of *0.02 M sodium hydroxide* is required to change the colour of the solution.

Water-soluble oxidisable substances. 10 ml of the filtrate obtained in the test for Water-soluble acidic or alkaline substances, add 1 ml of *1 M sulphuric acid* and 0.1 ml of *0.02 M potassium permanganate*; the solution is not completely decolorised within 10 minutes.

Ammonia. To 10 ml of the filtrate obtained in the test for Water-soluble acidic or alkaline substances add 1 ml of *1 M sodium hydroxide* and boil; the vapours do not turn red *litmus paper* blue.

Chlorides. Boil 1.0 g with 20 ml of *ethanol (90 per cent)* under a reflux condenser for 5 minutes, cool, add 40 ml of *water* and 0.5 ml of *nitric acid* and filter. To the filtrate, add 0.15 ml of 1.0 per cent w/v solution of *silver nitrate* in *ethanol (90 per cent)*. After 5 minutes, protected from light, any opalescence produced is not more intense than that obtained by adding 0.15 ml of a 1 per cent w/v solution of *silver nitrate* in *ethanol (90 per cent)* to a mixture of 0.2 ml of *0.02 M hydrochloric acid*, 20 ml of *ethanol (90 per cent)*, 40 ml of *water* and 0.5 ml of *nitric acid (150 ppm)*.

Wool fat content. 72.5 to 77.5 per cent.

Weigh 30 g in a tared porcelain dish containing a glass rod, heat on a water-bath with continuous stirring to constant weight and weigh the residue.

Storage. Store protected from light and moisture.

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X

Xanthan Gum 3973
Xylometazoline Hydrochloride 3974
Xylometazoline Nasal Drops 3975
Xylometazoline Hydrochloride and Sorbitol Nasal Drops 3976
Xylose 3977

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Xanthan Gum

Xanthan Gum is high-molecular-mass anionic polysaccharide produced by fermentation of carbohydrates with *Xanthomonas campestris*. It consists of a principal chain of b(1→4)-linked D-glucose units with trisaccharide side chains, on alternating anhydroglucose units, consisting of 1 glucuronic acid unit included between 2 mannose units. Most of the terminal units contain a pyruvate moiety and the mannose unit adjacent to the principal chain may be acetylated at C-6.

Xanthan Gum has a relative molecular mass of approximately 1×10^6. It exists as the sodium, potassium or calcium salt.

Xanthan Gum contains not less than 1.5 per cent of pyruvoyl groups, ($C_3H_3O_2$; Mol. Wt. 71.1), calculate on the dried basis.

Category. Pharmaceutical aid (Excipient).

Description. A white or yellowish-white, free-flowing powder.

Identification

A. Suspend 1 g in 15 ml of *0.1 M hydrochloric acid* in a flask, close the flask with a fermentation bulb containing *barium hydroxide solution* and heat carefully for 5 minutes. The *barium hydroxide solution* shows a white turbidity.

B. To 300 ml of *water*, previously heated to 80° and stirred rapidly with a mechanical stirrer in a 400 ml beaker, add, at the point of maximum agitation, a dry blend of 1.5 g of carob bean gum and 1.5 g of the substance under examination. Stir until the mixture forms a solution, and then continue stirring for 30 minutes or longer. Do not allow the water temperature to drop below 60° during stirring. Discontinue stirring and allow the mixture to stand for at least 2 hours. A firm rubbery gel forms after the temperature drops below 40° but no such gel forms in a 1 per cent control solution of the sample prepared in the same manner but omitting the carob bean gum.

Tests

pH (2.4.24). 6.0 to 8.0 determined in 1 per cent w/v solution.

Viscosity (2.4.28). Not less than 600 mPas.

Add 3.0 g within 45-90 seconds into 250 ml of a 1.2 per cent w/v solution of *potassium chloride* in a 500-ml beaker stirring with a low-pitch propeller-type stirrer rotating at 800 rpm. When adding the substance take care that agglomerates are destroyed. Add an additional quantity of 44 ml of *water*, to rinse any adhering residue from the walls of the beaker. Stir the preparation at 800 rpm for 2 hours whilst maintaining the temperature at 24° ± 1°. Determine the viscosity within 15 min at 24° ± 1° using a rotating viscometer set at 60 rpm and equipped with a rotating spindle 12.7 mm in diameter and 1.6 mm high which is attached to a shaft 3.2 mm in diameter. The distance from the top of the cylinder to the lower tip of the shaft being 25.4 mm, and the immersion depth being 50.0 mm.

2-Propanol. Not more than 750 ppm

Determine by gas chromatography (2.4.13).

Internal standard solution. Dilute 0.50 g of *2-methyl-2-propanol* to 500 ml with *water*.

Test solution. To 200 ml of *water* in a 1000-ml round bottomed flask, add 5.0 g of the substance under examination and 1 ml of a 1.0 per cent w/v emulsion of *dimeticone* in *liquid paraffin*, stopper the flask and shake for 1 hour. Distil 90.0 ml, mix the distillate with 4.0 ml of the internal standard solution and dilute to 100.0 ml with *water*.

Reference solution. Dilute a suitable quantity of *2-propanol*, accurately weighed, with *water* to obtain a solution having a known concentration of *2-propanol* of about 1 mg per ml. To 4.0 ml of the solution add 4.0 ml of the internal standard solution and dilute to 100.0 ml with *water*.

Chromatographic system
- a glass or stainless steel column 1.8 m x 4 mm, packed with styrene-divinylbenzene copolymer (149-177 µm),
- temperature:
  column. 165°,
  injection port and detector 200°,
- a flame ionisation detector,
- flow rate: 30 ml per minute, using nitrogen as the carrier gas.

Inject 5 µl of the reference solution. The test is not valid unless the relative retention times with reference to 2-propanol for 2-methyl-2-propanol is about 1.5.

Inject 5 µl of the reference solution and the test solution .

Other polysaccharides. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel*.

Mobile phase. A mixture of 10 volumes of 1.6 per cent w/v solution of *sodium dihydrogen phosphate*, 40 volumes of *butanol* and 50 volumes of *acetone*.

Test solution. To 10 mg of the substance under examination in a thick-walled centrifuge test tube add 2 ml of a 23 per cent w/v solution of *trifluoroacetic acid*, shake vigorously to dissolve the forming gel, stopper the test tube, and heat the mixture at 120° for 1 hour. Centrifuge the hydrolysate, transfer the clear supernatant liquid carefully into a 50 ml flask, add 10 ml of *water* and evaporate the solution to dryness under reduced pressure. Take up the residue thus obtained in 10 ml of *water* and evaporate to dryness under reduced pressure. Wash 3 times with 20 ml of *methanol* and evaporate under reduced pressure. To the resulting clear film which has no odour of acetic acid, add 0.1 ml of *water* and 1 ml of *methanol*. Centrifuge to separate the amorphous precipitate. Dilute the supernatant liquid, if necessary, to 1 ml with *methanol*.

Reference solution. Dissolve 10 mg of *glucose* and 10 mg of *mannose* in 2 ml of *water* and dilute to 10 ml with *methanol*.

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Apply to the plate 5 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 15 cm. Dry the plate in air and spray with 2 per cent w/v solution of *diphenylamine* in *methanol* to which added 0.5 ml of *aniline* and 2.5 ml of *orthophosphoric acid*. Heat the plate at 120° for 5 minutes and examine in day light. The principal spot in the chromatogram obtained with the test solution shows 2 spots corresponding to the spots due to glucose and mannose in the chromatogram obtained with the reference solution. In addition, 1 weak reddish and 2 faint bluish-grey bands may be visible just above the starting line. 1 or 2 bluish-grey bands may also be seen in the upper quarter of the chromatogram. No other bands are visible.

The test is not valid unless the chromatogram obtained with the reference solution shows two clearly separated greyish-brown spots due to glucose and mannose in the middle third.

Total ash (2.3.19). Not less than 6.5 and not more than 16.0 per cent, determined on 1.0 g.

Loss on drying (2.4.19). Not more than 15.0 per cent, determined on 1.0 g by drying in an oven at 105° for 2.5 hours.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 10^3 CFU per g and total fungal count is not more than 10^2 CFU per g determined by plate count. 1 g is free from *Escherichia coli*.

Assay.

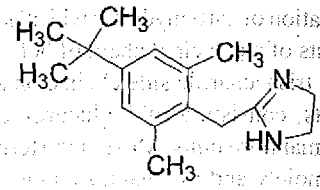
Test solution. Dissolve a quantity of the substance under examination containing 120 mg of the dried substance in 20 ml of *water*.

Reference solution. Dissolve 45 mg of *pyruvic acid* in 500 ml of *water*.

Place 10.0 ml of the test solution in a 50 ml round-bottomed flask, add 20.0 ml of *0.1 M hydrochloric acid* and weigh. Boil on a water-bath under a reflux condenser for 3 hours. Weigh and adjust to the initial mass with *water*. In a separating funnel mix 2.0 ml of the solution with 1.0 ml of *dinitrophenylhydrazine-hydrochloric solution*. Allow to stand for 5 minutes and add 5.0 ml of *ethyl acetate*. Shake and allow the solids to settle. Collect the upper layer and shake with three quantities, each of 5.0 ml, of *sodium carbonate solution*. Combine the aqueous layers and dilute to 50.0 ml with *sodium carbonate solution*, mix. Treat 10.0 ml of the reference solution at the same time and in the same manner as for the test solution.

Immediately measure the absorbance of the two solutions at the maximum at about 375 nm (2.4.7), using *sodium carbonate solution* as the compensation liquid. The absorbance of the test solution is not less than that of the reference solution, which corresponds to a content of pyruvic acid of not less than 1.5 per cent.

Xylometazoline Hydrochloride



$C_{16}H_{24}N_2,HCl$ Mol. Wt. 280.8

Xylometazoline Hydrochloride is 2-(4-*tert*-butyl-2,6-dimethylbenzyl)-2-imidazoline hydrochloride.

Xylometazoline Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{16}H_{24}N_2,HCl$, calculated on the dried basis.

Category. Sympathomimetic amine.

Description. A white or almost white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *xylometazoline hydrochloride IPRS* or with the reference spectrum of xylometazoline hydrochloride.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.05 per cent w/v solution in *0.1 M hydrochloric acid* shows an absorption maximum at about 265 nm and a minimum at about 257 nm with two inflections at about 270 nm and 275 nm; absorbance at about 265 nm, about 0.5.

C. To 1 ml of a 0.05 per cent w/v solution, add 0.2 ml of a 5 per cent w/v solution of *sodium nitroprusside* and 0.1 ml of *5 M sodium hydroxide*, allow to stand for 10 minutes and add 2 ml of *sodium bicarbonate solution*; a violet colour is produced.

D. It gives the reactions of chlorides (2.3.1).

Tests

pH (2.4.24). 5.0 to 6.6, determined in a 5.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of *water*. Allow to stand for 1 hour before injection.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with *water*. Dilute 2.0 ml of the solution to 100.0 ml with *water*.

Reference solution (b). Dissolve 5 mg, each of, *N-(2-aminoethyl)-2-[4-(1,1-dimethylethyl)-2,6- dimethylphenyl] acetamide IPRS (xylometazoline impurity A IPRS)* and the

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substance under examination in 50.0 ml of *water*. Dilute 10.0 ml of the solution to 50.0 ml with *water*.

Reference solution (c). Dilute 5.0 ml of reference solution (b) to 50.0 ml with *water*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a 0.14 per cent w/v solution of *potassium dihydrogen phosphate* adjusted to pH 3.0 with *orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
5	70	30
20	15	85
35	15	85
37	70	30
47	70	30

The relative retention time with reference to xylometazoline for xylometazoline impurity A is about 0.79.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to xylometazoline and xylometazoline impurity A is not less than 2.5.

Inject reference solution (a), (c) and the test solution. In the chromatogram obtained with the test solution the area of the peak due to xylometazoline impurity A is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent) and the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent). The sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Iron (2.4.14). Moisten the residue obtained in the test for Sulphated ash with 5 ml of *hydrochloric acid*, evaporate to dryness and dissolve in sufficient *water* to produce 50 ml. 10 ml of the resulting solution complies with the limit test for iron (50 ppm).

Sulphates (2.3.17). 0.75 g complies with the limit test for sulphates (200 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.5 g in 50 ml of *anhydrous glacial acetic acid*, add 10 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, using *1-naphtholbenzein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02808 g of $C_{16}H_{24}N_2$, HCl.

Storage. Store protected from light and moisture.

Xylometazoline Nasal Drops

Xylometazoline Hydrochloride Nasal Drops; Xylometazoline Hydrochloride Nasal Solution

Xylometazoline Nasal Drops are a solution of Xylometazoline Hydrochloride in Purified Water.

Xylometazoline Nasal Drops contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of xylometazoline hydrochloride, $C_{16}H_{24}N_2$, HCl.

Usual strengths. 0.05 per cent w/v; 0.1 per cent w/v.

Identification

A. To a volume containing 50 mg of Xylometazoline Hydrochloride add 5 ml of *1 M sodium hydroxide*, extract with 10 ml of *dichloromethane*, evaporate to dryness and dissolve the residue in 0.5 ml of *dichloromethane*.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *xylometazoline hydrochloride IPRS* treated in the same manner or with the reference spectrum of xylometazoline.

B. To a volume containing 0.5 mg of Xylometazoline Hydrochloride add 0.2 ml of a 5 per cent w/v solution of *sodium nitroprusside* and 0.1 ml of *5 M sodium hydroxide*, allow to stand for 10 minutes and add 1 ml of *sodium bicarbonate solution*; a violet colour is produced.

Tests

pH (2.4.24). 5.6 to 6.6.

N-(2-Aminoethyl)-4-tert-butyl-2,6-xylylacetamide. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

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Mobile phase. A mixture of 200 volumes of *methanol* and 3 volumes of *strong ammonia solution*.

Test solution. Add a volume containing 10 mg of Xylometazoline Hydrochloride to 30 ml of *water*, add 5 ml of *5 M sodium hydroxide*, mix, extract with three quantities, each of 20 ml, of *dichloromethane*, evaporate the combined extracts to dryness and dissolve the residue in 1 ml of *dichloromethane*.

Reference solution. A 0.03 per cent w/v solution of *N-(2-aminoethyl)-4-tert-butyl-2,6-xylylacetamide IPRS* in *dichloromethane*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air, spray with a solution containing 0.3 g of *ninhydrin* in a mixture of 100 ml of *1-butanol* and 3 ml of *glacial acetic acid*. Heat at 100° for 10 minutes, allow to cool, and spray with *dilute potassium iodobismuthate solution*. Any spot corresponding to *N-(2-aminoethyl)-4-tert-butyl-2,6-xylyl- acetamide* in the chromatogram obtained with test solution is not more intense than the spot in the chromatogram obtained with reference solution.

Other tests. Comply with the tests stated under Nasal Preparations.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Mix the contents of 10 containers. Transfer a suitable volume of the sample containing 10 mg of Xylometazoline Hydrochloride into 50 ml- volumetric flask and dilute to volume with the mobile phase and mix.

Reference solution. Dissolve 20 mg of *xylometazoline hydrochloride IPRS* in 10 ml of *methanol* and dilute to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as hypersil),
- mobile phase: a mixture of 60 volumes of buffer solution prepared by dissolving 2.5 g of *anhydrous ammonium sulphate* in 1000 ml of *water*, adjusted to pH 2.7 with *1M orthophosphoric acid* and 40 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{24}N_2$, HCl in the nasal drops.

Storage. Store protected from light and moisture.

Xylometazoline Hydrochloride and Sorbitol Nasal Drops

Xylometazoline Hydrochloride and Sorbitol Nasal Drops are a solution of Xylometazoline Hydrochloride and Sorbitol solution (70 per cent, non-crystallising) in aqueous vehicle. It may contain sutiable preservative.

Xylometazoline Hydrochloride and Sorbitol Nasal Drops contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of Xylometazoline Hydrochloride, $C_{16}H_{24}N_2$,HCl and 70 per cent Sorbitol Solution (Non crystallizing), $C_6H_{14}O_6$.

Category. Nasal Decongestant.

Usual strengths. Xylometazoline Hydrochloride 0.05 per cent w/v; 0.1 per cent w/v and Sorbitol Solution (70 per cent) 2.0 per cent w/v.

Identification

A. In the Assay for xylometazoline hydrochloride, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

B. To 3.0 ml of a freshly prepared 10 per cent w/v solution of *catechol*, add 6 ml of *sulphuric acid*, cool in ice. Add 0.3 ml of nasal drops and heat gently over a flame for 30 seconds. A pink colour is produced, which becomes deep brownish red.

Tests

pH (2.4.24). 5.6 to 6.2.

Other tests. Comply with the tests stated under Nasal Preparations.

Assay. Determine by liquid chromatography (2.4.14).

For Xylometazoline Hydrochloride —

Test solution. Transfer a weighed qunatity of Nasal Drops in volumetric flask to obtain a solution of 0.01 per cent w/v of Xylometazoline Hydrochloride in mobile phase and filter.

Reference solution. Transfer 100 mg of an accurately weighed qunatity of *xylometazoline hydrochloride IPRS* in 100.0 ml of volumetric flask, add 10 ml of *methanol* and dissolve, then make up the volume with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil HS),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 2.5 g of *anhydrous ammonium*

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sulphate in 1000 ml of *water*, adjusted to pH 2.7 with *1 M orthophosphoric acid* and 40 volumes of *acetonitrile*,

— flow rate: 1 ml per minute,

— spectrophotometer set at 225 nm,

— injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Determine the weight per ml of the sample (2.4.29) and calculate the content of $C_{16}H_{24}N_2,HCl$, weight in volume.

For Sorbitol —

Weigh accurately about 2.0 g of the sample in a 250 ml round-bottom flask, add 20 ml of 2.14 per cent w/v solution of *sodium periodate* and 2 ml of *1 M sulphuric acid*. Boil under a water bath for 15 minutes. Cool, and add 3.0 g of *sodium bicarbonate*, 25 ml of *0.1 M sodium arsenite*, mix and add 5 ml of a 20 per cent w/v solution of *potassium iodide*. Allow to stand for 15 minutes. Titrate with *0.05 M iodine* until the first trace of yellow colour appears.

Carry out blank titration.

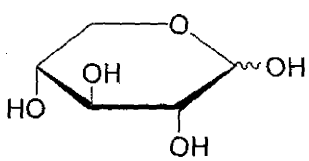
1 ml of *0.05 M iodine* is equivalent to 0.001822 g of $C_6H_{14}O_6$.

Determine the weight per ml of the sample (2.4.29) and calculate the content of $C_6H_{14}O_6$, weight in volume.

Storage. Store protected from light.

Xylose

D-Xylose; D-Xylopyranose



$C_5H_{10}O_5$ Mol. Wt. 150.1

Xylose contains not less than 98.0 per cent and not more than 102.0 per cent of $C_5H_{10}O_5$, calculated on the dried basis.

Category. Diagnostic aid in intestinal malfunction.

Description. Colourless needles or a white, crystalline powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with a suspension of *silica gel G* in a 0.3 per cent w/v solution of *sodium acetate* to form a uniform layer 0.5 mm thick.

Mobile phase. A mixture of 70 volumes of *glacial acetic acid*, 60 volumes of *chloroform* and 10 volumes of *water*.

Test solution. Dissolve 0.5 g of the substance under examination in 10.0 ml of *water*.

Reference solution (a). A 5.0 per cent w/v solution of *xylose IPRS* in *water*.

Reference solution (b). A mixture of equal volumes of the test solution and reference solution (a).

Apply to the plate 2 µl of each solution. Develop the plate in a continuous elution tank for about 4 hours. Dry the plate in warm air, spray with a solution in *acetone* containing 1 per cent w/v solution of *diphenylamine*, 1 per cent v/v of *aniline* and 1 per cent v/v of *phosphoric acid* and heat for 10 minutes at 130°. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The principal spot in the chromatogram obtained with reference solution (b) appears as a single, compact spot.

B. When heated with *potassium cupri-tartrate solution* it produces a copious precipitate of cuprous oxide.

Tests

Appearance of solution. A 10.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and colourless (2.4.1).

Acidity. Dissolve 5.0 g in 50 ml of *carbon dioxide-free water*. Not more than 0.2 ml of *0.1 M sodium hydroxide* is required to neutralise the solution using *dilute phenolphthalein solution* as indicator.

Specific optical rotation (2.4.22). +18.5° to +19.5°, determined at 20° in a 10.0 per cent w/v solution containing 0.4 per cent v/v of *5 M ammonia*.

Arsenic (2.3.10). Dissolve 10.0 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (1 ppm).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method A (20 ppm).

Iron (2.3.14). A solution of 4.0 g in 25 ml of *water* complies with the limit test for iron (10 ppm).

Chlorides (2.3.12). Dissolve 3.0 g in 20 ml of *water*. 5 ml of the resulting solution complies with the limit test for chlorides (330 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 100° at a pressure not exceeding 0.7 kPa.

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Assay. *Carry out the following procedure keeping strict control of time between steps.*

Weigh accurately about 1.0 g of the substance under examination, dissolve in a saturated solution of *benzoic acid* in a 100-ml volumetric flask and dilute to volume with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with the same solvent. To 1.0 ml of the resulting solution, in two different test-tubes, add 5 ml of *4-bromoaniline solution* into each tube and mix. Loosely stopper one tube, place in a water-bath maintained at 70° for 10 minutes, remove, cool rapidly to room temperature and mix. Keep the tube in the dark for 70 minutes and measure the absorbance at the maximum at about 520 nm (2.4.7), using the untreated solution in the second test tube as the blank. Simultaneously, carry out the operation using 1.0 ml of a 0.01 per cent w/v solution of *xylose IPRS* in a saturated solution of *benzoic acid* beginning at the words "add 5 ml of *4-bromoaniline solution*.....".

Calculate the content of $C_5H_{10}O_5$.

Storage. Store protected from moisture.

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Z

Zaleplon 3981
Zaleplon Capsules 3982
Zanamivir 3983
Zidovudine 3985
Zidovudine Capsules 3986
Zidovudine Injection 3987
Zidovudine Oral Solution 3988
Zidovudine Tablets 3989
Zidovudine, Lamivudine and Nevirapine Tablets 3990
Zinc Oxide 3992
Zinc Oxide Cream 3993
Zinc Oxide and Salicylic Acid Paste 3993
Zinc Stearate 3994
Zinc Sulphate Eye Drops 3994
Zinc Undecenoate 3995
Zinc Undecenoate Ointment 3995
Ziprasidone Hydrochloride Monohydrate 3996
Zoledronic Acid 3998
Zoledronic Acid Injection 3998
Zolmitriptan 3999
Zolmitriptan Nasal Spray 4001
Zolmitriptan Tablets 4002
Zolpidem Tartrate 4003
Zolpidem Tartrate Prolonged-release Tablets 4004
Zolpidem Tablets 4005
Zonisamide 4006
Zonisamide Capsules 4007
Zopiclone 4008
Zopiclone Tablets 4009
Zuclopenthixol Acetate 4010

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Sulphated ash (2.3.18). Not more than 0.2 per cent.

Water (2.3.43). Not more than 2.0 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. Equal volumes of *acetonitrile* and of *water.*

Test solution. Dissolve 10 mg of the substance under examination in 100.0 ml of the solvent mixture. Further dilute 5.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution (a). A 0.05 per cent w/v solution of *zaleplon IPRS* and 0.00005 per cent w/v solution, each of, *zaleplon related compound A IPRS* [(*E*)-*N*-{3-[3-(dimethylamino) acryloyl] phenyl}-N-ethylacetamide] and *zaleplon related compound B IPRS* (*N*-[3-(3-cyanopyrazolo[*1,5*-α]pyrimidin-5-yl)phenyl-*N*-ethylacetamide) in the solvent mixture.

Reference solution (b). A 0.005 per cent w/v solution of *zaleplon IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 10 cm × 4.0 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- mobile phase: a mixture of 180 volumes of a buffer solution prepared by dissolving 0.3 g of *ammonium formate* in 1000 ml of *water*, adjusted to pH 4.0 with *formic acid* and 70 volumes of *acetonitrile*,
- flow rate: 1ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 10 µl.

Name	Relative retention time
Zaleplon	1.0
Zaleplon related compound B	1.2

Inject reference solution (a).The test is not valid unless the resolution between zaleplon and zaleplon related compound B is not less than 2.0.

Inject reference solution (b). The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Inject reference solution (b) and the test solution.

Calculate the content of $C_{17}H_{15}N_5O$.

Storage. Store protected from light, at a temperature not exceeding 30°.

Zaleplon Capsules

Zaleplon Capsules contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of zaleplon, $C_{17}H_{15}N_5O$.

Usual strengths. 5mg;10 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle) (Use sinkers, if required).

Medium. 900 ml of *water.*

Speed and time. 75 rpm and 20 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *zaleplon IPRS* in sufficient *methanol* and dilute with the dissolution medium to obtain a solution having a known concentration similar to the expected concentration of the test solution.

Chromatographic system

- a stainless steel column 5.0 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 35°,
- mobile phase: a mixture of 67 volumes of a buffer solution prepared by dissolving 0.77 g *ammonium acetate* in 1000 ml of *water* and 33 volumes of *methanol*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{15}N_5O$ in the medium.

Q. Not less than 85 per cent of the stated amount of $C_{17}H_{15}N_5O$.

Related substances. Determine by liquid chromatography (2.4.14).

Use solvent mixture, test solution and chromatographic system as described under Assay.

Reference solution (a). A 0.05 per cent w/v solution of *zaleplon IPRS* in the solvent mixture.

Reference solution(b). A 0.00005 per cent w/v solution of *zaleplon related compound B IPRS* in reference solution (a).

Reference solution(c). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture. Further dilute 1.0 ml of the solution to 100.0 ml with the same solvent.

Reference solution(d). Dilute 10.0 ml of reference solution (a) in 50.0 ml with the solvent mixture.

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Name	Relative retention time
Cyanopyrazolamine[1,2]	0.16
Zaleplonrelated compound A[2,3]	0.76
Zaleplon	1.0
Zaleplonrelated compound B[2]	1.05
Unknown impurity	---

[1]3-aminopyrazole-4-carbonitrile,

[2]This is a process impurity that is included in the table for identification purposes only. It is controlled in the drug substance and is not to be reported or included in the total degradation products for the drug product,

[3]*N*-[3-(3-cyanopyrazolo[*1,5-α*]pyrimidin-5-yl)phenyl-*N*-ethylacetamide.

Inject reference solution (c). The test is not valid unless the signal-to-noise ratio of the principal peak is not less than 10.0.

Inject reference solution (b).The test is not valid unless the resolution between the peaks due to zaleplon and zaleplon related compound B is not less than 1.5, the tailing factor for zaleplon peak is not more than 1.5 and the relative standard deviation for the replicate injections for zaleplon peak is not more than 2.0 per cent.

Inject reference solution (d) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.002 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent). The sum of the areas of all the peaks is not more than 0.01times the area of the principal peak in the chromatogram obtained with reference solution (d) (1.0 per cent).

Uniformity of content. Complies with the test stated under Capsules.

Determine by liquid chromatography (2.4.14), using the chromatographic system, solvent mixture and reference solution as described under Assay.

Test solution. Transfer one capsule in 50-ml volumetric flask, disperse in minimum quantity of *water* and add sufficient quantity of solvent mixture, shake for 10 minutes, filter. Dilute to volume with the solvent mixture, mix well and filter, rejecting the first few ml of filtrate. Dilute further, if necessary with the solvent mixture.

Calculate the content of $C_{17}H_{15}N_5O$ in the capsule.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 10 volumes of *acetonitrile* and 40 volumes of *water*.

Test solution. Weigh a quantity of the mixed contents of 20 capsules containing about 25 mg of Zaleplon in to a 250-ml volumetric flask, disperse in 50 ml of the solvent mixture, with the aid of ultrasound for 10 minutes and dilute to volume with the same solvent.

Reference solution. A 0.01 per cent w/v solution of *zaleplon IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 0.3 g of *ammonium formate* in 1000 ml of *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate:1.4 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 15 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
30	50	50
31	95	5
40	95	5

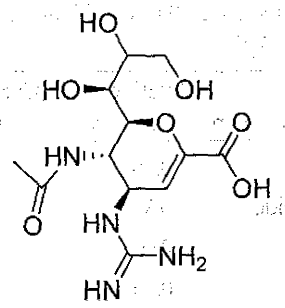
Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{15}N_5O$, in the capsules.

Storage. Store protected from light, at a temperature not exceeding 30°.

Zanamivir



, x H20

$C_{12}H_{20}N_4O_7, xH_2O$ Mol. Wt. 332.3 (anhydrous form)

Zanamivir is *D*-glycero-*D*-galacto-Non-2-enonic acid, 5-(acetylamino)-4-[(aminoiminomethyl)amino]-2,6-anhydro-3,4,5-trideoxy.

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Zanamivir contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{12}H_{20}N_4O_7$, calculated on the anhydrous basis.

Category. Neuraminidase inhibitor.

Description. A white crystalline powder, it contains variable quantities of water.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *zanamivir IPRS* or with the reference spectrum of zanamivir.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak chromatogram obtained with reference solution (a).

Tests

Specific optical rotation (2.4.22). + 36.0° to + 38.0°, determined in 1.0 per cent w/v solution at 20°.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 45 mg of the substance under examination in 40 ml of *water* and dilute to 100.0 ml with *acetonitrile*.

Reference solution. A solution containing 0.045 per cent w/v of *zanamivir IPRS* and 0.0009 per cent w/v of *talo-zanamivir IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with polyamine bonded to cross linked polyvinyl alcohol polymer (5 µm) (Such as Shodex Asahipak NH_2P-50),
- mobile phase: a mixture of 60 volumes of *acetonitrile* and 40 volumes of *0.0075M sulphuric acid*, adjusted to pH 6.2 with *ammonia*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm and 234 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
Imidazole[1]	0.26	0.4
Imidazole carboximidamide[2]	0.30	0.3
O-Triazinyl zanamivir[3]	0.60	—
Zanamivir urea analog[4]	0.70	—
4-Amino zanamivir[5]	0.77	—
4-Biguanide zanamivir[6]	0.83	0.63
Zanamivir	1.0	—
talo-Zanamivir[7]	1.14	—
Zanamivir dimer[8]	2.75	—

[1]*1H*-imidazole (No individual limit, included in the determination of total impurities),

[2]*1H*- imidazole -1-carboximidamide,

[3]5-Acetamido-9-*O*-[4-amino-6-(1*H*-pyrazol-1-yl)-1,3,5-triazin-2-yl]-2,6-anhydro-3,4,5-trideoxy-4-guanidino-*D-glycero-D-galacto*-non-2-enonic acid,

[4]5-Acetamido-9-*O*-[4-amino-6-(1*H*-pyrazol-1-yl)-1,3,5-triazin-2-yl]-2,6-anhydro-3,4,5-trideoxy-4-ureido-*D-glycero-D-galacto*-non-2-enonic acid,

[5]5-Acetamido-9-*O*-[4-amino-6-(1*H*-pyrazol-1-yl)-1,3,5-triazin-2-yl]-2,6-anhydro-3,4,5-trideoxy-4-amino-*D-glycero-D-galacto*-non-2-enonic acid,

[6]5-Acetamido-9-*O*-[4-amino-6-(1*H*-pyrazol-1-yl)-1,3,5-triazin-2-yl]-2,6-anhydro-3,4,5-trideoxy-4-(1-biguanidyl)-*D-glycero-D-galacto*-non-2-enonic acid,

[7]5-Acetamido-2,6-anhydro-3,4,5-trideoxy-4-guanidino-*D-glycero-D-talo*-non-2-enonic acid (No individual limit, included in the determination of total impurities),

[8]4,4'-(2-Amino-4-oxo-1,3,5-triazapent-2-ene-1,5-diyl)bis(5-acetamido-2,6-anhydro-3,4,5-trideoxy-*D-glycero-D-galacto*-non-2-enonic acid.

Inject the reference solution at 234 nm. The test is not valid unless the resolution between the peaks due to talo-zanamivir and zanamivir is not less than 1.5.

Inject the test solution at 210 nm. Calculate the percentage of imidazole impurity by using following expression.

$$\frac{A_I \times C}{A_I \times C + A_Z} \times 100$$

where, A_I = peak response of imidazole;
A_Z = peak response of zanamivir;
C = correction factor.

Inject the test solution at 234 nm. The area of any peak corresponding to imidazole carboximidamide is not more than 0.01 per cent and the area of any peak corresponding to 4-biguanide zanamivir is not more than 0.2 per cent, calculated by using the expression.

$$\frac{A_C \times C}{A_C \times C + A_Z} \times 100$$

Where, A_C = peak response of imidazole carboximidamide or 4-biguanide zanamivir,
A_Z = sum of the responses of all the peaks including the zanamivir,
C = correction factor for imidazole carboximidamide or 4-biguanide zanamivir.

In the chromatogram obtained with the test solution at 234 nm, the area of any peak corresponding to *O*-triazinyl zanamivir is not more than 0.3 per cent, the area of any peak corresponding to zanamivir urea analog and 4-amino zanamivir, each of, is not more than 0.2 per cent, the area of any peak corresponding to zanamivir dimer is not more than 0.5 per cent and the area of any other secondary peak is not more than 0.1 per cent, calculated by area normalization. The sum of all the impurities is not more than 1.2 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). 4.0 per cent to 9.0 per cent, using method 3.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 45 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (a). A 0.0045 per cent w/v solution of *zanamivir IPRS* in the mobile phase.

Reference solution (b). A solution containing 0.00025 per cent w/v of *talo-zanamivir IPRS* and 0.005 per cent w/v *zanamivir IPRS* in the mobile phase.

Use chromatographic system as described under Related substances with the following modification.

- spectrophotometer set at 234 nm,

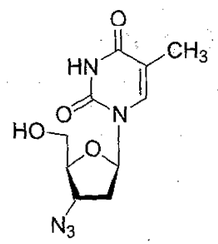
Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to talo-zanamivir and zanamivir is not less than 1.5 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 1.5 in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{12}H_{20}N_4O_7$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Zidovudine



$C_{10}H_{13}N_5O_4$ Mol. Wt. 267.2

Zidovudine is 1-(3-azido-2,3-dideoxy-β-D-ribofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione.

Zidovudine contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{10}H_{13}N_5O_4$, calculated on the anhydrous basis.

Category. Antiretroviral.

Description. A white or almost white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *zidovudine IPRS* or with the reference spectrum of zidovudine.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

C. Melting range (2.4.21). 122° to 125°

Tests

Specific optical rotation (2.4.22). + 60.5° to + 63.0°, determined in a 1.0 per cent w/v solution in *ethanol (95 per cent)*.

Related substances. A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 90 volumes of *dichloromethane* and 10 volumes of *methanol*.

Test solution. Dissolve 0.2 g of the substance under examination in 10.0 ml of *methanol*.

Reference solution. A solution containing 0.01 per cent w/v, each of, *zidovudine IPRS* and *triphenylmethanol* in *methanol*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise to about three-fourths of the height of the plate. Dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot observed in the chromatogram obtained with the test solution corresponding to triphenylmethanol is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.5 per cent). No other secondary spot in the chromatogram obtained with the test solution is more intense than the principal spot in the chromatogram obtained with the reference solution (0.5 per cent).

Spray the plate with a mixture of 0.5 g of *carbazole* in 95 ml of *ethanol (95 per cent)* and 5 ml of *sulphuric acid*, heat for 10 minutes at 120° and compare the intensities of any secondary spots observed in the chromatogram obtained with the test solution with those of the principal spots in the chromatogram obtained with the reference solution. No spot corresponding to triphenylmethanol (R_f value about 2.3 relative to the R_f of

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zidovudine) is more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.5 per cent). No secondary spot in the chromatogram obtained with the test solution is more intense than the principal spot in the chromatogram obtained with the reference solution.

B. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in 100.0 ml of *methanol*.

Reference solution (a). A 0.001 per cent w/v solution of *zidovudine IPRS* in *methanol*.

Reference solution (b). A solution containing 0.1 per cent w/v of *zidovudine IPRS*, 0.001 per cent w/v, each of, *zidovudine impurity B IPRS*, *zidovudine impurity C IPRS*, *stavudine IPRS* and β-thymidine in *methanol*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. *water*
  B. *methanol*,
- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 265 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
15	35	65
30	35	65
35	5	95
40	5	95
45	90	10

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to zidovudine and zidovudine impurity B is not less than 1.5 and the tailing factor for zidovudine is not more than 1.5.

Inject reference solution (a) and the test solution. The area of any peak corresponding to zidovudine impurity B is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent), the area of any peak corresponding to zidovudine impurity C is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent) and the area of any peak due to β-thymidine and stavudine is not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent). The sum of the percentages of related substances by tests A and B is not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.25 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in a suitable quantity of *methanol* in a 50-ml volumetric flask and make up to volume with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). Weigh 100 mg of *zidovudine IPRS*, dissolve in a suitable quantity of *methanol* in a 50-ml volumetric flask and make up to volume with the mobile phase (solution A). Dilute 5.0 ml of solution A to 50.0 ml with the mobile phase.

Reference solution (b). Transfer 2.0 ml of a 0.005 per cent w/v solution of *zidovudine impurity B IPRS* in the mobile phase to a 50-ml volumetric flask, add 5.0 ml of solution A and make up to volume with the mobile phase.

Chromatographic system
- a stainless steel column 15 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 70 volumes of *water* and 30 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to zidovudine and zidovudine impurity B is not less than 2.0.

Inject reference solution (a). The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{10}H_{13}N_5O_4$.

Storage. Store protected from light.

Zidovudine Capsules

Zidovudine Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of zidovudine, $C_{10}H_{13}N_5O_4$.

Usual strength. 100 mg.

Identification

A. Transfer a quantity of the mixed contents of the capsules containing about 15 mg of Zidovudine to a 100-ml volumetric

flask. Add about 80 ml of a mixture of 75 volumes of *methanol* and 25 volumes of *water*, shake for 10 minutes and dilute to volume with the same solvent mixture, mix and filter. Dilute 10 ml of the filtrate to 100 ml with the same solvent mixture. When examined in the range 200 nm to 300 nm (2.4.7), the resulting solution shows an absorption maximum at about 265 nm.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (c).

Tests

Dissolution (2.5.2).
Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter promptly through a membrane filter disc having an average pore diameter not more than 1.0 µm, rejecting the first few ml of the filtrate and dilute a suitable volume of the filtrate, if necessary with *water*.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A known quantity of *zidovudine IPRS* is dissolved in 1 ml of *methanol* and suitably diluted with *water* to obtain a solution having a similar concentration as that of the test solution.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 20 volumes of *methanol* and 80 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{13}N_5O_4$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{10}H_{13}N_5O_4$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the mixed contents of 20 capsules containing about 100 mg of Zidovudine and transfer to a 100-ml volumetric flask. Add about 60 ml of the mobile phase, mix with the aid of ultrasound for 10 minutes, dilute to volume with the mobile phase, mix and filter. Dilute 5.0 ml of the filtrate to 50.0 ml with the mobile phase.

Reference solution (a). A 0.1 per cent w/v solution of *zidovudine IPRS* in the mobile phase.

Reference solution (b). A 0.1 per cent w/v solution of *thymine* in *methanol*.

Reference solution (c). Transfer 10.0 ml of the reference solution (a) and 2.0 ml of reference solution (b) to a 100-ml volumetric flask and make up to the volume with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 20 volumes of *methanol* and 80 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 10 µl.

the relative retention times are about 0.2 for thymine and 1.0 for zidovudine, the resolution between zidovudine and thymine is not less than 5.0

Inject reference solution (c). The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (c) and the test solution and measure the responses for the thymine peak. The content of thymine in the capsules should not be more than 3.0 per cent.

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances.

Inject reference solution (c) and the test solution and measure the responses for the principal peak.

Calculate the content of $C_{10}H_{13}N_5O_4$ in the capsules.

Storage. Store protected from moisture.

Zidovudine Injection

Zidovudine Injection is a sterile solution of Zidovudine in Water for Injections.

Zidovudine Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of $C_{10}H_{13}N_5O_4$.

Usual strengths. 10 mg per ml.

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Description. A clear, colourless solution.

Identification

A. When examined in the range 220 nm to 360 nm (2.4.7), a 0.0015 per cent w/v solution in a mixture of 75 volumes of *methanol* and 25 volumes of *water* shows absorption maxima similar to those obtained with a solution of *zidovudine IPRS* of the same concentration.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution (a).

Tests

pH (2.4.24). 3.5 to 7.0, in a mixture containing a volume of injection containing 150 mg of zidovudine and 5 ml of *0.12 M potassium chloride*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a measured volume of the injection containing 25 mg of Zidovudine, to 25 ml with *methanol*.

Reference solution (a). A 0.1 per cent w/v solution of *zidovudine IPRS* in *methanol*.

Reference solution (b). A solution containing 0.1 per cent w/v of *zidovudine IPRS* and 0.001 per cent w/v of *zidovudine impurity C (thymine)*.

Chromatographic system
- a stainless steel column 25 cm × 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 80 volumes of *water* and 20 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 10 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to zidovudine and thymine is not less than 4.0, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution the area of any peak corresponding to thymine is not greater than twice the area of the corresponding peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Bacterial endotoxins (2.2.3). Not more than 1.0 Endotoxin Unit per mg of zidovudine.

Assay. Determine by liquid chromatography (2.4.14), as given in the test for Related substances.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{10}H_{13}N_5O_4$ in the injection.

Storage. Store protected from light and moisture.

Zidovudine Oral Solution

Zidovudine Oral Solution is a solution of Zidovudine in a suitable flavoured vehicle.

Zidovudine Oral Solution contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of zidovudine, $C_{10}H_{13}N_5O_4$.

Usual strength. 50 mg per 5 ml.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 40 volumes of *1-butanol*, 30 volumes of *heptane*, 30 volumes of *acetone* and 10 volumes of *strong ammonia solution*.

Test solution. Dilute the preparation under examination with *methanol* to obtain a solution containing 5 mg of zidovudine per ml.

Reference solution. A 0.5 per cent w/v solution of *zidovudine IPRS* in a mixture of 75 volumes of *methanol* and 25 volumes of *water*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (c).

Tests

pH (2.4.24). 3.0 to 4.0, determined in a mixture containing a volume of the preparation under examination containing 150 mg of zidovudine and 5 ml of *0.12 M potassium chloride*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Transfer a measured volume of the preparation under examination containing about 100 mg of zidovudine to

a 100-ml volumetric flask, dissolve and dilute to volume with the mobile phase and mix. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). Weigh 100 mg of *zidovudine IPRS* and transfer to a 100-ml volumetric flask, add about 50 ml of the mobile phase, mix with the aid of ultrasound to dissolve, dilute to volume with the mobile phase and mix.

Reference solution (b). Weigh 20 mg of *thymine IPRS* and transfer to a 200-ml volumetric flask, add about 150 ml of the mobile phase, mix with the aid of ultrasound to dissolve, dilute to volume with the mobile phase and mix.

Reference solution (c). Transfer 10.0 ml of the reference solution (a) and 2.0 ml of reference solution (b) to a 100-ml volumetric flask and make up to the volume with the mobile phase.

Chromatographic system
- a stainless steel column 12.5 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 90 volumes of *0.04 M sodium acetate*, 9 volumes of *methanol*, 1 volume of *acetonitrile* and 0.2 volume of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 10 µl.

Inject reference solution (c). The test is not valid unless the relative retention times are about 0.12 for thymine and 1.0 for zidovudine, the resolution between zidovudine and thymine is not less than 4.0, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (c) and the test solution and measure the responses for the thymine peak. The content of thymine in the oral solution should not be more than 3.0 per cent.

Other tests. Comply with the tests stated under Oral Liquids.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances.

Inject reference solution (c) and the test solution.

Calculate the content of $C_{10}H_{13}N_5O_4$ in the Oral Solution.

Storage. Store protected from light and moisture.

Zidovudine Tablets

Zidovudine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of zidovudine, $C_{10}H_{13}N_5O_4$.

Usual strengths. 100 mg; 300 mg.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak due to Zidovudine in the chromatogram obtained with reference solution (a).

B. Remove the coating from a few tablets and crush them in a mortar so that no large pieces remain.

Shake a quantity of powdered tablets containing 0.1 g of Zidovudine with 40 ml of *methanol*, filter and evaporate the filtrate to dryness. The residue complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *zidovudine IPRS* or with the reference spectrum of zidovudine.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter promptly through a membrane filter disc having an average pore diameter not greater than 1.0 µm, rejecting the first few ml of the filtrate and dilute a suitable volume of the filtrate, if necessary with *water*.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A known quantity of *zidovudine IPRS* is dissolved in 1 ml of *methanol* and suitably diluted with *water* to obtain a solution having a similar concentration as that of the test solution.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil BDS C18),
- mobile phase: a mixture of 90 ml of *methanol* and 40 ml of *acetonitrile* and a buffer prepared by dissolving 3.0 g of *sodium acetate* and 3.0 g of *sodium 1-octanesulphonate* in 900 ml of *water*, adjusted to pH 5.3 with *glacial acetic acid*,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 10 µl.

Inject the reference solution. The tailing factor is not more than 2.0 for zidovudine peak and the relative standard deviation for replicate injections is not more than 2.0 per cent.

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Inject the reference solution and the test solution and measure the peak responses of the major peak.

Calculate the content of $C_{10}H_{13}N_5O_4$ in the medium.

Q. Not less than 80 per cent of the stated amount of $C_{10}H_{13}N_5O_4$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse the powdered tablets containing about 1.5 g of Zidovudine in 50 ml of *water*, shake for 30 minutes. Add about 150 ml of *methanol*, sonicate for 10 minutes and dilute to 500.0 ml with *water*. Dilute 4.0 ml of the solution to 100.0 ml with *water*, filter.

Reference solution (a). A 0.01 per cent w/v solution of *3'-chloro-3'-deoxythymidine (zidovudine impurity B IPRS)* in *methanol*.

Reference solution (b). A 0.02 per cent w/v solution of *thymine IPRS (zidovudine impurity C IPRS)* in *methanol*.

Reference solution (c). Dissolve about 30 mg of *zidovudine IPRS* in 3.0 ml of *methanol*, add 2.5 ml of reference solution (a), 5.0 ml of reference solution (b) and dilute to 250.0 ml with *water*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 3.0 g of *sodium acetate* and 1.3 g of *sodium 1-octanesulphonate* in 900 ml of *water*. Add 90 ml of *methanol* and 40 ml of *acetonitrile*, adjusted to pH 5.3 with *glacial acetic acid*, filter,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 20 µl.

The relative retention time with reference to zidovudine for zidovudine impurity C is about 0.17 and for zidovudine impurity B is about 1.2.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to zidovudine and zidovudine impurity B is not less than 2.5, the tailing factor of the principal peak is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (c) and the test solution. In the chromatogram obtained the test solution, the area of the peak due to zidovudine impurity C is not more than 1.5 per cent, multiplying with response factor of 1.7 and the area of any other secondary peaks is not more than 0.2 per cent. The sum of areas of all the secondary peaks is not more than 2.0 per cent.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances.

Inject reference solution (c) and the test solution.

Calculate the content of $C_{10}H_{13}N_5O_4$ in the tablets.

Storage. Store protected from light and moisture.

Zidovudine, Lamivudine and Nevirapine Tablets

Zidovudine, Lamivudine and Nevirapine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of zidovudine, $C_{10}H_{13}N_5O_4$, lamivudine, $C_8H_{11}N_3O_3S$ and nevirapine, $C_{15}H_{14}N_4O$.

Usual strength. Zidovudine, 300 mg, Lamivudine, 150 mg and Nevirapine , 200 mg.

Identification

A. In the Assay, the three principal peaks in the chromatogram obtained with the test solution correspond to the peaks due to zidovudine, lamivudine and nevirapine in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 75 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter promptly through a membrane filter disc having an average pore diameter not greater than 1.0 µm, rejecting the first few ml of the filtrate and dilute a suitable volume of the filtrate, if necessary with *water*.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Weigh 0.3 g of *zidovudine IPRS*, 0.15 g of *lamivudine IPRS* and 0.2 g of *nevirapine IPRS* and transfer to a 100 ml volumetric flask. Add about 20 ml of *methanol*, mix with the aid of ultrasound to dissolve and dilute to volume with a mixture of equal volumes of *methanol* and *water*. Dilute 5.0 ml of the solution to 50.0 ml with *0.1 M hydrochloric acid*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil BDS C18),

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– mobile phase: a mixture of 35 volumes of *methanol* and 65 volumes of a buffer solution prepared by dissolving 0.68 g of *potassium dihydrogen phosphate* and 1.0 g of *sodium octanesulphonate* in 1000.0 ml of *water* to which 1 ml of *triethylamine* is added and adjusted to pH 2.5 with *phosphoric acid*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 266 nm,
– injection volume: 10 µl.

Inject the reference solution. The tailing factor for the individual zidovudine, lamivudine and nevirapine peaks is not more than 2.0 and the relative standard deviation for replicate injections of all the analyte peaks is not more than 2.0 per cent.

Inject the reference solution and the test solution and measure the peak responses of the major peaks due to zidovudine, lamivudine and nevirapine.

Calculate the contents of $C_{10}H_{13}N_5O_4$, $C_8H_{11}N_3O_3S$ and $C_{15}H_{14}N_4O$ in the medium.

Q. Not less than 70 per cent of the stated amounts of $C_{10}H_{13}N_5O_4$, $C_8H_{11}N_3O_3S$ and $C_{15}H_{14}N_4O$.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 60 volumes of *water* and 40 volumes of *methanol*.

Test solution. Disperse a quantity of the powdered tablets containing 50 mg of Lamivudine, in the solvent mixture and dilute to 100.0 ml with the solvent mixture, filter.

Reference solution (a). A solution containing 0.0001 per cent w/v of *lamivudine diastereomer IPRS*, 0.001 per cent w/v of *zidovudine impurity B IPRS*, 0.05 per cent w/v of *lamivudine IPRS* and 0.1 per cent w/v of *zidovudine IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.03 per cent w/v of *lamivudine IPRS*, 0.04 per cent w/v *of nevirapine IPRS* and 0.04 per cent w/v of *zidovudine IPRS* in the solvent mixture.

Reference solution (c). Dissolve 4 mg of *nevirapine impurity A IPRS* in 40 ml of *methanol* with the aid of ultrasound and dilute to 100.0 ml with *water*.

Reference solution (d). Dilute 5.0 ml of reference solution (b) and 5.0 ml of reference solution (c) to 100.0 ml with the solvent mixture.

Chromatographic system
– a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Prontosil C18H),
– sample temperature: 5°,
– mobile phase: A. a mixture of 100 volumes of 0.23 per cent w/v solution of *ammonium dihydrogen phosphate*, adjusted to pH 3.5 with *orthophosphoric acid* and 0.2 volume of *methanol*,
B. *acetonitrile*,
– flow rate: 1 ml per minute,
– a gradient programme using the conditions given below,
– spectrophotometer set at 266 nm,
– injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	99	1
10	99	1
20	90	10
25	85	15
42	70	30
55	70	30
57	99	1
65	99	1

Name	Relative retention time	Correction factor
Lamivudine impurities		
Carboxylic acid impurity[1]	0.23	2.33*
Lamivudine diastereomer[2]	0.57	1.43*
Nevirapine impurities		
Nevirapine impurity A[3]	1.45	—
Nevirapine impurity B[4]	1.22	0.83#
Nevirapine impurity C[5]	1.67	1.03#
Zidovudine impurities		
Zidovudine impurity A[6]	0.76	0.86$
Zidovudine	1.0	—
Zidovudine impurity B[7]	1.02	1.06$
Zidovudine impurity C[8]	0.39	0.86$
Thymidine[9]	0.66	0.93$
Zidovudine threo isomer[10]	0.96	1.06$
Zidovudine thymidine adduct[11]	1.28	0.98$

*Correction factor with respect to Lamivudine,
#Correction factor with respect to Nevirapine impurity A,
$Correction factor with respect to Zidovudine,
[1]4-amino-2-oxo-pyrimidinyl-1,3-oxoathiolane-2-carboxylic acid,
[2](2S-*cis*)-(-)-1-[(2*R*, 5*R*)-2-(Hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine;
[3]5,11-dihydro-6*H*-11-ethyl-4-methyl-dipyrido(3,2-b:2',3'-e)(1,4)diazepin-6-one,
[4](5,11-dihydro-4-methyl-6*H*-dipyrido(3,2-b:2',3'-e)(1,4)diazepin-6-one),
[5]5,11-dihydro-4-methyl-6*H*-11-propyl-dipyrido(3,2-b:2',3'-e)(1,4)diazepin-6-one,
[6]3'-azido-3'deoxy-3'-Azido-3'deoxythymidine; stavudine,

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[7]3'-chloro-3'deoxythymidine,

[8]2,4-dihydroxy-5-methy pyrimidine; thymine,

[9]1-(2-deoxy-f'-D-ribifuranosyl)-5-methyl uracil,

[10]1-(3-azido-2,3-dideoxy-f'-D-threo-pentafuranosyl)-thymidine,

[11]1-(3-(3-(3-azido-2,3-dideoxy-pentofuranosyl))-5-methyl-2,6-dioxo-3,6-dihydropyrimidin-1-yl)-2,3-dideoxypentofuranosyl)-5-methylpyrimidine-2,4-dione.

The retention time of lamivudine, zidovudine, nevirapine and nevirapine impurity A peaks are about 18.5 minutes, 31 minutes, 40.6 minutes and 45 minutes respectively.

Inject reference solution (a) and (d). The test is not valid unless the resolution between the peaks due to lamivudine diastereomer and lamivudine is not less than 1.5 and between zidovudine and zidovudine impurity B is not less than 1.5 in the chromatogram obtained with reference solution (a), the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 5.0 per cent, for the peaks due to lamivudine, nevirapine related compound A and zidovudine in the chromatogram obtained with reference solution (d).

Inject reference solution (d) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to carboxylic acid impurity, lamivudine distereomer, each of, is not more than1.66 times the area of the peak due to lamivudine in the chromatogram obtained with reference solution (d) (0.5 per cent), the area of any peak corresponding to nevirapine impurity A, nevirapine impurity B and nevirapine impurity C, each of, is not more than 1.25 times the area of peak due to nevirapine impurity A in the chromatogram obtained with reference solution (d) (0.5 per cent), the area of any peak corresponding to zidovudine impurity A (stavudine), zidovudine impurity B, thymidine, zidovudine threo isomers and zidovudine thymidine adduct, each of, is not more than 1.25 times the area of the peak due to zidovudine in the chromatogram obtained with reference solution (d) (0.5 per cent), the area of any peak corresponding to zidovudine impurity C (thymine) is not more than 2.5 times the area of the peak due to zidovudine in the chromatogram obtained with reference solution (d) (1.0 per cent), the area of any other secondary peak is not more than 1.66 times the area of the peak due to lamivudine in the chromatogram obtained with reference solution (d) (0.5 per cent). The sum of all the impurities is not more than 2.5 per cent.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 100 mg of Zidovudine and transfer to a 200-ml volumetric flask. Add about 20 ml of *methanol*, mix with the aid of ultrasound for 10 minutes, dilute to volume with *water*, mix and filter. Further dilute 10.0 ml of the filtrate to 25.0 ml with *water*.

Reference solution. Weigh 100 mg of *zidovudine IPRS*, 50 mg of *lamivudine IPRS* and 65 mg of *nevirapine IPRS* and transfer to a 200-ml volumetric flask. Add about 20 ml of *methanol*, mix with the aid of ultrasound to dissolve, dilute to volume with *water* and mix. Further dilute 10.0 ml of the solution to 25.0 ml with *water*.

Use the chromatographic system as described under Dissolution.

Inject the reference solution. The test is not valid unless the tailing factor for the individual peaks due to zidovudine, lamivudine and nevirapine is not more than 2.0 and the relative standard deviation for replicate injections of all the analyte peaks is not more than 2.0 per cent.

Inject the reference solution and the test solution and measure the responses for the major peaks.

Calculate the contents of $C_{10}H_{13}N_5O_4$, $C_8H_{11}N_3O_3S$ and $C_{15}H_{14}N_4O$ in the tablets.

Storage. Store protected from moisture.

Zinc Oxide

ZnO Mol. Wt. 81.4

Zinc Oxide contains not less than 99.0 per cent and not more than 100.5 per cent of ZnO, calculated on the ignited basis.

Category. Mild astringent; topical protectant.

Description. A soft, white or faintly yellowish white amorphous powder, free from grittiness. It gradually absorbs carbon dioxide from air.

Identification

A. It becomes yellow when strongly heated; the yellow colour disappears on cooling.

B. Dissolve 0.1 g in 1.5 ml of *2 M hydrochloric acid* and dilute to 5 ml with *water*. The solution gives the reaction of zinc salts (2.3.1).

Tests

Alkalinity. Shake 1.0 g with 10 ml of boiling *water*, add 0.1 ml of *phenolphthalein solution* and filter. If the filtrate is red, no more than 0.3 ml of *0.1 M hydrochloric acid* is required to discharge the colour.

Carbonate and substances insoluble in acids. Dissolve 1.0 g in 15 ml of *2 M hydrochloric acid*; no effervescence is produced and the solution is not more opalescent than opalescence standard OS2 (2.4.1), and colourless (2.4.1).

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Arsenic (2.3.10). Dissolve 2.0 g in 15 ml of *brominated hydrochloric acid AsT* and 45 ml of *water* and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (5 ppm).

Iron (2.3.14). Dissolve 0.1 g in a mixture of 5 ml of *water* and 1 ml of *hydrochloric acid* and dilute to 40 ml with *water*. The resulting solution complies with the limit test for iron (400 ppm). Use 0.5 ml of *thioglycollic acid* in the test.

Lead. Dissolve 2 g in a mixture of 20 ml *water* and 5 ml of *glacial acetic acid* and add 0.25 ml of *potassium chromate solution*; the solution remains clear.

Loss on ignition (2.4.20). Not more than 1.0 per cent, determined on 2.0 g by igniting at 500°.

Assay. Dissolve 0.15 g in 10 ml of *2 M acetic acid* and dilute to 50 ml with *water*. To the resulting solution add about 50 mg of *xylenol orange triturate* and sufficient *hexamine* to produce violet-pink colour. Add a further 2 g of *hexamine* and titrate with *0.1 M disodium edetate* until the solution becomes yellow.

1 ml of *0.1 M disodium edetate* is equivalent to 0.008138 g of ZnO.

Storage. Store protected from moisture.

Zinc Oxide Cream

Zinc Cream

Zinc Oxide Cream contains 32 per cent w/w of Zinc Oxide in a suitable water-in-oil emulsified base.

Zinc Oxide Cream contains not less than 30.0 per cent and not more than 34.0 per cent w/w of zinc oxide, ZnO.

Identification

The residue obtained in the Assay is yellow when hot and white when cool.

Tests

Other tests. Comply with the tests stated under Creams.

Assay. Weigh 0.5 g in a porcelain dish, heat gently over a small flame until the base is completely volatilised or charred. Increase the heat until all the carbon is removed. Dissolve the residue in 10 ml of *2 M acetic acid* and add sufficient *water* to produce 50 ml. To the resulting solution add about 50 mg of *xylenol orange triturate* and sufficient *hexamine* to produce violet-pink colour. Add a further 2 g of *hexamine* and titrate with *0.1 M disodium edetate* until the solution becomes yellow.

1 ml of *0.1 M disodium edetate* is equivalent to 0.008138 g of ZnO.

Zinc Oxide and Salicylic Acid Paste

Zinc Oxide and Salicylic Acid Paste contains not less than 22.5 per cent and not more than 25.5 per cent w/w of zinc oxide, ZnO and not less than 1.9 per cent and not more than 2.1 per cent w/w of salicylic acid, $C_7H_6O_3$.

Zinc oxide, finely sifted	240 g
Salicylic acid, finely sifted	20 g
Starch, finely sifted	240 g
White Soft Paraffin	500 g

Extemporaneous Preparation. Melt the soft paraffin, incorporate the Zinc Oxide, the Salicylic Acid and the starch and stir until cold.

Usual strength. 24 per cent of zinc oxide and 2 per cent of salicylic acid w/w.

Identification

A. Shake 1 g of substance under examination in 10 ml of *water*, and filter. To the filtrate, add 1.0 ml of *ferric chloride solution*; an intense reddish violet colour is produced. To the solution, add 1 ml of *acetic acid*; the colour is not dispersed. To the solution, add 2.0 ml of *2 M hydrochloric acid*; the colour is dispersed and a white crystalline precipitate is formed.

B. Heat 0.5 g gently in a porcelain dish over a very small flame until the base is completely volatile or charred. Increase the heat until all the carbon is removed. The residue is yellow; when hot and white; when cool.

Tests

Other tests. Comply with the tests stated under Topical Semi-Solid Preparations.

Assay. *For Salicylic Acid* — Shake 500 mg of the paste in 10 ml of *1 M hydrochloric acid* and add 10 ml of *ether* until fully dispersed. Decant and reserve the aqueous layer. Extract the ether layer with two further 10 ml quantities of *1 M hydrochloric acid*, combine the aqueous extracts with the reserve aqueous layer, wash with 10 ml of *ether* and reserve for the Assay of Zinc Oxide. Combine the ether extract, add 15 ml of *petroleum spirit (boiling range, 40° to 60°)* and extract with successive quantities of 20 ml, 10 ml and 10 ml of a mixture of equal volumes of *ethanol (95 per cent)* and *1 M sodium hydroxide*. Dilute the combined extracts to 100 ml with *2 M hydrochloric acid*, further dilute 15 ml of the resulting solution to 50 ml with the same solvent and measure the absorbance of the final solution at the maximum at 302 nm (2.4.7) Calculate the content of $C_7H_6O_3$ using 260 as a specific absorbance at 302 nm.

For Zinc Oxide — To the combined aqueous extracts obtained in the Assay for Salicylic Acid, add 20 ml of *1 M sodium hydroxide* and 50 mg of *xylenol orange triturate*. To the

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resulting solution, add sufficient *hexamine* to change the colour of the solution to red and then a further 3 g of *hexamine* and titrate with *0.1 M disodium edetate*. Carry out a blank titration.

1 ml of *0.1 M disodium edetate* is equivalent to 0.008139 g of zinc oxide, ZnO.

Storage. Store protected from moisture.

Zinc Stearate

$(C_{17}H_{35}COO)_2Zn$ Mol. Wt. 632.3

Zinc Stearate consists mainly of zinc stearate but many contain variable proportions of zinc palmitate $(C_{15}H_{31}COO)_2Zn$, and zinc oleate $(C_{17}H_{33}COO)_2Zn$.

Zinc Stearate contains not less than 10.0 per cent and not more than 12.0 per cent of zinc, Zn.

Category. Pharmaceutical aid (dusting powder).

Description. A fine, white, bulky, amorphous powder, free from grittiness.

Identification

A. To 5.0 g add 50 ml of *ether* and 40 ml of a 7.5 per cent v/v solution of *nitric acid* in *distilled water* and heat under a reflux condenser until dissolution is complete. Allow to cool, separate the aqueous layer and shake the ether layer with two quantities, each of 4 ml, of *distilled water*. Combine the washings with the aqueous layer, wash with 15 ml of *ether* and heat on a water-bath until ether is completely eliminated. Allow to cool and dilute to 50.0 ml with *distilled water* (solution A). Evaporate the ether layer to dryness and dry the residue at 105°. The freezing point of the residue is not lower than 53° (2.4.11).

B. Neutralise 5 ml of solution A to red *litmus paper* with *10 M sodium hydroxide*. The solution gives the reactions of zinc salts (2.3.1).

Tests

Appearance of solution. Solution A is not more intensely coloured than reference solution YS6 (2.4.1).

Acidity or alkalinity. Shake 1.0 g with 5 ml of *ethanol (95 per cent)* and add 20 ml of *carbon dioxide-free water* and 0.1 ml of *phenol red solution*. Not more than 0.3 ml of *0.1 M hydrochloric acid* or 0.1 ml of *0.1 M sodium hydroxide* is required to change the colour of the solution.

Alkalis and alkaline earths. Add 1.0 g to a mixture of 25 ml of *water* and 5 ml of *hydrochloric acid*, boil, filter immediately and wash with 25 ml of hot *water*. Add *dilute ammonia solution* to make the filtrate just alkaline and then add *ammonium sulphide solution* in excess to precipitate the zinc as zinc sulphide completely. Filter, add 0.5 ml of *sulphuric acid* to the filtrate, evaporate to dryness and ignite to constant weight; the residue weighs not more than 20 mg.

Arsenic (2.3.10). Mix 5.0 g with 10 ml of *bromine solution* and evaporate to dryness on a water-bath. Ignite gently, dissolve the cooled residue, ignoring any carbon, in 50 ml of *water* and 14 ml of *brominated hydrochloric acid AsT* and remove the excess of bromine with 2 ml of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). Heat 5.0 g with 40 ml of *2 M acetic acid* and allow to cool. Filter, wash the residue with two successive quantities, each of 5 ml, of warm *water* and dilute the combined filtrate and washings to 100.0 ml with *water*. 12 ml of the solution complies with the limit test for heavy metals, Method D (20 ppm). Use 1.0 ml of *lead standard solution (10 ppm Pb)* to prepare the standard.

Chlorides (2.3.12). 10 ml of solution A complies with the limit test for chlorides (250 ppm).

Sulphates (2.3.17). Dilute 1 ml of solution A to 50 ml with *water*. Dilute 12.5 ml of the solution to 15 ml with *water*. It complies with the limit test for sulphates (0.6 per cent).

Assay. Weigh 1.0 g and boil with 50 ml of *2 M acetic acid* until the fatty acid layer which separates is clear, adding more *water* if necessary to maintain the original volume. Cool, filter and wash the filter and the flask thoroughly with *water* until the last washing is not acidic to blue *litmus paper*. To the combined filtrate and washings add about 50 mg of *xylenol orange triturate* and sufficient *hexamine* to produce violet-pink colour. Add a further 2 g of *hexamine* and titrate with *0.1 M disodium edetate* until the colour changes to yellow.

1 ml of *0.1 M disodium edetate* is equivalent to 0.00654 g of Zn.

Zinc Sulphate Eye Drops

Zinc Sulphate Eye Drops are a sterile solution containing 0.25 per cent w/v of Zinc Sulphate in Purified Water.

Zinc Sulphate Eye Drops contain not less than 0.22 per cent and not more than 0.28 per cent w/v of zinc sulphate, $ZnSO_4$, $7H_2O$.

Description. A clear, colourless solution.

Identification

It gives the reactions of zinc salts and of sulphates (2.3.1).

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Tests

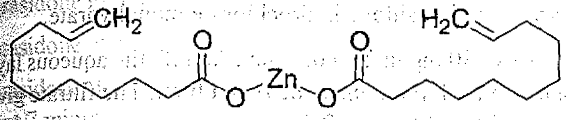
Other tests. Comply with the tests stated under Eye Drops.

Assay. To 5.0 ml add 50 ml of *water* and 5 ml of *ammonia buffer pH 10.9* and titrate with *0.01 M disodium edetate* using *mordant black II solution* as indicator.

1 ml of *0.01 M disodium edetate* is equivalent to 0.002875 g of $ZnSO_4, 7H_2O$.

Storage. Store in containers of glass or any other non-metallic material and sealed so as to exclude micro-organisms.

Zinc Undecenoate



$C_{22}H_{38}O_4Zn$ Mol. Wt. 431.9

Zinc Undecenoate is zinc di(undec-10-enoate).

Zinc Undecenoate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{22}H_{38}O_4Zn$, calculated on the dried basis.

Category. Antifungal (topical).

Description. A white or almost white, fine powder.

Identification

A. To 2.5 g add 10 ml of *water* and 10 ml of *1 M sulphuric acid* and extract with two quantities, each of 10 ml, of *ether*. Reserve the aqueous layer for test C. Wash the combined ether extracts with *water* and evaporate to dryness. To the residue add 2 ml of freshly distilled *aniline* and boil under a reflux condenser for 10 minutes, cool and add 30 ml of *ether*. Extract with three quantities, each of 20 ml, of *2 M hydrochloric acid* and then with 20 ml of *water*. Evaporate the ether extract to dryness on a water-bath. The residue, after recrystallising twice from *ethanol (70 per cent)* and drying at a pressure not exceeding 2 kPa for 3 hours, melts at about 67° (2.4.21).

B. Dissolve 0.1 g in a mixture of 2 ml of *1 M sulphuric acid* and 5 ml of *glacial acetic acid* and add, dropwise, 0.25 ml of *potassium permanganate solution*; the pink colour of permanganate is discharged.

C. A mixture of 1 ml of the aqueous layer reserved in test A and 4 ml of *water* gives the reaction of zinc salts (2.3.1).

D. Melting range (2.4.21). 115° to 121°.

Tests

Alkalinity. Mix 1.0 g with 5 ml of *ethanol (95 per cent)* and 0.5 ml of *phenol red solution*, add 50 ml of *carbon dioxide-free water* and examine immediately; no reddish colour is produced.

Alkalis and alkaline earths. Add 1.0 g to a mixture of 25 ml of *water* and 5 ml of *hydrochloric acid*, boil, filter immediately and wash with 25 ml of hot *water*. Add *dilute ammonia solution* to make the filtrate just alkaline and then add *ammonium sulphate solution* in excess to precipitate the zinc as zinc sulphide completely. Filter, add 0.5 ml of *sulphuric acid* to the filtrate, evaporate to dryness and ignite to constant weight; the residue weighs not more than 20 mg.

Sulphates (2.3.17). To 0.25 g add a mixture of 10 ml of *distilled water* and 5 ml of *2 M hydrochloric acid*. Cool, filter and dilute to 20 ml with *distilled water*. The resulting solution complies with the limit test for sulphates (600 ppm).

Degree of unsaturation. Dissolve 0.1 g in a mixture of 5 ml of *2 M hydrochloric acid* and 30 ml of *glacial acetic acid* and titrate with *0.05 M bromine* using 0.05 ml of *ethoxychrysoidine hydrochloride solution*, added towards the end of the titration, as indicator. Not less than 9.1 ml and not more than 9.4 ml of *0.05 M bromine* is required to discharge the red colour.

Loss on drying (2.4.19). Not more than 1.5 per cent, determined on 0.5 g by drying in an oven at 105°.

Assay. Weigh 0.35 g, add 25 ml of *2 M acetic acid*, heat to boiling, cool and dilute to 50 ml with *water*. Add about 50 mg of *xylenol orange triturate* and sufficient *hexamine* to produce a violet-pink colour. Add a further 2 g of *hexamine* and titrate with *0.1 M disodium edetate* until the colour changes to yellow.

1 ml of *0.1 M disodium edetate* is equivalent to 0.04319 g of $C_{22}H_{38}O_4Zn$.

Storage. Store protected from light and moisture.

Zinc Undecenoate Ointment

Zinc Undecylenate Ointment

Zinc Undecenoate Ointment contains 20 per cent w/v of Zinc Undecenoate in a suitable ointment basis.

Zinc Undecenoate Ointment contains not less than 18.0 per cent and not more than 22.0 per cent of zinc undecenoate, $C_{22}H_{38}O_4Zn$, and not less than 4.5 per cent and not more than 5.5 per cent of free undecenoic acid, $C_{11}H_{20}O_2$.

Tests

Other tests. Comply with the tests stated under Ointments.

Assay. *For Zinc Undecenoate* — Weigh 2.0 g, add 20 ml of *dilute hydrochloric acid* and boil under a reflux condenser for at least 20 minutes or until the fatty layer is clear. Filter while hot and wash the residue with hot *water*. Cool the

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combined filtrate and washings, neutralise to *litmus paper* with *dilute ammonia solution*, add 3 ml of *dilute hydrochloric acid* and 5 g of *hexamine* and titrate with *0.05 M disodium edetate* using *xylenol orange solution* as indicator, until the colour changes to yellow.

1 ml of *0.05 M disodium edetate* is equivalent to 0.02160 g of $C_{22}H_{38}O_4Zn$.

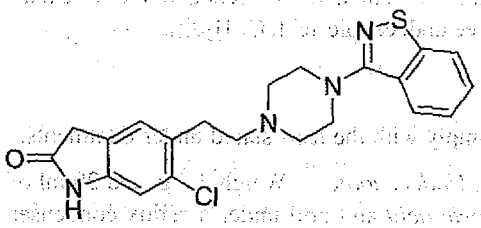
For free undecenoic acid — Weigh 5.0 g, add 100 ml of *dilute hydrochloric acid* and heat to 70° with constant stirring. Cool and transfer to a separator with the aid of four quantities, each of 25 ml of *ether* and add the rinsings to the mixture in the separator. Dilute the aqueous phase to 300 ml, saturate it with *sodium chloride* and shake the mixture. Transfer the aqueous layer to a second separator and extract with another 100 ml of *ether*. Wash the combined ether extracts with successive quantities, each of 10 ml, of *water* until the washings are free from chloride. Transfer the ether solution to a beaker and evaporate on a water-bath to about 5 ml. Add 20 ml of *carbon tetrachloride*, mix, transfer the mixture to a small separator and drain the *carbon tetrachloride* layer into a 100-ml volumetric flask. Rinse the beaker with three quantities, each of 5 ml, of *carbon tetrachloride* and transfer the rinsings to the volumetric flask, dilute to volume with carbon tetrachloride and mix. Evaporate 50.0 ml of the resulting solution to about 5 ml, add 100 ml of *ethanol (95 per cent)*, previously neutralised, and 0.15 ml of *phenolphthalein solution* and titrate the total undecenoic acid with *0.1 M sodium hydroxide*.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01843 g of $C_{11}H_{20}O_2$.

Calculate the content of free undecenoic acid from the difference between the total undecenoic acid and the undecenoic acid equivalent to the determined zinc undecenoate (the content of zinc undecenoate multiplied by 0.8533 gives the equivalent of undecenoic acid).

Storage. Store protected from light and moisture.

Ziprasidone Hydrochloride Monohydrate



, HCl, H_2O

$C_{21}H_{22}Cl_2N_4OS,H_2O$ Mol. Wt. 467.4

Ziprasidone Hydrochloride Monohydrate is 5-[2-[4-(1,2-Benzisothiazol-3-yl)piperazin-1-yl]ethyl]-6-chloro-1,3-dihydroindol-2(*H*)-one hydrochloride monohydrate.

Ziprasidone Hydrochloride Monohydrate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{21}H_{22}Cl_2N_4OS$, calculated on the anhydrous basis.

Category. Neuroleptic.

Description. A white or slightly pink powder. It shows polymorphism (2.5.11).

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ziprasidone hydrochloride monohydrate IPRS* or with the reference spectrum of ziprasidone hydrochloride monohydrate.

B. Dissolve 30 mg in 2 ml of water. Acidify the aqueous layer with 0.15 ml of *dilute nitric acid* and filter. The filtrate gives reaction (A) of chlorides (2.3.1)

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Carry out the test protected from light, and prepare solutions immediately before use.

Solvent mixture A. 40 volumes of *water* and 60 volumes of *methanol*.

Solvent mixture B. 0.04 volume of *hydrochloric acid*, 20 volumes of *water* and 80 volumes of *methanol*.

Test solution (a). Dissolve 23 mg of the substance under examination in solvent mixture A and dilute to 100.0 ml with solvent mixture A.

Test solution (b). Dissolve 23 mg of the substance under examination in solvent mixture B and dilute to 50.0 ml with solvent mixture B.

Reference solution. Dilute 1.0 ml of test solution (b) to 100.0 ml with solvent mixture B. Dilute 1.0 ml of the solution to 10.0 ml with solvent mixture B.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 60 volumes of buffer solution prepared by dissolving 6.8 g *potassium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid* and 40 volumes of *methanol*,

  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,

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– spectrophotometer set at 229 nm,
– injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
20	100	0
21	0	100
24	0	100
28	100	0

Name	Relative retention time	Correction factorr
Ziprasidone impurity A[1]	0.4	0.7
Ziprasidone impurity B[2]	0.8	—
Ziprasidone impurity C[3]	0.9	—
Ziprasidone (Retention time: about 7 minutes)	1.0	—

[1]3-piperazin-1-yl-1,2-benzisothiazole,
[2]5-[2-[4-(1,2-benzisothiazol-3-yl)piperazin-1-yl]ethyl]-6-chloro-1*H*-indole-2,3- dione,
[3]2-[2-amino-5-[2-[4-(1,2-benzisothiazol-3-yl)piperazin-1-yl]ethyl]-4-chlorophenyl]acetic acid.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution, test solution (a) and test solution (b). In the chromatogram obtained with the test solution (b), the area of any peak corresponding to ziprasidone impurity B is not more than twice the area of the corresponding peak in the chromatogram obtained with the reference solution (0.2 per cent), the area of any peak corresponding to ziprasidone impurity A is not more than 1.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.15 per cent) and in the chromatogram obtained with test solution (a) the area of any peak corresponding to ziprasidone impurity C is not more than 0.75 times the area of the principal peak in the chromatogram obtained with the reference solution (0.15 per cent). In the chromatogram obtained with test solution (b) the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent). Ignore any peak with an area 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent), disregard any peak due to ziprasidone impurity C obtained with test solution (b) and any peak with retention time more than 20 minutes.

Ziprasidone impurity D and E. Determine by liquid chromatography as described under Related substances with the following modifications.

Chromatographic system
– column temperature: 35°,
– mobile phase: a mixture of 40 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water,* adjusted to pH 6.0 with 28 per cent w/v solution of *potassium hydroxide,* 5 volumes of *methanol* and 55 volumes of *acetonitrile,*
– flow rate: 1 ml per minute,

Name	Relative retention time	Correction factorr
Ziprasidone (retention time: about 4.5 minutes)	1.0	—
Ziprasidone impurity D[1]	2.0	1.4
Ziprasidone Impurity E[2]	3.0	0.5

[1]5,5-bis[2-[4-(1,2-benzisothiazol-3-yl)piperazin-1-yl]ethyl]-6,6-dichloro-3-hydroxy-1,1,3,3-tetrahydro-2*H*,2*H*-3,3-biindole-2,2-dione,
[2]3-(1,2-benzisothiazol-3-yl)-5-[2-[4-(1,2-benzisothiazol-3-yl)piperazin-1-yl]ethyl]-6-chloro-1,3-dihydro-2*H*-indol-2-one.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and test solution (b). Run the chromatogram 11 times the retention time of ziprasidone. In the chromatogram obtained with test solution (b), the area of any peak corresponding to ziprasidone impurity D and E is not more than 1.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.15 per cent). The area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent), disregard any peak eluting before the peak due to ziprasidone.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0. 1 per cent.

Water (2.3.43). 3.7 per cent to 5.0 per cent, determined on 0.25 g.

Assay. Determine by liquid chromatography as described under Related substances with the following modifications.

Reference solution. A 0.023 per cent w/v solution of the *ziprasidone hydrochloride IPRS* in solvent mixture A.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 3000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and test solution (a).

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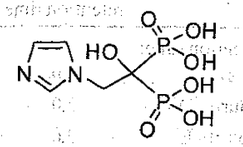
Calculate the content of $C_{21}H_{22}Cl_2N_4OS$.

Storage. Store protected from light.

Zoledronic Acid

Zoledronic Acid Monohydrate



$C_5H_{10}N_2O_7P_2, H_2O$ Mol. Wt. 290.1

Zoledronic Acid is [1-hydroxy-2-(1*H*-imidazol-1-yl)ethylidene]diphosphonic acid, monohydrate.

Zoledronic Acid contains not less than 98.0 per cent and not more than 102.0 per cent of $C_5H_{10}N_2O_7P_2$, calculated on the anhydrous basis.

Category. Bone resorption inhibitor.

Description. A white to almost white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *zoledronic acid IPRS* or with the reference spectrum of zoledronic acid.

B. In the assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 2.0 to 4.0, determined in a 0.02 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of the mobile phase.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil BDS),
- mobile phase: mix 1 ml of *triethylamine* with 500 ml of *water*, adjusted to pH 3.2 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution, area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (1.0 per cent). The sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent). Ignore any peak with an area less than 0.1 times the area of the peak in the chromatogram obtained with the reference solution (0.1 per cent).

Water (2.3.43). 5.0 to 8.0 per cent, determined on 1.0 g in a mixture of equal volumes of *acetic acid* and *formamide*.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of the mobile phase. Dilute 20.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution. A 0.02 per cent w/v solution of *zoledronic acid IPRS* in the mobile phase.

Use the chromatographic system as described under Related substances.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_5H_{10}N_2O_7P_2$.

Storage. Store protected from light and moisture.

Labelling. The label states whether or not the material is intended for use in the manufacture of parenteral preparations.

Zoledronic Acid Injection

Zoledronic Acid Injection is prepared by dissolving the contents of a sealed container containing Zoledronic Acid with or without auxiliary substances in the requisite amount of water for injection as directed on the label.

The constituted solution complies with the requirements for Clarity of solution and Particulate matter stated under Parenteral Preparations (Injections).

Usual strength. 4 mg per vial.

Storage. The constituted solution should be used immediately after preparation but, in any case, within the period recommended by the manufacturer.

Zoledronic Acid Injection contains not less than 90.0 per cent and not more than 110.0 per cent of stated amount of zoledronic acid, $C_5H_{10}N_2O_7P_2$.

The contents of the sealed container comply with the requirements stated under Parenteral Preparations (Powders for Injection) and with the following requirements.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.5 to 7.0, determined in a solution constituted as directed on the label, in Water for Injections.

Other tests. Comply with the tests stated under Parental Preparations (Injections).

Bacterial endotoxins (2.2.3). Not more than 17.0 Endotoxin Units per mg of Zoledronic acid.

Sterility (2.2.11). Complies with the test for sterility.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve the contents of sealed container in sufficient mobile phase to give a concentration 0.16 mg per ml.

Reference solution. A 0.016 per cent w/v solution of *zoledronic acid IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil BDS),
- mobile phase: mix 1 ml of *triethylamine* with 500 ml of *water*, adjusted to pH 3.2 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

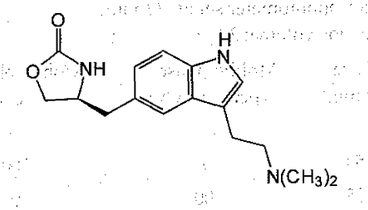
Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_5H_{10}N_2O_7P_2$ in the Injection.

Storage. Store the sealed container at a temperature not exceeding 30°. Use the prepared solution within the period recommended by the manufacturer and stored strictly in accordance with the instructions of the manufacturer.

Zolmitriptan



$C_{16}H_{21}N_3O_2$ Mol. Wt. 287.4

Zolmitriptan is (*S*)-4-[[3-(2-dimethylamino)ethyl]-*H*-indol-5-yl]methyl]–2-oxazolidinone.

Zolmitriptan contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{16}H_{21}N_3O_2$, calculated on the dried basis.

Category. Antimigraine.

Description. A white to cream, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *zolmitriptan IPRS* or with the reference spectrum of zolmitriptan.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 50 mg of the substance under examination in 50.0 ml of mobile phase A.

Reference solution (a). A 0.001 per cent w/v solution of *zolmitriptan IPRS* in mobile phase A.

Reference solution (b). A 0.0001 per cent w/v solution of *zolmitriptan impurity C IPRS* in reference solution (a).

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as X-Terra C18),
- column temperature. 40°,
- mobile phase. A. a mixture of 70 volumes of buffer solution prepared by dissolving 0.3 g of *potassium dihydrogen phosphate* and 0.5 g of *sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 9.8 with *sodium hydroxide solution*, 20 volumes of *methanol* and 10 volumes of *acetonitrile*,

B. a mixture of 30 volumes of buffer solution and 70 volumes of *acetonitrile*,

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- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
50	0	100
55	100	0

Name	Relative retention time
Zolmiltriptan impurity B[1]	0.26
Zolmiltriptan impurity A[2]	0.34
Zolmiltriptan impurity D[3]	0.54
Zolmiltriptan impurity C[4]	0.80
Zolmiltriptan	1.0

[1](s)-2-amino-3-(4-aminophenyl)propanol,

[2](s)-4-(4-aminobenzyl)-1.3-oxozolidin-2-one,

[3]N-methyl-2-[5-(2-oxo-1,3oxozolidin-4-yl-methyl)-1H-indol-3-yl]ethyl amine,

[4] (s)-4-(-4-nitrobenzyl)-1.3-oxozolidin-2-one.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to zolmitriptan impurity C and zolmitriptan is not less than 1.5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.15 times the area of the peak in the chromatogram obtained with reference solution (a) (0.15 per cent) and the sum of areas of all the secondary peaks is not more than 0.5 times the area of the peak in the chromatogram obtained with reference solution (a) (0.5 per cent).

(R)- 4-[(3[2-(dimethyl amino)ethyl]-1H-indol-5-yl]methyl)]-2-oxazolidinone (R-isomer). Not more than 0.15 per cent.

Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 10 mg of the substance under examination in mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (a). A 0.05 per cent w/v solution of *zolmitriptan IPRS* in the mobile phase.

Reference solution (b). Dissolve 7.5 mg of R-isomer in 100.0 ml of the mobile phase. Dilute 0.5 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml, each of, reference solution (a) and reference solution (b) to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.0 mm, packed with immobilised α-1 acid glycoprotein on spherical silica particles (Such as Chiral-AGP) (5 µm),
- mobile phase. dissolve 6.8 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.9 with *triethylamine*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 222 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to *R*-isomer and zolmitriptan is not less than 1.5.

Inject reference solution (b) and the test solution.

Calculate the content of R-isomer.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in 50.0 ml of mobile phase A. Dilute 5.0 ml of the solution to 50.0 ml with mobile phase A.

Reference solution. A 0.005 per cent w/v solution of *zolmitriptan IPRS* in mobile phase A.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as X-Terra C18),
- column temperature. 40°,
- mobile phase: A. a mixture of 70 volumes of buffer solution prepared by dissolving 0.3 g of *potassium dihydrogen phosphate* and 0.5 g of *sodium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 9.8 with *sodium hydroxide solution*, 20 volumes of *methanol* and 10 volumes of *acetonitrile*,

  B. a mixture of 30 volumes of buffer solution and 70 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
25	50	50
27	100	0

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Inject the reference solution. The test is not valid unless the column efficiency is not less than 5000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{21}N_3O_2$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Zolmitriptan Nasal Spray

Zolmitriptan Nasal Spray is a solution of Zolmitriptan in a suitable liquid in a container fitted with an appropriate nasal delivery system.

Zolmitriptan Nasal Spray contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount per inhalation of zolmitriptan, $C_{16}H_{21}N_3O_2$, by actuation of the valve.

Usual strength. 5 mg per metered dose (50 mg per ml).

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to that of the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.5 to 5.5.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a quantity of the nasal spray containing 50 mg of Zolmitriptan to 50.0 ml with the mobile phase.

Reference solution. A 0.0001 per cent w/v solution of *zolmitriptan IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 85 volumes of buffer solution prepared by dissolving 7.8 g of *sodium dihydrogen orthophosphate monohydrate* in 1000 ml of *water*, adjusted to pH 7.5 with *triethylamine* and 15 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than 15 times the area of the principal peak in the chromatogram obtained with the reference solution (1.5 per cent).

Other tests. Comply with the tests stated under Nasal Preparations.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a quantity of nasal spray containing 50 mg of Zolmitriptan to 50.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.01 per cent w/v solution of *zolmitriptan IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 80 volumes of *0.05 M ammonium acetate* in 0.1 per cent v/v of *ammonia solution*, adjusted to pH 4.0 with *trifluoroacetic acid*, 10 volumes of *methanol*, and 10 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{16}H_{21}N_3O_2$ in the nasal spray.

Storage. Store protected from light.

Labelling. The label states that the amount of active ingredient delivered by each actuation of the valve and the number of deliveries available from the container.

Zolmitriptan Tablets

Zolmitriptan Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of zolmitriptan, $C_{16}H_{21}N_3O_2$.

Usual strengths. 1 mg; 2.5 mg; 5 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (b).

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Tests

Dissolution (2.5.2)

Apparatus No. 2 (Paddle),

Medium. 500 ml of *0.1 M hydrochloric acid*,

Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter. Reject the first few ml of the filtrate and dilute a suitable volume of the filtrate with dissolution medium. Measure the absorbance of the resulting solution at the maximum at about 223 nm (2.4.7). Calculate the content of $C_{16}H_{21}N_3O_2$ in the medium from the absorbance obtained from a solution of known concentration of *zolmitriptan IPRS*.

Q. Not less than 80 per cent of the stated amount of $C_{16}H_{21}N_3O_2$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of powdered tablets containing about 25 mg of Zolmitriptan, dissolve in 60 ml of the mobile phase and dilute to 100.0 ml with the mobile phase, filter.

Reference solution. A 0.0025 per cent w/v solution of *zolmitriptan IPRS* in the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Develosil ODS UG-5),
- column temperature. 40°,
- mobile phase. a mixture of 70 volumes of buffer solution prepared by dissolving 1.15 g of *ammonium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 9.8 with *ammonia* solution, 20 volumes of *methanol* and 10 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 40 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent) and the sum of areas of all the secondary peaks is not more than 20 times the area of the principal peak in the chromatogram obtained with the reference solution (2.0 per cent).

(R)- 4-[(3[2-(dimethyl amino)ethyl]-1H-indol-5-yl]methyl)]-2-oxazolidinone (R-isomer). Not more than 0.15 per cent.

Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 10 mg of Zolmitriptan, disperse in 20.0 ml of mobile phase and filter.

Reference solution (a). A 0.0075 per cent w/v solution of *R*- isomer in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 25 mg of *zolmitriptan IPRS* in 50.0 ml of the mobile phase.

Reference solution (d). Mix 1.0 ml of the reference solution (c), 2.0 ml of the reference solution (b) and dilute to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.0 mm, packed with immobilised α-1 acid glycoprotein on spherical silica particles (Such as Chiral-AGP) (5 µm),
- mobile phase: dissolve 6.8 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 6.95 with *triethylamine*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 222 nm,
- injection volume: 20 µl.

Inject reference solution (d). The test is not valid unless the resolution between R-isomer and zolmitriptan is not less than 1.5.

Inject reference solution (b) and the test solution.

Calculate the content of R-isomer.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography as described under Assay with the following modification.

Test solution. Disperse one tablet in the mobile phase and dilute to obtain a solution containing 0.0025 per cent w/v of zolmitriptan and filter.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 25 mg of Zolmitriptan, in 100 ml of the mobile phase and filter. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). A 0.05 per cent w/v solution of *zolmitriptan IPRS* in the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as X-terra-C18),

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- mobile phase: a mixture of 65 volumes of buffer solution prepared by dissolving 1.15 g of *ammonium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 9.85 with equal volumes of *ammonia* and *acetonitrile*, 20 volumes of *methanol* and 15 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 225 nm,
- injection volume: 10 µl.

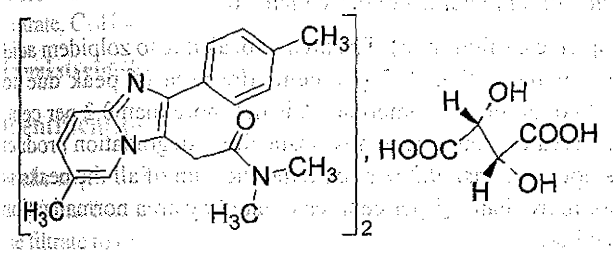
Inject reference solution (b). The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (b) and the test solution.

Calculate the content of $C_{16}H_{21}N_3O_2$ in the tablet.

Storage. Store protected from light.

Zolpidem Tartrate



$(C_{19}H_{21}N_3O)_2, C_4H_6O_6$ Mol. Wt. 765.0

Zolpidem Tartrate is bis[*N*,*N*-dimethyl-2-[6-methyl-2-(4-methylphenyl)imidazo[1,2-*a*]pyridine-3-yl]acetamide] tartrate.

Zolpidem Tartrate contains not less than 98.5 per cent and not more than 101.0 per cent of $(C_{19}H_{21}N_3O)_2, C_4H_6O_6$, calculated on the anhydrous basis.

Category. Sedatives.

Description. A white or almost white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *zolpidem tartrate IPRS* or with the reference spectrum of zolpidem tartrate.

B. A 10 per cent w/v solution in *methanol* gives reaction (c) of tartrates (2.3.1).

Tests

Appearance of solution. Dissolve 1.0 g of the substance under examination in 100 ml of a 0.5 per cent w/v solution of *tartaric acid*. The solution is clear (2.4.1), and not more intensely coloured than reference solution YS6 or BYS6 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in 50.0 ml of the mobile phase.

Reference solution (a). A 0.01 per cent w/v solution of *N,N-dimethyl-2-[7-methyl-2-(4-methylphenyl) imidazo[1,2-a] pyridin-3-yl acetamide IPRS (zolpidem impurity A IPRS)* in the mobile phase.

Reference solution (b). A 0.05 per cent w/v solution of *zolpidem tartrate IPRS* in the mobile phase.

Reference solution (c). Dilute 1.0 ml of the reference solution (b) to 100.0 ml with the mobile phase.

Reference solution (d). To 2.0 ml of reference solution (b), add 10.0 ml of reference solution (a).

Chromatographic system
- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (4 µm),
- mobile phase: 18 volumes of *acetonitrile*, 23 volumes of *methanol* and 59 volumes of 0.56 per cent w/v solution of *orthophosphoric acid*, adjusted to pH 5.5 with *triethylamine*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (d). The test is not valid unless the resolution between the peaks due to zolpidem impurity A and zolpidem tartrate is not less than 2.0.

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.2 times the area of the peak in the chromatogram obtained with reference solution (c) (0.2 per cent) and the sum of the areas of all the secondary peaks is not more than the area of the peak in the chromatogram obtained with reference solution (c) (1.0 per cent). Ignore any peak with a relative retention time of 0.16 to the zolpidem peak, corresponding to tartaric acid and any secondary peak with an area less than 0.05 times the area of the peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 3.0 per cent, determined on 0.5 g.

Assay. Dissolve 0.3 g in a mixture of 20 ml of *anhydrous acetic acid* and 20 ml of *acetic anhydride*. Titrate with *0.1 M perchloric acid*, determining the end point potentiometrically (2.4.25). Carry out a blank titration

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1 ml of *0.1 M perchloric acid* is equivalent to 0.03824 g of $(C_{19}H_{21}N_3O)_2, C_4H_6O_6$.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Zolpidem Tartrate Prolonged-release Tablets

Zolpidem Tartrate Prolonged-release Tablets manufactured by different manufacturers, whilst complying with the requirements of the monograph, are not interchangeable, as the dissolution profile of the products of different manufacturers may not be the same.

Zolpidem Tartrate Prolonged release Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of zolpidem tartrate, $C_{42}H_{48}N_6O_8$.

Usual strengths. 6.25 mg, 12.5 mg

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution (b) corresponds to the peak in the chromatogram obtained with reference solution (b).

Tests

Dissolution (2.5.2). Complies with the test stated under Tablets.

Uniformity of content. Complies with the test stated under tablets.

Determine by liquid chromatography (2.4.14), using the chromatographic conditions as described under Assay.

Test solution. Transfer one tablet to 25-ml volumetric flash add about 20.0 ml of a mixture of *ethanol* and *0.01 M hydrochloric acid* (4:2) and disperse with the aid of ultrasound for 20 minutes. Cool and dilute to volume with the same diluent and filter, rejecting the first few ml of the filtrate. Dilute 10.0 ml of the filtrate to 25.0 ml with mobile phase.

Inject reference solution (b) and test solution.

Calculate the content of $C_{42}H_{48}N_6O_8$ in the tablet.

Related substances. Determine by liquid chromatography (2.4.14).

Using the buffer solution, mobile phase, reference solution (a), test solution (b) and chromatographic system as described under Assay.

Reference solution(b). Dissolve a suitable quantity of *zolpidem related compound A IPRS* in the reference solution (a) to obtain a solution containing 0.0001 per cent w/v of *zolpidem related compound A*. Dilute 1.0 ml of the solution to 5.0 ml with mobile phase.

Name	Relative retention time	Correction factor
Zolpidem acid[1]	0.3	0.8
Zolpidem related compound A[2]	0.9	1.0
Zolpidem	1.0	—
Any unspecified degradation product	—	1.0
Total impurities	—	—

[1]2-(6-Methyl-2-p-tolylimidazo[1,2-a]pyridin-3-yl)acetic acid,

[2]*N*,*N*-Dimethyl-2-(7-methyl-2-*p*-tolylimidazo[1,2-a]pyridin-3-yl) acetamide.

Inject reference solution (b). The test is not valid unless the resolution between the peak due to zolpidem related compound A and zolpidem is not less than 1.5, the relative standard deviation for replicate injections is not more than 2.0 per cent and tailing factor is not more than 3.0.

Inject test solution (b). The area of peak due to zolpidem acid is not more than 0.2 per cent, the area of peak due to zolpidem related compound A is not more than 0.2 per cent, the area of peak due to any unspecified degradation product is not more than 0.2 per cent, and the sum of all the peaks is not more than 0.5 per cent, calculated by area normalization method.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution (a). Weigh and powder 20 tablets. Disperse a quantity of powder containing 50 mg of Zolpidem Tartrate in 70 ml of a mixture of *ethanol* and *0.01 M hydrochloric acid* (5:2) stir for 1 hour and dilute to 100.0 ml with *ethanol*. Shake well and filter.

Test solution (b). Dilute test solution (a), suitably with mobile phase, to obtain a solution containing 0.01 per cent w/v of Zolpidem Tartrate.

Reference solution (a). A 0.05 per cent w/v of *zolpidem tartrate IPRS* in a mixture of *ethanol* and *0.01 M hydrochloric acid* (4:1).

Reference solution (b). A 0.01 per cent w/v of *zolpidem tartrate IPRS* from the reference solution (a) in mobile phase.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature 40°,
- mobile phase: a mixture of 11 volumes of a buffer solution prepared by dissolving 3.3 ml of *orthophosphoric acid*

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in 1000 ml of *water*, adjusted to pH 5.5 with *triethylamine*,
- 4 volumes of *acetronitrile* and 5 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 15 µl.

Inject the reference solution (b). The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 and the tailing factor is not more than 3.0.

Inject the reference solution (b) and the test solution (b).

Calculate the content of $C_{42}H_{48}N_6O_8$ in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Zolpidem Tablets

Zolpidem Tartrate Tablets

Zolpidem Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of zolpidem tartrate, $C_{42}H_{48}N_6O_8$.

Usual strengths. 5 mg; 10 mg.

Identification

Shake a quantity of the powdered tablets containing 0.1 g of Zolpidem Tartrate with 40 ml of *methanol*, filter and evaporate the filtrate to dryness. The residue complies with the following test.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *zolpidem tartrate IPRS* or with the reference spectrum of zolpidem tartrate.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.01 M hydrochloric acid*,
Speed and time. 50 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve 10 m g of *zolpidem tartrate IPRS* in sufficient *methanol* to produce 100.0 ml. Dilute the resulting solution with the dissolution medium to obtain a solution containing 0.0005 per cent w/v of zolpidem tartrate.

Use the chromatographic system described under test for Related substances.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{42}H_{48}N_6O_8$.

Q. Not less than 75 per cent of the stated amount of $C_{42}H_{48}N_6O_8$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 25 mg of Zolpidem Tartrate with 25 ml of the mobile phase and dilute to 50.0 ml with the same solvent and filter.

Reference solution (a). A 0.01 per cent w/v solution of *N,N-dimethyl-2-[7-methyl-2-(4-methylphenyl) imidazo[1,2-a] pyridin-3-yl acetamide IPRS (zolpidem impurity A IPRS)* in the mobile phase.

Reference solution (b). A 0.05 per cent w/v solution of *zolpidem tartrate IPRS* in the mobile phase.

Reference solution (c). Dilute 1.0 ml of the reference solution (b) to 100.0 ml with the mobile phase.

Reference solution (d). To 2.0 ml of reference solution (b), add 10.0 ml of reference solution (a).

Chromatographic system
- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (4 µm),
- mobile phase: 18 volumes of *acetonitrile*, 23 volumes of *methanol* and 59 volumes of 0.56 per cent w/v solution of *orthophosphoric acid*, adjusted to pH 5.5 with *triethylamine*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (d). The test is not valid unless the resolution between the peaks due to zolpidem impurity A and zolpidem tartrate is not less than 2.0.

Inject reference solution (c) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent) and the sum of the areas of all the secondary peaks is not more than twice the area of the peak in the chromatogram obtained with reference solution (c) (2.0 per cent). Ignore any peak with a relative retention time of 0.16 to the zolpidem peak, corresponding to tartaric acid and

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any secondary peak with an area less than 0.05 times the area of the peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay, using the following solution as the test solution.

Test solution. Disperse one tablet in the mobile phase, mix and dilute to obtain a solution containing 0.005 per cent w/v of zolpidem tartrate and filter.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 25 mg of Zolpidem Tartrate, disperse in 25 ml of the mobile phase and dilute to 50.0 ml with same solvent and filter. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. A 0.005 per cent w/v solution of *zolpidem tartrate IPRS* in the mobile phase.

Use the chromatographic system described under test for Related substances.

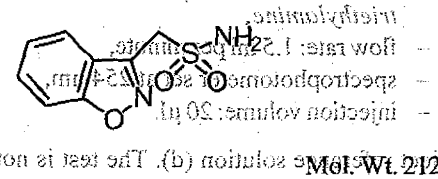
Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{42}H_{48}N_6O_8$ in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Zonisamide



$C_8H_8N_2O_3S$ Mol. Wt. 212.2

Zonisamide is 1-(1,2-benzoxazol-3-yl)methanesulfonamide.

Zonisamide contains not less than 98.0 per cent and not more than 102.0 per cent of $C_8H_8N_2O_3S$, calculated on the anhydrous basis.

Description. A white to off-white powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained from *zonisamide IPRS* or with the reference spectrum of zonisamide.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution. A solution containing 0.0001 per cent w/v of *zonisamide IPRS* and 0.00015 per cent w/v of *zonisamide impurity A IPRS* (*1,2-benzisoxazole-3-methane sulphonic acid IPRS*) in the mobile phase.

Use the chromatographic system as described under Assay.

The relative retention time with reference to zonisamide for zonisamide impurity A is 0.51.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to zonisamide and zonisamide impurity A is not less than 10.0.

Inject the reference solution and the test solution. Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any peak corresponding to zonisamide impurity A is not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.15 per cent); the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (0.3 per cent).

Heavy metals (2.3.13). 2.0 g complies with the limit test of heavy metals, Method B (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 0.8 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 10 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution. A 0.01 per cent w/v solution of *zonisamide IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 10 volumes of *acetonitrile*, 10 volumes of *methanol* and 80 volumes of a 0.136 per

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cent w/v solution of *monobasic potassium phosphate*, adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 240 nm,
- injection volume: 20 µl

Inject the reference solution. The test is not valid unless the column efficiency is not less than 5000 theoretical plates, the tailing factor is not more than 1.8 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_8N_2O_3S$.

Storage. Store protected from moisture.

Zonisamide Capsules

Zonisamide Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of zonisamide, $C_8H_8N_2O_3S$.

Usual strengths. 25 mg; 50 mg; 100 mg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle) (Use sinkers, if required),
Medium. 900 ml of *water*,
Speed and time. 75 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter through 0.45 µm pore size filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a quantity of *zonisamide IPRS* in minimum quantity of *acetonitrile* and dilute with dissolution medium to obtain a solution having a known concentration similar to the test solution.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of *acetonitrile*, 10 volumes of *methanol* and 140 volumes of a buffer solution prepared by diluting 52 ml of 40 per cent *tetrabutylammonium hydroxide solution* in 948 ml of *water*, adjusted to pH 7.5 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation of replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_8N_2O_3S$.

Q. Not less than 70 per cent of the stated amount of $C_8H_8N_2O_3S$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of mixed contents of 20 capsules containing about 0.4 g of Zonisamide add 20 ml of *water* and disperse with the aid of ultrasound for 30 minutes and dilute to 100.0 ml with the mobile phase. Mix well and filter. Further dilute 8.0 ml of resulting solution to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve a quantity of *zonisamide IPRS* in minimum quantity of *acetonitrile* and dilute with mobile phase to obtain a solution having concentration of 0.032 per cent w/v of zonisamide.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase and further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve a 20 mg of *zonisamide impurity C IPRS* in the 10.0 ml of *methanol* and dilute to 50.0 ml with the mobile phase.

Reference solution (d). Equal volumes of reference solution (a) and reference solution (c).

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of *acetonitrile*, 10 volumes of *methanol* and 140 volumes of a buffer solution prepared by diluting 52 ml of 40 per cent *tetrabutylammonium hydroxide solution* in 948 ml of *water*, adjusted to pH 7.5 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 238 nm,
- injection volume: 20 µl.

Name	Relative retention time
Zonisamide	1.0
Zonisamide impurity C[1]	1.3
Methyl zonisamide[2]	1.6
Zonisamide impurity A[3]	2.5

[1] *N'*-(benzisoxazol-3-ylmethylsulfonyl)-*N*,*N*-dimethylformimidamide, for identification and system suitability,

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[2]1-benzisoxazol-3-yl-*N*-methylmethanesulphonamide,

[3]1,2-benzisoxazole-3-methane sulphonic acid.

Inject reference solution (d). The test is not valid unless the resolution between the peaks due to zonisamide and zonisamide impurity C is not less than 2.0 and the tailing factor is not more than 1.5.

Inject reference solution (b) and the test solution. Run the chromatogram 6 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to methyl zonisamide and zonisamide impurity A is not more than 2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent). The area of any other secondary peak is not more than 2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Ignore any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh a quantity of the mixed contents of 20 capsules containing 100 mg of Zonisamide, and transfer to a 100.0 ml volumetric flask. Add about 80.0 ml of *methanol* and disperse with the aid of ultrasound for about 15 minutes, cool and dilute to volume with *methanol*. Mix well and filter. Dilute 1.0 ml of the filtrate to 10.0 ml with mobile phase.

Reference solution. Dilute 1.0 ml of 0.1 per cent w/v solution of *zonisamide IPRS* in *methanol* to 10.0 ml with mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of *acetonitrile*, 40 volumes of *methanol* and 160 volumes of *0.1 per cent v/v solution of trifluoroacetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 237 nm,
- injection volume: 10 µl.

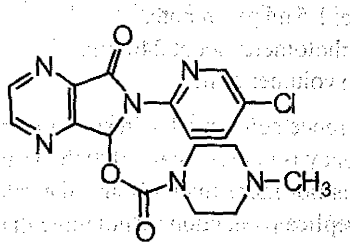
Inject the reference solution. The test is not valid unless tailing factor is not more than 2.0 and the relative standard deviation of replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution. Run the chromatogram 1.5 times the retention time of the principal peak.

Calculate the content of $C_8H_8N_2O_3S$ in the capsules.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Zopiclone



$C_{17}H_{17}ClN_6O_3$ Mol. Wt. 388.8

Zopiclone is (*RS*)-6-(5-chloro-2-pyridyl)-6,7-dihydro-7-oxo-5*H*-pyrrolo[3,4-*b*]pyrazin-5-yl 4-methylpiperazine-1-carboxylate.

Zopiclone contains not less than 98.5 per cent and not more than 100.5 per cent of $C_{17}H_{17}ClN_6O_3$.

Category. Non-benzodiazepine hypnotic.

Description. A white or slightly yellowish powder.

Identification

Tests A and C may be omitted if test B is carried out. Test B may be omitted if tests A and C are carried out.

A. When examined in the range 220 nm to 350 nm (2.4.7), a 0.001 per cent w/v solution in 0.35 per cent solution of *hydrochloric acid* shows absorption maxima at about 303 nm and specific absorbance at the absorption maximum is 340 to 380.

B. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *zopiclone IPRS* or with the reference spectrum of zopiclone.

C Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 2 volumes of *triethylamine*, 50 volumes of *acetone* and 50 volumes of *ethyl acetate*.

Test solution. Dissolve 10 mg of the substance under examination in *dichloromethane* and dilute to 10.0 ml with *dichloromethane*.

Reference solution. A 0.1 per cent w/v solution of *zopiclone IPRS* in *dichloromethane*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Solution A. A 5.0 per cent w/v solution in *dimethylformamide*.

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Appearance of solution. Solution A is not more opalescent than opalescence standard OS2 (2.4.1).

Optical rotation (2.4.22). – 0.05° to + 0.05°, determined in 10.0 ml of solution A, diluted to 50.0 ml with *dimethylformamide.*

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use.

Test solution. Dissolve 40 mg of the substance under examination in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 3.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (c). A 0.04 per cent w/v solution of *zopiclone impurity A IPRS (zopiclone oxide IPRS)* in the mobile phase. To 10.0 ml of the solution, add 1.0 ml of the test solution and dilute to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 38 volumes of *acetonitrile* and 62 volumes of a solution containing 0.81 per cent w/v solution of *sodium laurylsulphate* and 0.16 per cent w/v solution of *sodium dihydrogen phosphate*, adjusted to pH 3.5 with a 10 per cent v/v solution of *orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 303 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to zopiclone impurity A and zopiclone is not less than 3.0.

Inject reference solution (a), (b) and the test solution. Run the chromatogram 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent) and not more than 2 such peaks have an area more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Dissolve 0.3 g in a mixture of 10 ml of *anhydrous acetic acid* and 40 ml of *acetic anhydride.* Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.03888 g of $C_{17}H_{17}ClN_6O_3$.

Storage. Store protected from light.

Zopiclone Tablets

Zopiclone Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of zopiclone, $C_{17}H_{17}ClN_6O_3$.

NOTE — Carry out all of the following procedures protected from light.

Usual strengths. 3.75 mg; 7.5 mg.

Identification

Shake a quantity of the powdered tablets containing 37.5 mg of Zopiclone with 30 ml of *acetone* and dilute to 50 ml with *acetone* and filter. Evaporate the filtrate to dryness on a water-bath and dry the residue in an oven at 60° for 2 hours. On the residue, determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *zopiclone IPRS* or with the reference spectrum of zopiclone.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 500 ml of *0.1 M hydrochloric acid.*
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Dilute a suitable volume of the filtrate, if necessary with dissolution medium. Measure the absorbance of the resulting solution at the maximum at about 305 nm (2.4.7). Calculate the content of $C_{17}H_{17}ClN_6O_3$ in the medium from the absorbance obtained from a solution of known concentration of *zopiclone IPRS.*

Q. Not less than 70 per cent of the stated amount of $C_{17}H_{17}ClN_6O_3$.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Disperse a quantity of the powdered tablets containing 37.5 mg of Zopiclone with 50 ml of the mobile phase with the aid of ultrasound for 20 minutes and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

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Reference solution (b). A solution containing 0.0001 per cent w/v, each of, *zopiclone IPRS, zopiclone oxide IPRS* and *2-amino-5-chloropyridine IPRS* in the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 37.5 volumes of *acetonitrile* and 62.5 volumes of a solution containing 0.5 per cent w/v solution of *sodium dodecyl sulphate* and 0.01 per cent w/v solution of *sodium dihydrogen orthophosphate*, adjusted to pH 4.0 with *orthophosphoric acid*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 303 nm,
- injection volume: 20 µl.

The retention times for 2-amino-5-chloropyridine, zopiclone oxide and zopiclone are about 7 minutes, about 20 minutes and about 26 minutes respectively.

Inject reference solution (b) and (c). The test is not valid unless the resolution between the peaks due to zopiclone and zopiclone oxide is not less than 3.0 in the chromatogram obtained with reference solution (b) and in the chromatogram obtained with reference solution (c), the signal-to-noise ratio of the principal peak is not less than 10.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution, the area of the peak due to 2-amino-5-chloropyridine is not more than twice the area of the peak due to 2-amino-5-chloropyridine in reference solution (b) (0.5 per cent). The area of any other secondary peak is not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent) and the sum of areas of all the secondary peaks is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in reference solution (a) (0.1 per cent).

Uniformity of content. Complies with the test stated under Tablets as described under Assay using the following solution as the test solution.
Determine by liquid chromatography (2.4.14).

Test solution. Disperse 1 tablet with the mobile phase to obtain a solution containing 0.0075 per cent w/v of Zopiclone.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 37.5 mg of Zopiclone with 60 ml of the mobile phase with the aid of ultrasound for 20 minutes and dilute to 500.0 ml with the mobile phase, filter.

Reference solution. A 0.0075 per cent w/v solution of *zopiclone IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 1 volume of *acetonitrile* and 1 volume of a solution containing 0.5 per cent w/v of *sodium dodecyl sulphate* and 0.1 per cent w/v of *sodium dihydrogen orthophosphate*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 303 nm,
- injection volume: 10 µl.

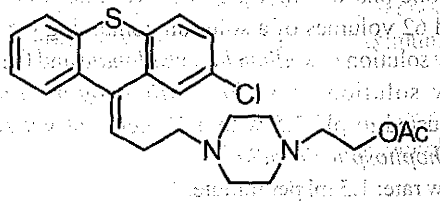
Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.4 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{17}ClN_6O_3$ in the tablets.

Storage. Store protected from light.

Zuclopenthixol Acetate



$C_{24}H_{27}ClN_2O_2S$ Mol. Wt.443.0

Zuclopenthixol Acetate is (*Z*)-2-{4-[3-(2-chlorothioxanthene-9-ylidene)propyl]piperazin-1-yl}ethyl acetate.

Zuclopenthixol Acetate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{24}H_{27}ClN_2O_2S$, calculated on the dried basis.

Category. Neuroleptic.

Description. A yellowish, viscous oil.

Identification

A. When examined in the range 210 nm to 350 nm (2.4.7), a 0.0015 per cent w/v solution in *ethanol (95 per cent)* exhibits two maxima at 230 nm and 268 nm. The absorbances at the maxima are about 1.18 and 0.51 respectively.

B. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *zuclopenthixol*

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acetate IPRS or with the reference spectrum of zuclopenthixol acetate.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel F254*.

NOTE — Protected from light.

Mobile phase. A mixture of 10 volumes of *diethylamine*, 40 volumes of *dichloromethane* and 50 volumes of *cyclohexane*.

Test solution. A 0.25 per cent w/v solution of the substance under examination in *dichloromethane*.

Reference solution (a). A 0.00025 per cent w/v solution of the substance under examination in *dichloromethane*.

Reference solution (b). A 0.000125 per cent w/v solution of the substance under examination in *dichloromethane*.

Reference solution (c). A 0.0005 per cent w/v solution of *2-chlorothioxanthone IPRS* in *dichloromethane*.

Reference solution (d). A 0.000625 per cent w/v solution of *zuclopenthixol hydrochloride IPRS* in *dichloromethane* (containing a few drops of *diethylamine*).

Apply to the plate 4 µl of each solution. After removal of the plate, dry the plate in air, spray with a mixture of equal volumes of *sulphuric acid* and *ethanol*, heat at 110° for 5 minutes and examine under ultraviolet light at 365 nm immediately. In the chromatogram obtained with the test solution, any spot corresponding to 2-chlorothioxanthone (zuclopenthixol acetate impurity B) is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent) and any spot corresponding to zuclopenthixol (zuclopenthixol acetate impurity C) is not more intense than the spot in the chromatogram obtained with reference solution (d) (0.25 per cent). Any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.1 per cent) and not more than three such spots are more intense than the spot in the chromatogram obtained with reference solution (b) (0.05 per cent).

Trans-Isomer. Determine by liquid chromatography (2.4.14).

NOTE — Protected from light.

Test solution. A 0.04 per cent w/v of the substance under examination in *dichloromethane*.

Reference solution (a). A 0.00046 per cent w/v solution of *trans-clopenthixol acetate dihydrochloride IPRS* in *dichloromethane*.

Reference solution (b). A solution containing 0.02 per cent w/v of the substance under examination and 0.023 per cent w/v of *trans-clopenthixol acetate dihydrochloride IPRS* in *dichloromethane*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with silica (5 µm) (Such as Spherisorb S 5W),
- mobile phase: a mixture of 0.03 volume of *13.5 M ammonia*, 45 volumes of *dichloromethane*, 45 volumes of *heptane* and 50 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 15 µl.

Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is not less than 2.6.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to trans-clopenthixol acetate (zuclopenthixol acetate impurity A) is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.4 per cent, determined on 1.0 g by drying at 60° at a pressure not exceeding 0.7 kPa for 3 hours.

Assay. Dissolve 0.2 g in 50 ml of *anhydrous acetic acid*, determining the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02215 g of $C_{24}H_{27}ClN_2O_2S$.

Storage. Store protected from light, at a temperature not exceeding -20°.

Zuclopenthixol Acetate Injection

Zuclopenthixol Acetate Injection is a sterile solution of Zuclopenthixol Acetate in a suitable vegetable oil.

Zuclopenthixol Acetate Injection contains not less than 95.0 per cent and not more than 105.0 per cent of $C_{24}H_{27}ClN_2O_2S$.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel F254*.

NOTE — Protect the solutions from light.

Mobile phase. A mixture of 3 volumes of *diethylamine* and 90 volumes of *cyclohexane*.

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Test solution. Dilute a volume of the injection with *ethanol (95 per cent)* to obtain a solution containing 0.5 per cent w/v of Zuclopenthixol Acetate.

Reference solution (a). A 0.5 per cent w/v solution of *zuclopenthixol acetate dihydrochloride IPRS* in *ethanol (95 per cent).*

Reference solution (b). A solution containing 0.5 per cent w/v, each of, *zuclopenthixol acetate dihydrochloride IPRS* and *zuclopenthixol decanoate dihydrochloride IPRS* in *ethanol (95 per cent).*

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air, spray with a 1.0 per cent w/v solution of *sodium molybdate* in *sulphuric acid* heat at 110° for 20 minutes and examine in daylight. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel F254.*

NOTE— Protect the solutions from light.

Mobile phase. A mixture of 10 volumes of *diethylamine*, 40 volumes of *dichloromethane* and 50 volumes of *cyclohexane.*

Test solution. Dissolve a quantity of the injection containing 0.1 g of Zuclopenthixol Acetate in *dichloromethane* and dilute to 50.0 ml with *dichloromethane.*

Reference solution (a). A 0.001 per cent w/v solution of *2-chlorothioxanthone IPRS* in *dichloromethane.*

Reference solution (b). A 0.002 per cent w/v solution of *zuclopenthixol hydrochloride IPRS* in *dichloromethane* (containing a few drops of *diethylamine*).

Reference solution (c). A 0.0004 per cent w/v solution of *zuclopenthixol hydrochloride IPRS* in *dichloromethane* (containing a few drops of *diethylamine*).

Reference solution (d). A 0.0002 per cent w/v solution of *zuclopenthixol hydrochloride IPRS* in *dichloromethane* (containing a few drops of *diethylamine*).

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 15 cm. Dry the plate in air, spray with a mixture of equal volumes of *sulphuric acid* and *ethanol*, heat at 110° for 5 minutes and examine under ultraviolet light at 365 nm. In the chromatogram obtained with the test solution, any spot corresponding to 2-chlorothioxanthone is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent). Any spot corresponding to zuclopenthixol is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent). Any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (0.2 per cent) and not more than one other secondary spot more intense than the spot in the chromatogram obtained with reference solution (d) (0.1 per cent).

***Trans*-Isomer.** Determine by liquid chromatography (2.4.14).

NOTE— Protect the solutions from light.

Test solution. Dissolve a quantity of the injection containing 20 mg of Zuclopenthixol Acetate in *dichloromethane* and dilute to 50.0 ml with *dichloromethane.*

Reference solution (a). Dissolve 23 mg of *trans-clopenthixol acetate dihydrochloride IPRS* (equivalent to 20 mg of *trans*-clopenthixol acetate) in *dichloromethane* containing a few drops of *diethylamine* and dilute to 50.0 ml with same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *dichloromethane.*

Reference solution (b). A mixture of equal volumes of the test solution and undiluted reference solution (a).

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with silica (5 µm) (Such as Spherisorb S 5W),
- mobile phase : a mixture of 0.08 volume of *13.5 M ammonia*, 45 volumes of *dichloromethane*, 45 volumes of *n-heptane*, and 50 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 50 µl.

Inject reference solution (b). The test is not valid unless the resolution between the principal peaks is not less than 2.6.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to *trans*-clopenthixol acetate is not more than the area of the peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Internal standard solution. A 0.2 per cent w/v solution of *flupenthixol propionate dihydrochloride IPRS* in *dichloromethane.*

Test solution. Dissolve a quantity of the injection containing 20 mg of Zuclopenthixol Acetate in 25 ml of *dichloromethane*, add 5.0 ml of the internal standard solution and dilute to 50.0 ml with *dichloromethane.*

Reference solution. Dissolve 20 mg of *zuclopenthixol acetate dihydrochloride IPRS* in 25 ml of *dichloromethane* containing a few drops of *diethylamine*, add 5 ml of the internal standard solution and dilute to 50.0 ml with *dichloromethane.*

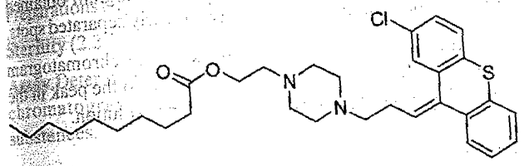
Use chromatographic system as described under test for *trans*-Isomer.

Inject the reference solution and the test solution.

Calculate the content of $C_{24}H_{27}ClN_2O_2S$ in the injection.

Storage. Store protected from light.

Zuclopenthixol Decanoate



$C_{32}H_{43}ClN_2O_2S$ Mol. Wt. 555.2

Zuclopenthixol Decanoate 2-[4-[3-[(9*Z*)-2-Chloro-9*H*-thioxanthen-9-ylidene]propyl]piperazin-1-yl]ethyl decanoate.

Zuclopenthixol Decanoate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{32}H_{43}ClN_2O_2S$, calculated on dried basis.

Category. Antipsychotic.

Description. A yellowish, viscous oily liquid.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *zuclopenthixol decanoate IPRS* or with the reference spectrum of zuclopenthixol decanoate.

Tests

Appearance of solution. A 5.0 per cent w/v solution in *ethanol (95 per cent)* is clear (2.4.1)

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Carry out all the following procedures protected from light, and prepare solutions immediately before use.

Test solution. Dissolve 25 mg of the substance under examination in 70 ml of *acetonitrile* with the aid of ultrasound and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the *acetonitrile.*

Reference solution (b). A 0.00025 per cent w/v solution of *zuclopenthixol impurity* B in *acetonitrile.*

Reference solution (c). Dissolve the contents of a vial of *zuclopenthixol for system suitability IPRS* (zuclopenthixol decanoate containing impurities A, B and C) in 1.0 ml of *methanol.*

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 25 volumes of a buffer solution prepared by dissolving 8.9 gm of *docusate sodium* in 700 ml of *water*, stirring for about 6-8 hours, and dilute to 1000 ml with the same solvent, 75 volumes of *ethanol* and 0.1 volume of *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Name	Relative retention time
Zuclopenthixol decanoate impurity C[1]	0.4
Zuclopenthixol decanoate impurity B[2]	0.5
Zuclopenthixol decanoate (Retention time: about 12 minutes)	1.0
Zuclopenthixol decanoate impurity A[3]	1.1

[1]2-[4-[3-[(9*Z*)-2chloro-9*H*-thioxanthen-9-ylidene]propyl]piperazin-1-yl]ethanol,

[2]2chloro-9*H*-thioxanthen-9-one,

[3]2-[4-[3-[(9*E*)-2chloro-9*H*-thioxanthen-9-ylidene]propyl]piperazin-1-yl]ethyl decanoate.

Inject the reference solution (c). The test is not valid unless peak-to-valley ratio is not less than 2.0, where *Hp* is the height above the baseline of the peak due to zuclopenthixol decanoate impurity C and *Hv* is the height above the baseline of the lowest point of the curve separating this peak from the peak due to zuclopenthixol decanoate impurity B and not less than 2.5, where *Hp* is the height above the baseline of the peak due to zuclopenthixol decanoate impurity A and *Hv* is the height above the baseline of the lowest point of the curve separating this peak from the peak due to zuclopenthixol decanoate.

Inject reference solution (a), (b) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to zuclopenthixol decanoate impurity A is not more than 1.3 times the area of principal peak in the chromatogram obtained with reference solution (a) (1.3 per cent). The area of any peak corresponding to zuclopenthixol decanoate impurity B is not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent). The area of any peak correspond

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to zuclopenthixol decanoate impurity C is not more than 0.3 times the area of principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). The area of any other secondary peak is not more 0.1 times than the area of principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent). The sum of the areas of all the secondary peaks is not more than 1.5 times the area of principal peak in thechromatogram obtained with the reference solution (a) (1.5per cent). Ignore any peak with an area less than 0.05 times the area of principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 3 hours.

Assay. Dissolve 0.25 g in 50 ml of *anhydrous acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02776 g of $C_{32}H_{43}ClN_2O_2S$.

Storage.Store protected from light and moisture, at a temperature not exceeding -20°. It is recommended that the contents ofan opened container be used as soon as possible and any unused part be protected by an atmosphere of inert gas.

Zuclopenthixol Decanoate Injection

Zuclopenthixol Decanoate Injection is a sterile solution of Zuclopenthixol Decanoate in a suitable vegetable oil.

Zuclopenthixol Decanoate Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of zuclopenthixol decanoate, $C_{32}H_{43}ClN_2O_2S$.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF 254*. Heat the plate at 110° for 30 minutes before use.

NOTE — Protect the solutions from light.

Mobile phase. A mixture of 3 volumes of *diethylamine* and 90 volumes of *cyclohexane*.

Test solution. Dilute a volume of the injection with *ethanol (95 per cent)* to obtain a solution containing 0.5 per cent w/v of Zuclopenthixol Decanoate.

Reference solution (a). A 0.5 per cent w/v solution of *zuclopenthixol decanoate dihydrochloride IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A solution containing 0.5 per cent w/v, each of, *zuclopenthixol decanoate dihydrochloride IPRS* and *zuclopenthixol acetatedihydrochloride IPRS* in *ethanol (95 per cent)*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air, spray with 1 per cent w/v solution of *sodium molybdate* in *sulphuric acid*, heat at 110° for 20 minutes and examine in day light. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a).The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Protect the solutions from light.

Test solution. Dilute a volume of the injection containing 500 mg of Zuclopenthixol Decanoate to 100.0 ml with *acetonitrile*.

Reference solution (a). A 0.015 per cent w/v solution of *zuclopenthixol hydrochloride IPRS* in *acetonitrile*.

Reference solution (b). A 0.0025 per cent w/v solution of *2-chlorothioxanthone IPRS* in *acetonitrile*.

Reference solution (c). A 0.005 per cent w/v solution of trans-*clopenthixol decanoate IPRS* in *acetonitrile*.

Reference solution (d). A solution containing 0.015 per cent w/v of *zuclopenthixol hydrochloride IPRS*, 0.0025 per cent w/v of *2-chlorothioxanthone IPRS* and 0.005 per cent w/v of trans-*clopenthixol decanoate IPRS* in the test solution.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 26 volumes of a buffer solution prepared by dissolving 8.9 g of *dioctyl sodium sulfosuccinate* in *water*, stirring for about 6-8 hours, and dilute to 1000 ml with *water*, 74 volumes of *ethanol (95 per cent)* and 0.1 volume of *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

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Inject reference solution (d). The test is not valid unless the chromatogram shows clearly separated peaks.

Inject reference solution (a), (b), (c) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to zuclopenthixol is not more than the area of principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent), the area of any peak corresponding to *2*-chlorothioxanthone is not more than the area of principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the area of any peak corresponding to trans-clopenthixol decanoate is not more than the area of principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Sterility (2.2.11). Complies with the test for sterility.

Assay. Determine by liquid chromatography (2.4.14), using chromatographic system as described under Related substances.

NOTE — Protect the solutions from light.

Test solution. Dilute a volume of the injection containing 500 mg of Zuclopenthixol Decanoate to 100.0 ml with *acetonitrile.*

Reference solution. A 0.57 per cent w/v solution of *zuclopenthixol decanoate dihydrochloride IPRS* in *acetonitrile.*

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{32}H_{43}ClN_3O_2S$ in the injection.

1 mg of Zuclopenthixol Decanoate dihydrochloride is equivalent to 0.8852 mg of Zuclopenthixol Decanoate.

Storage. Store protected from light.

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VITAMINS, MINERALS, AMINO ACIDS, FATTY ACIDS ETC.

General Monographs

Oil-Soluble Vitamins Capsules 4021
Oil-Soluble Vitamins Oral Solution 4024
Oil-Soluble Vitamins Tablets 4027
Water-Soluble Vitamins Capsules 4030
Water-Soluble Vitamins Tablets 4035

Monographs

Alfacalcidol 4038
Arginine 4039
Ascorbic Acid 4039
Ascorbic Acid Injection 4040
Ascorbic Acid Tablets 4040
Biotin 4041
Calcipotriol Anhydrous 4042
Calcipotriol Ointment 4043
Calcitriol 4044
Calcitriol Capsules 4045
Calcium Carbonate 4045
Calcium Carbonate Tablets 4046
Calcium Chloride 4046
Calcium Chloride Injection 4047
Calcium Citrate Malate 4048
Calcium Folinate 4049
Calcium Folinate Injection 4050
Calcium Gluconate 4051
Calcium Gluconate Injection 4052
Calcium Gluconate Tablets 4053
Calcium Lactate 4054
Calcium Lactate Tablets 4054
Calcium Levulinate 4055

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Calcium Levulinate Injection 4055
Calcium Pantothenate 4055
Calcium Pantothenate Tablets 4056
Dibasic Calcium Phosphate 4057
Tribasic Calcium Phosphate 4058
Calcium and Vitamin D_3 Tablets 4059
Cholecalciferol 4060
Cholecalciferol Injection 4061
Cholecalciferol Tablets 4061
Cholecalciferol Concentrate (Powder Form) 4062
Chromium Picolinate 4064
Citric Acid 4065
Citric Acid Monohydrate 4066
Copper Gluconate 4067
Cyanocobalamin 4068
Cyanocobalamin Injection 4069
Ergocalciferol 4070
Ergocalciferol Tablets 4071
Ferrous Ascorbate 4072
Ferrous Ascorbate and Folic Acid Suspension 4073
Ferrous Ascorbate and Folic Acid Tablets 4074
Ferrous Fumarate 4075
Ferrous Fumarate Tablets 4076
Ferrous Gluconate 4077
Ferrous Gluconate Tablets 4078
Ferrous Sulphate 4078
Dried Ferrous Sulphate 4079
Ferrous Sulphate Tablets 4080
Folic Acid 4081
Folic Acid Tablets 4082
Folic Acid and Methylcobalamin Tablets 4083
Glutamic Acid 4084
Glycine 4085

Inositol 4085
Iron and Ammonium Citrate 4087
Iron and Folic Acid Syrup 4087
Iron and Folic Acid Tablets 4088
Levocarnitine 4089
Levocarnitine Tablets 4091
Alpha Lipoic Acid 4091
Lutein 4092
Lysine Hydrochloride 4093
Heavy Magnesium Carbonate 4094
Light Magnesium Carbonate 4095
Magnesium Chloride 4096
Heavy Magnesium Oxide 4096
Light Magnesium Oxide 4097
Magnesium Sulphate 4098
Magnesium Sulphate Injection 4098
Magnesium Trisilicate 4099
Methylcobalamin 4100
Nicotinamide 4101
Nicotinamide Tablets 4102
Nicotinic Acid 4102
Nicotinic Acid Tablets 4103
D-Panthenol 4104
Pepsin 4104
Phenylalanine 4105
Phytomenadione 4106
Phytomenadione Injection 4106
Potassium Chloride 4107
Potassium Chloride for Injection 4108
Potassium Citrate 4109
Potassium Iodide 4110
Potassium Sorbate 4110
Pyridoxine Hydrochloride 4111

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Pyridoxine Tablets 4112
Riboflavin 4113
Riboflavin Sodium Phosphate 4115
Riboflavin Tablets 4116
Selenious Acid 4117
Selenomethionine 4117
Sodium Citrate 4119
Thiamine Hydrochloride 4119
Thiamine Injection 4121
Thiamine Tablets 4121
Thiamine Mononitrate 4123
Threonine 4124
Tocopheryl Acetate 4125
Tryptophan 4126
Valine 4127
Vitamin A Capsules 4128
Vitamin A Concentrate Oil 4129
Vitamin A Concentrate Powder 4129
Water-Miscible Vitamin A Concentrate 4130
Vitamin A Paediatric Oral Solution 4131
Vitamins A and D Capsules 4132
Concentrated Vitamin D Solution 4132
Concentrated Vitamins A and D Solution 4132
Zinc Chloride 4133
Zinc Chloride Injection 4133
Zinc Citrate 4134
Zinc Sulphate 4134
Zinc Sulphate Monohydrate 4135
Zinc Sulphate Oral Solution 4135
Zinc Sulphate Dispersible Tablets 4135

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Oil-Soluble Vitamins Capsules

Oil-Soluble Vitamins Capsules contain two or more of the following oil-soluble vitamins: Vitamin A, Vitamin D as Ergocalciferol (Vitamin D_2) or Cholecalciferol (Vitamin D_3), Vitamin E, Phytonadione (Vitamin K_1), and Beta Carotene. Capsules contain not less than 90.0 per cent and not more than 165.0 per cent of the labeled amounts of vitamin A ($C_{20}H_{30}O$) as retinol or esters of retinol in the form of retinyl acetate ($C_{22}H_{32}O_2$) or retinyl palmitate ($C_{36}H_{60}O_2$); vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$).

Oil-Soluble Vitamins Capsules contain no other vitamins or any minerals. They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

Tests

Microbial contamination (2.2.9). The total aerobic viable count is not more than 3000 cfu per g, the total combined molds and yeasts count is not more than 300 cfu per g. 1 g is free from *Escherichia coli*. 10 g is free from *salmonella*.

Other tests. Comply with the tests stated under Capsules.

Assay

Vitamin A. Determine by liquid chromatography (2.4.14).

NOTE — Where the use of a vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation. Vitamin A IPRS is retinyl acetate. It is to be used where Vitamin A IPRS is specified. Use low-actinic glassware throughout this procedure.

Test solution (a). Weigh 20 capsules. Open the capsules without the loss of shell material and transfer the contents to a 100-ml beaker, equivalent to 5 capsules, to a container having a polytef-lined screw-cap. *(NOTE — For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent to 5 Capsules, to a container having a polytef-lined screw cap.)* Add 10 ml of *dimethyl sulphoxide* and 15 ml of *n-hexane*, and shake for 45 minutes on a wrist-action shaker in a water-bath maintained at 60°. *(NOTE — Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly).* Centrifuge at 3000 rpm for 10 minutes, and transfer the hexane layer by means of a pipet to a 100-ml volumetric flask. Add 15 ml of *n-hexane* to the dimethyl sulphoxide layer, shake thoroughly for 5 minutes, and transfer the n- hexane layer by means of a pipet to the 100-ml volumetric flask. Repeat this extraction with 3 additional 15-ml portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume.

Test solution (b). Dilute a suitable volume of test solution (a) with *n-hexane* to obtain a solution having concentration 0.0015 per cent w/v of Vitamin A as Retinol.

Reference solution (a). A 0.0015 per cent w/v solution of *retinyl acetate from vitamin A IPRS* in *n-hexane*.

Reference solution (b). A 0.0015 per cent w/v solution of *retinyl palmitate IPRS* in *n-hexane*.

Reference solution (c). A mixture of equal volumes of reference solution (a) and reference solution (b) with *n-hexane* to obtain a solution having concentration 0.00075 per cent w/v each of *retinyl acetate* and *retinyl palmitate*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with aminopropylsilane bonded to porous silica (3µm),
- mobile phase: *n-hexane*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 325nm,
- injection volume: 40 µl.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate is not less than 10.0 and the relative standard deviation for replicate injections is not more than 3.0 per cent in the chromatogram obtained with reference solution (c).

Inject reference solution (a) and test solution (b).

NOTE — Measure the peak area of all-trans-retinyl acetate from the Standard solution and the peak area of all-trans-retinyl acetate or all-trans-retinyl palmitate from the Test solution (b).

Calculate the content of vitamin A, as retinol ($C_{20}H_{30}O$) in the capsules.

NOTE — The molar responses of retinyl acetate and retinyl palmitate are equivalent.

1 mg of retinyl acetate $C_{22}H_{32}O_2$ is equivalent to 0.872 mg of vitamin A, as retinol $C_{20}H_{30}O$.

NOTE — One Vitamin A IPRS Unit = 0.3 µg of all-trans-retinol(vitamin A alcohol) or 0.344 µg of all-trans-retinyl acetate (vitamin A acetate) or 0.55 µg of all-trans-retinyl palmitate (vitamin Apalmitate), and 1 µg of retinol (3.3 Vitamin A IPRS Units)= 1 retinol equivalent (RE).

Vitamin D. Determine by liquid chromatography (2.4.14).

NOTE — Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical

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form present in the formulation and the relevant IPRS. Use low-actinic glassware throughout this procedure.

Test solution. Proceed as directed for test solution (a) in Vitamin A. Transfer not less than 20.0 ml of the solution retained as specified in the direction for the test solution (b) in Vitamin A, to a suitable container, and evaporate, if necessary, in vacuum at room temperature and dilute with *n-hexane* to obtain a solution having a concentration of 0.0002 per cent w/v of Cholecalciferol or Eergocalciferol.

Reference solution (a). A 0.0002 per cent w/v solution of *cholecalciferol IPRS or ergocalciferol IPRS* in *n-hexane.*

Reference solution (b). Heat a volume of reference solution (a) at 60° for 1 hour to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with aminopropylsilane bonded to porous silica (3 µm),
- mobile phase: a mixture of 99 volumes of *n-hexane* and 1 volume of *isopropyl alcohol,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 100 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to the vitamin D form present and its corresponding precursor is not less than 10.0 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 3.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Cholecalciferol or Ergocalciferol (Vitamin D)

$$= \frac{r_u}{r_s} \times \frac{C_s}{C_u} \times F \times 100$$

where, r_u = peak area of cholecalciferol or ergocalciferol from the test solution;

r_s = peak area of cholecalciferol or ergocalciferol from reference solution (a);

C_s = concentration of Cholecalciferol IPRS or Ergocalciferol IPRS in reference solution (a) (µg per ml);

C_u = nominal concentration of cholecalciferol orergocalciferol in the test solution. (µg per ml);

F = correction factor to account for the average amount of previtamin D present in the test solution, 1.09.

Calculate the content of vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the capsules.

NOTE — 1 Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol.

Vitamin E. Determine by liquid chromatography (2.4.14).

NOTE — Where vitamin E (alpha tocopherol, alpha tocopheryl acetate,or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant IPRS. Use low-actinic glassware throughout this procedure.

Solvent mixture. A 1 per cent v/v solution of *orthophosphoric acid* in *water.*

Test solution. Proceed as directed for test solution (a) in Vitamin A. Transfer not less than 20.0 ml of the solution retained as specified in the direction for test solution (b) in Vitamin A, to a suitable container, and evaporate if necessary under vacuum at room temperature to dryness. Transfer the contents to a suitable volumetric flask with the aid of *methanol*, and dilute with *methanol* to volume to obtain a solution of 0.2 per cent w/v of Vitamin E (alpha-tocopherol, alpha-tocopheryl acetate, or alpha-tocopheryl acid succinate).

Reference solution (a). A solution containing 0.2 per cent w/v each of *alpha tocopherol IPRS* and *alpha tocopheryl acetate IPRS*, or *alpha tocopheryl acid succinate IPRS* in the *methanol.*

Reference solution (b). A 0.065 per cent w/v solution of *ergocalciferol IPRS* in *methanol.* Dilute 1.0 ml of the solution to a 100-ml volumetric flask containing 100 mg of *alpha tocopheryl acetate IPRS.* Dissolve in 30 ml of *methanol*, with the aid of sonication if necessary, and dilute with *methanol* to volume. Store of the solution in a refrigerator.

Chromatographic system
- a stainless steel column 10 cm x 8.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: mixture of 95 volumes of *methanol* and 5 volumes of the solvent mixture,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 100 µl.

The relative retention times with reference to alpha tocopheryl acetate for ergocalciferol is about 0.5.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due ergocalciferol and alpha tocopheryl acetate is not less than 12.0 and the tailing factor is not less than 0.8 and not more than 1.2 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 3.0 per cent in the chromatogram obtained with reference solution (a).

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Inject reference solution (a) and the test solution.

Calculate the content of vitamin E, as alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the capsules.

NOTE — 1 mg of dl-alpha tocopherol = 1.1 Vitamin E Units, 1 mg of dl-alpha tocopheryl acetate = 1 Vitamin E Unit, 1 mg of dl-alpha tocopheryl acid succinate = 0.89 Vitamin E Unit, 1 mg of d-alpha tocopherol = 1.49 Vitamin E Units, and 1 mg of d-alpha tocopheryl acetate = 1.36 Vitamin E Units, 1 mg of d-alpha tocopheryl acid succinate = 1.21 Vitamin E Units. In terms of d-alphatocopherol equivalents, 1 mg of d-alpha tocopheryl acetate = 0.91, 1 mg of d-alpha tocopheryl acid succinate = 0.81, 1 mg of dl-alphatocopherol = 0.74, 1 mg of dl-alpha tocopheryl acetate = 0.67, and 1 mg of dl-alpha tocopheryl acid succinate = 0.60.

Phytonadione. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Test solution. Proceed as directed for test solution (a) in Vitamin A. Transfer not less than 20.0 ml of the solution retained as specified in the direction for test solution (b) in Vitamin A, to a suitable container, and evaporate if necessary under vacuum at room temperature to dryness. Transfer the residue to a suitable volumetric flask with the aid of *methanol*, and dilute with *methanol* to volume to obtain a solution of 0.002 per cent w/v of Phytonadione.

Reference solution (a). A 0.002 per cent w/v solution of *phytonadione IPRS* in *methanol*.

Reference solution (b). A solution containing 0.065 per cent w/v of *alpha tocopherol acetate IPRS* and 0.002 per cent w/v of *phytonadione IPRS* in *methanol*.

Chromatographic system

- stainless steel column 10 cm x 8.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: mixture of 95 volumes of *methanol* and 5 volumes of *water*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 100 µl.

The relative retention times with reference to phytonadione for alpha-tocopheryl acetate is about 0.68.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due alpha-tocopheryl acetate and phytonadione is not less than 5.0 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 3.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and test solution.

Calculate the content of phytonadione ($C_{31}H_{46}O_2$) in the capsules.

Beta Carotene

NOTE — Use low-actinic glassware throughout this procedure.

Solution A. 0.001 per cent w/v solution of *iodine* in *cyclohexane. (NOTE —prepare this solution fresh daily)*

Test solution A (for preparations containing beta carotene in oil solutions). Proceed as directed in Vitamin A except use *cyclohexane* instead of *n-hexane* as the extraction solvent, and dilute the filtered extracts with *cyclohexane* to obtain a solution having concentration 0.0002 per cent w/v of Beta carotene.

Test solution B (for preparations containing beta carotene in dry powder). Remove the contents of not less than 20 Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Disperse a quantity of the powder containing 2 mg of Beta carotene, to a 500-ml saponification flask. Add 100 ml of *ethanol*, 6 ml of *potassium hydroxide solution* (dissolve 58.8 g of *potassium hydroxide* in 50 of *water*), and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 minutes with constant stirring. Cool to room temperature, add 170 ml of *hexane*, and stir for 30 minutes. Quantitatively transfer the contents of the flask to a 500-ml separatory-funnel with portions of *hexane*. Allow the layers to separate for 5-10 minutes, and transfer the upper organic layer to a 500-ml volumetric flask. Transfer the lower aqueous layer into the saponification flask; add 170 ml of *hexane*, and stir for an additional 20 minutes. Quantitatively transfer the contents of the saponification flask to the separatory-funnel with the aid of portions of *hexane*. Allow the layers to separate for 10 minutes. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory-funnel with small portions of *hexane*, and transfer the washings to the volumetric flask. Dilute the *hexane* extracts with *hexane* to volume, add 3 g of *anhydrous sodium sulphate*, shake, and allow to settle. Quantitatively transfer a volume of the solution, equivalent to 0.01 per cent w/v of beta carotene, to a 50-ml volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add *cyclohexane*. Add 2 ml of *solution A* and heat for 15 minutes in a water-bath maintained at 65°. Cool rapidly, and dilute with *cyclohexane* to volume.

Measure the absorbance of the resulting solution at 452 nm (2.4.7). Calculate the content of $C_{40}H_{56}$, taking 223 as specific absorbance at the maximum at 452 nm. Carry out a blank *cyclohexane*

NOTE — 1 IU of betacarotene = 0.6 µg of alltrans - beta carotene

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Storage. Store protected from light and moisture.

Labelling. Label the Capsules to state that the product is Oil-Soluble Vitamins Capsules. The label also states the quantity of each vitamin/dosage unit and, where necessary, the chemical form in which it is present. Where the product contains vitamin E, the label also indicates whether it is the *d*- or *dl*- form.

Oil-Soluble Vitamins Oral Solution

Oil-Soluble Vitamins Oral Solution contain, two or more of the following oil-soluble vitamins: Vitamin A, as retinol or esters of retinol in the form of retinyl acetate or retinyl palmitate, Vitamin D as Ergocalciferol (Vitamin D_2) or Cholecalciferol (Vitamin D_3), Vitamin E as alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate, Phytonadione (Vitamin K_1), and Beta Carotene. Oral Solution contain not less than 90.0 per cent and not more than 150.0 per cent of the labeled amounts of vitamin A, as retinol equivalent ($C_{20}H_{30}O$); vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E, as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$).

Tests

Ethanol (2.3.45). Not less than 90.0 per cent and not more than 120.0 per cent of the stated amount of ethanol, determined by method III.

Microbial contamination (2.2.9). The total aerobic viable count is not more than 3000 cfu per ml, the total combined molds and yeasts count is not more than 300 cfu per ml. 1 g is free from *Escherichia coli*. 10 ml is free from *salmonella*.

Other tests. Comply with the tests stated under Oral Solution.

Assay

Vitamin A. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Test solution. Dissolve a suitable quantity of the oral solution in *n-hexane* to obtain a solution having concentration 0.0013 per cent w/v solution of Retinol.

Reference solution (a). A 0.0013 per cent w/v solution of *retinol from retinyl acetate IPRS* in *n-hexane*.

Reference solution (b). A 0.0013 per cent w/v solution of *retinol from retinyl palmitate IPRS* in *n-hexane*.

Reference solution (c). A mixture of, equal volumes of reference solution (a) and reference solution (b).

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with aminopropylsilane bonded to porous silica (3µm) (Such as Zorbax NH2),
- mobile phase: *n-hexane*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 325nm,
- injection volume: 40 µl.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate is not less than 10.0 and the relative standard deviation for replicate injections is not more than 3.0 per cent obtained with reference solution (c).

Inject reference solution (a) or (b) and the test solution.

Calculate the content of vitamin A, as retinol ($C_{20}H_{30}O$) in the oral solution.

NOTE — One Vitamin A Unit= 0.3 µg of all-trans-retinol (vitamin A alcohol) or 0.344 µg of all-trans-retinyl acetate (vitamin A acetate) or 0.55 µg of all-trans-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 Vitamin A Units)= 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-trans-betacarotene.

Vitamin D. Determine by liquid chromatography (2.4.14).

NOTE — Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant IPRS. Use low-actinic glassware throughout this procedure.

Test solution (a). Dissolve a suitable quantity of the oral solution in *n-hexane* to obtain a solution having concentration 0.002 per cent w/v solution of Cholecalciferol or Ergocalciferol.

Test solution (b). Transfer 5.0 ml of test solution (a) to a container having a polytef-lined screw cap and heat, with constant shaking, for 1 hour in a water-bath maintained at 60° to obtain a solution containing vitamin D (Cholecalciferol or Ergocalciferol) and its corresponding precursor. Cool, and dilute with *n-hexane* to obtain a solution containing 0.0002 per cent w/v of Cholecalciferol or Ergocalciferol.

Reference solution (a). A 0.0002 per cent w/v solution of *cholecalciferol IPRS* or *ergocalciferol IPRS* in *n-hexane*.

Reference solution (b). Heat a volume of reference solution (a) at 60° for 1 hour to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with aminopropylsilane bonded to porous silica (3 µm) (Such as Zorbax NH2),
- mobile phase: a mixture of 99 volumes of *n-hexane* and 1 volume of *isopropyl alcohol*,

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– flow rate: 1 ml per minute,
– spectrophotometer set at 265 nm,
– injection volume: 100 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to vitamin D form present and its corresponding precursor is not less than 10.0 and the relative standard deviation for replicate injections is not more than 3.0 per cent obtained with reference solution (b).

Inject reference solution (a) and test solution (b).

Cholecalciferol or Ergocalciferol (Vitamin D)

$$= \frac{r_u}{r_s} \times \frac{C_s}{C_u} \times F \times 100$$

where, r_u = peak area of cholecalciferol or ergocalciferol from test solution (b);
r_s = peak area of cholecalciferol or ergocalciferol from reference solution (a);
C_s = concentration of Cholecalciferol IPRS or Ergocalciferol IPRS in reference solution (a) (µg per ml);
C_u = nominal concentration of cholecalciferol or ergocalciferol in test solution (b). (µg per ml);
F = correction factor to account for the average amount of previtamin D present in test solution (b), 1.09.

Calculate the content of vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the oral solution.

NOTE — 1 Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol.

Vitamin E. Determine by liquid chromatography (2.4.14).

NOTE — Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant IPRS. Use low-actinic glassware throughout this procedure.

Buffer solution. A 1 per cent v/v solution of *orthophosphoric acid* in *water.*

Test solution. Dissolve a suitable quantity of the oral solution in *methanol* to obtain a solution having concentration 0.2 per cent w/v solution of Alpha Tocopherol, Alpha Tocopheryl Acetate or Alpha Tocopheryl Acid Succinate.

Reference solution. A solution containing 0.2 per cent w/v of *alpha tocopherol IPRS, alpha tocopheryl acetate IPRS* or *alpha tocopheryl acid succinate IPRS* in *methanol.*

Chromatographic system
– a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Sunfire C18),
– mobile phase: mixture of 95 volumes of *methanol* and 5 volumes of the buffer solution,
– flow rate: 1 ml per minute,
– spectrophotometer set at 291 nm,
– injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of vitamin E, as alpha tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the oral solution.

NOTE — 1 mg of dl-alpha tocopherol = 1.1 Vitamin E Units, 1 mg of dl-alpha tocopheryl acetate = 1 Vitamin E Unit, 1 mg of dl-alpha tocopheryl acid succinate = 0.89 Vitamin E Unit, 1 mg of d-alpha tocopherol = 1.49 Vitamin E Units, and 1 mg of d-alpha tocopheryl acetate = 1.36 Vitamin E Units, 1 mg of d-alpha tocopheryl acid succinate = 1.21 Vitamin E Units. In terms of d-alpha tocopherol equivalents, 1 mg of d-alpha tocopheryl acetate = 0.91, 1 mg of d-alpha tocopheryl acid succinate = 0.81, 1 mg of dl-alpha tocopherol = 0.74, 1 mg of dl-alpha tocopheryl acetate = 0.67, and 1 mg of dl-alpha tocopheryl acid succinate = 0.60.

Phytonadione (Vitamin K_1). Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Test solution. Dissolve a suitable quantity of the oral solution in *methanol* to obtain a solution having concentration 0.002 per cent w/v solution of Phytonadione.

Reference solution (a). A 0.002 per cent w/v solution of *phytonadione IPRS* in *methanol.*

Reference solution (b). A solution containing 0.065 per cent w/v of *alpha tocopheryl acetate IPRS* and 0.002 per cent w/v of *phytonadione IPRS* in *methanol.*

Chromatographic system
– a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Sunfire C18),
– mobile phase: a mixture of 95 volumes of *methanol* and 5 volumes of *water*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 254 nm,
– injection volume: 50 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due alpha-tocopheryl acetate and phytonadione is not less than 5.0 in the chromatogram obtained with reference solution (b) and the relative standard

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deviation for replicate injections is not more than 3.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of phytonadione ($C_{31}H_{46}O_2$) in the oral solution.

Beta Carotene. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Solvent mixture. A 0.005 per cent v/v solution of *butylated hydroxytoluene* in *ethanol.*

Test solution (a). Dissolve a quantity of the oral solution containing 20.0 mg of Beta Caroteneto in 250-ml volumetric flask. Add 250 mg of *butylated hydroxytoluene*, 120 ml of *methylene chloride*, and 100 ml of *ethanol.* Shake the flask until the sample is completely dissolved or suspended. Let the mixture stand in the dark until it reaches room temperature (about 2 hours). Add *methylene chloride* to volume, and shake again vigorously.

Test solution (b). Dilute a volume of test solution (a) with a mixture of, equal volume of *methylene chloride* and solvent mixture to obtain the final concentration 0.0003 per cent w/v of Beta Carotene. Pass through a membrane filter of 0.45-im pore size.

Reference solution (a). A 0.006 per cent w/v solution of *beta carotene IPRS* in *tetrahydrofuran.*

Reference solution (b). Transfer 5.0 ml of reference solution (a) into a 100-ml volumetric flask, add 5 ml of *tetrahydrofuran,* and dilute with solvent mixture to volume.

NOTE — Determine the concentration of reference solution (b) from the concentration of reference solution (c) as described below.

Reference solution (c). Transfer 5.0 ml of reference solution (a) into a 100-ml volumetric flask and dilute with *cyclohexane* to volume. Prepare in triplicate.

Measure the absorbance of the resulting solution at the maximum at about 457 nm (2.4.7). Calculate the concentration of total beta carotene (mg per ml) as all-trans-beta carotene ($C_{40}H_{56}$) in reference solution (c) taking 250 as the specific absorbance at 457 nm. Blank as a *cyclohexane.*

NOTE — The concentration of reference solution (c) equals the concentration of reference solution (b).

$$\text{Result} = \frac{A}{F}$$

where, A = average absorbance of the three preparations of reference solution (c);

F = absorptivity of pure all-trans-beta carotene in cyclohexane, 250.

Reference solution (d). Transfer 20 mg of *beta carotene IPRS* to a 50-ml volumetric flask, add 1 ml of *water,* 4 ml of *tetrahydrofuran*, and sonicate for 5 minutes. Dilute with solvent mixture to volume and sonicate for 5 minutes. Cool to room temperature, filter the suspension through a membrane filter of 0.45-µm pore size, and use the clear filtrate.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with spherical porous silica the surface of withch has been covalently modified with alkyl amide group and not endcapped (5 µm) (Such as Suplex pKb-100),
- column temperature: 30°,
- mobile phase: dissolve 50 mg of *butylated hydroxytoluene* into a 1000-ml volumetric flask, and dissolve with 20 ml of *2-propanol.* Add 0.2 ml of *N-ethyldiisopropylamine*, 25 ml of 0.2 per cent *ammonium acetate solution*, 455 ml of *acetonitrile* and about 450 ml of *methanol.* Allow the solution to reach room temperature, and dilute with *methanol* to volume,
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 448 nm,
- injection volume: 20 µl.

Name	Relative retention time	Correction factor
All-trans-alpha carotene	0.93	0.91
All-trans-beta carotene	1.00	—
9-cis-Beta carotene	1.07	—
13-cis-Beta carotene	1.17	0.83
15-cis-Beta carotene	1.21	0.71

Inject reference solution (b) and (d). The test is not valid unless the resolution between the peaks due to beta carotene and alpha carotene, between beta carotene and 9-cis-beta carotene is not less than 1.5 in the chromatogram obtained with reference solution (d), the tailing factor of beta carotene peak is not more than 2.0 and the relative standard deviation for replicate injections of beta carotene peak is not more than 2.0 per cent in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and test solution (b).

Calculate the content of beta carotene ($C_{40}H_{56}$) in the oral solution.

NOTE — Use concentration of all-trans-beta carotene in reference solution (c) as determined by the spectrometric procedure.

Storage. Store protected from light and tightly-closed containers, under an inert gas or with a minimum of headspace.

Labelling. The label states that the product is Oil-Soluble Vitamins Oral Solution. The label states the quantity of each

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vitamin present in a given volume of Oral Solution and, where necessary, the chemical form in which a vitamin is present. Where the product contains vitamin E, the label indicates whether it is the d- or dl- form.

Oil-Soluble Vitamins Tablets

Oil-Soluble Vitamins Tablets contain two or more of the following oil-soluble vitamins: Vitamin A (as retinyl acetate or retinyl palmitate), Vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), Vitamin E (as RRR-or *all-rac*-alpha-tocopherol, *RRR*-or *all-rac*-alpha-tocopheryl acetate, or RRR-or *all-rac*-alpha-tocopheryl acid succinate), Phytonadione (vitamin K_1), and Beta Carotene.

Oil-Soluble Vitamins Tablets contain not less than 90.0 per cent and not more than 165.0 per cent of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$), vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$), vitamin E as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$), phytonadione ($C_{31}H_{46}O_2$), and beta carotene ($C_{40}H_{56}$).

Oil-Soluble Vitamins Tablets contain no other vitamins or any minerals. They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

Identification

The retention times of the vitamin peaks of the test solutions correspond to those of the corresponding vitamin peaks of the reference solutions as obtained in the tests.

Tests

Microbial contamination (2.2.9). Total aerobic viable count is not more than 3000 CFU per g, the total combined molds and yeasts count is not more than 300 CFU per g. 1 g is free from *Escherichia coli* and 10 g is free from *Salmonella*.

Other tests. Comply with the tests stated under Tablets.

Assay

Vitamin A. Determine by liquid chromatography (2.4.14).

NOTE — Throughout the procedure protect the solutions from the atmosphere and light, preferably by the use of a blanket of inert gas and low-actinic glassware.

Where an ester form of vitamin A (Retinyl acetate or Retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation and the relevant IPRS.

Test solution (a). Transfer a portion of the powder, equivalent to 5 tablets, to a container having a polytef-lined screw-cap. Add 10 ml of *dimethyl sulphoxide* and 15 ml of *n-hexane*, shake for 45 minutes on a wrist-action shaker in a water bath maintained at 60°. *(NOTE — Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly).* Centrifuge at 3000 rpm for 10 minutes and transfer the hexane layer using a pipet to a 100-ml volumetric flask. Add 15 ml of *n-hexane* to the dimethyl sulphoxide layer, shake for 5 minutes and transfer the hexane layer using a pipet to the 100-ml volumetric flask. Repeat this extraction with 3 additional 15-ml portions of *n-hexane*. Dilute the extracts in the volumetric flask with *n-hexane* to volume.

Test solution (b). Dilute 10.0 ml of test solution (a) with *n-hexane* to obtain a solution of 0.0015 per cent w/v of Vitamin A as retinol.

Reference solution (a). A 0.0015 per cent w/v solution of retinol from *retinyl acetate*[1] *IPRS* in *n-hexane*.

Reference solution (b). A 0.0015 per cent w/v solution of retinol from *retinyl palmitate*[2] *IPRS* in *n-hexane*.

Reference solution (c). Mix equal volumes of reference solution (a) and reference solution (b).

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with aminopropylsilane bonded to porous silica (3 µm) (Such as Zorbax NH_2),
- mobile phase: *n-hexane*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 325 nm,
- injection volume: 40 µl.

Inject reference solution (a) or (b) and (c). The test is not valid unless the resolution between the peaks due to all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate is not less than 10.0 in the chromatogram obtained with reference solution (c) and the relative standard deviation for replicate injections is not more than 3.0 per cent in the chromatogram obtained with reference solution (a) or (b).

Inject reference solution (a) or (b) and the test solution (b).

Calculate the content of vitamin A as retinol ($C_{20}H_{30}O$) in the tablets.

NOTE — This chromatographic system can separate the 13-cis and all-trans-isomers of vitamin A, only the all-trans-isomer peak is used for the quantitation of vitamin A.

[1]Use the value of 0.872 to convert retinyl acetate to its retinol equivalent.

[2]Use the value of 0.546 to convert retinyl palmitate to its retinol equivalent.

Vitamin D. Determine by liquid chromatography (2.4.14).

NOTE — Throughout the procedure protect the solutions from the atmosphere and light, preferably by the use of a blanket of inert gas and low-actinic glassware.

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Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant IPRS.

Test solution (a). Transfer a portion of the powder, equivalent to 5 tablets, to a container having a polytef-lined screw-cap. Add 10 ml of *dimethyl sulphoxide* and 15 ml of *n-hexane*, and shake for 45 minutes on a wrist-action shaker in a water bath maintained at 60°. *(NOTE — Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly).* Centrifuge at 3000 rpm for 10 minutes, and transfer the hexane layer using a pipet to a 100-ml volumetric flask. Add 15 ml of *n-hexane* to the dimethyl sulphoxide layer, shake thoroughly for 5 minutes, and transfer the hexane layer using a pipet to the 100-ml volumetric flask. Repeat this extraction with 3 additional 15-ml portions of *n-hexane*. Dilute the extracts in the volumetric flask with *n-hexane* to volume.

Test solution (b). Transfer not less than 20.0 ml of test solution (a) to a suitable container, and, if necessary, evaporate under vacuum at room temperature to obtain a solution of 0.0002 per cent w/v of Vitamin D.

Reference solution (a). A 0.0002 per cent w/v solution of *cholecalciferol IPRS* or *ergocalciferol IPRS* in *n-hexane*.

Reference solution (b). Heat a volume of the reference solution (a) at 60° for 1 hour to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with aminopropylsilane bonded to porous silica (3 µm) (Such as Zorbax NH_2),
- mobile phase: a mixture of 99 volumes of *n-hexane* and 1 volume of *isopropyl alcohol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 100 µl.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to the vitamin D form present and its corresponding precursor is not less than 10.0 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 3.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and test solution (b).

Calculate the content of vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the tablets.

$$\frac{A_T \times C_R}{A_R \times C_T} \times F \times 100$$

where, A_T = peak response of cholecalciferol or ergocalciferol from the test solution;

A_R = peak response of cholecalciferol or ergocalciferol from the reference solution;

C_R = concentration of cholecalciferol IPRS or ergocalciferol IPRS in the reference solution;

C_T = nominal concentration of cholecalciferol or ergocalciferol in the test solution,

F = correction factor to account for the average amount of previtamin D present in the Sample solution, 1.09.

Vitamin E. Determine by liquid chromatography (2.4.14).

NOTE — Throughout the procedure protect the solutions from the atmosphere and light, preferably by the use of a blanket of inert gas and low-actinic glassware.

Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant Reference Standard.

Solvent mixture. A 1.0 per cent v/v solution of *orthophosphoric acid* in *water*.

Test solution (a). Transfer a portion of the powder, equivalent to 5 tablets, to a container having a polytef-lined screw-cap. Add 10 ml of *dimethyl sulphoxide* and 15 ml of *n-hexane*, shake for 45 minutes on a wrist-action shaker in a water bath maintained at 60°. *(NOTE — Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly).* Centrifuge at 3000 rpm for 10 minutes and transfer the hexane layer using a pipet to a 100-ml volumetric flask. Add 15 ml of *n-hexane* to the dimethyl sulphoxide layer, shake for 5 minutes and transfer the hexane layer using a pipet to the 100-ml volumetric flask. Repeat this extraction with 3 additional 15-ml portions of *n-hexane*. Dilute the extracts in the volumetric flask with *n-hexane* to volume.

Test solution (b). Transfer not less than 20.0 ml of test solution (a) to a suitable container, and, if necessary evaporate under vacuum at room temperature to dryness. Transfer the residue to a suitable volumetric flask with the aid of *methanol*, and dilute with *methanol* to volume to obtain a solution of 0.2 per cent w/v of Vitamin E (alpha-tocopherol, alpha-tocopheryl acetate, or alpha-tocopheryl acid succinate).

Reference solution (a). A 0.065 per cent w/v solution of *ergocalciferol IPRS* in *methanol*. Dilute 1.0 ml of the solution to a 100-ml volumetric flask containing 100 mg of *alpha tocopheryl acetate IPRS*. Dissolve in 30 ml of *methanol*, with the aid of sonication if necessary, and dilute with *methanol* to volume. Store this solution in a refrigerator.

Reference solution (b). A solution containing 0.2 per cent w/v each of *alpha tocopherol IPRS*, *alpha tocopheryl acetate IPRS* or *alpha tocopheryl acid succinate IPRS* in *methanol*.

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Chromatographic system
- a stainless steel column 10 cm x 8.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 95 volumes of *methanol* and 5 volumes of the solvent mixture,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 100 µl.

The relative retention times with reference to alpha tocopheryl acetate for ergocalciferol is about 0.5.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to ergocalciferol and alpha tocopheryl acetate is not less than 12.0, the tailing factor is not less than 0.8 and not more than 1.2 in the chromatogram obtained with reference solution (a) and the relative standard deviation for replicate injections is not more than 3.0 per cent in the chromatogram obtained with reference solution (b).

Inject reference solution (b) and test solution (b).

Calculate the content of vitamin E, as alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as 2*R*-alpha- tocopheryl ($C_{29}H_{50}O_2$) in the tablets.

$$\frac{A_T \times C_R}{A_R \times C_T} \times F \times 100$$

where, A_T = peak response of relevant vitamin E from the test solution;

A_R = peak response of relevant vitamin E from the reference solution;

C_R = concentration of *all-rac*-alpha-tocopherol from either alpha tocopherol IPRS or alpha tocopheryl acetate IPRS or *RRR*-alpha-tocopherol from alpha tocopheryl acid succinate IPRS in the reference solution b;

C_T = nominal concentration of the corresponding form of vitamin E as 2R-alpha-tocopherol in the test solution,

F = conversion factor for the content of *all-rac*-alpha-tocopherol to 2R-alpha-tocopherol, ½ (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources).

Phytonadione

NOTE — Use low-actinic glassware throughout this procedure.

Test solution (a). Transfer a portion of the powder, equivalent to 5 Tablets, to a container having a polytef-lined screw-cap. Add 10 ml of *dimethyl sulphoxide* and 15 ml of *n-hexane*, and shake for 45 minutes on a wrist-action shaker in a water bath maintained at 60°. (*NOTE — Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.*) Centrifuge at 3000 rpm for 10 minutes, and transfer the hexane layer by means of a pipet to a 100-ml volumetric flask. Add 15 ml of *n*-hexane to the dimethyl sulphoxide layer, shake thoroughly for 5 minutes, and transfer the hexane layer by means of a pipet to the 100-ml volumetric flask. Repeat this extraction with 3 additional 15-ml portions of *n-hexane*. Dilute the extracts in the volumetric flask with *n-hexane* to volume.

Test solution (b). Transfer not less than 20.0 ml of test solution (a) to a suitable container, and evaporate under vacuum at room temperature to dryness. Transfer the residue to a suitable volumetric flask with the aid of *methanol*, and dilute with *methanol* to volume to obtain a solution of 0.002 per cent w/v of Phytonadione.

Reference solution (a). A 0.002 per cent w/v solution of *phytonadione IPRS* in *methanol.*

Reference solution (b). A solution containing 0.065 per cent w/v of *alpha tocopheryl acetate IPRS* and 0.002 per cent w/v of *phytonadione IPRS* in *methanol.*

Chromatographic system
- a stainless steel column 10 cm x 8.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 95 volumes of *methanol* and 5 volumes of *water*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 100 µl.

The relative retention times with reference to phytonadione for alpha-tocopheryl acetate is about 0.68.

Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to alpha-tocopheryl acetate and phytonadione is not less than 5.0 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 3.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and test solution (b).

Calculate the content of phytonadione ($C_{31}H_{46}O_2$) in the tablets.

Beta Carotene

NOTE — Use low-actinic glassware throughout this procedure.

Weigh and powder 20 tablets. Disperse a quantity of the powder containing 2 mg of Beta carotene, to a 500-ml saponification flask. Add 100 ml of *ethanol*, 6 ml of *potassium hydroxide solution* (dissolve 58.8 g of *potassium hydroxide* in 50 ml of *water*), and a magnetic stirring bar. Attach an air

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condenser to the flask, and heat under reflux for 45 minutes with constant stirring. Cool to room temperature, add 170 ml of *hexane*, and stir for 30 minutes. Quantitatively transfer the contents of the flask to a 500-ml separatory funnel with portions of *hexane*. Allow the layers to separate for 5-10 minutes, and transfer the upper organic layer to a 500-ml volumetric flask. Transfer the lower aqueous layer into the saponification flask; add 170 ml of *hexane*, and stir for an additional 20 minutes. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of *hexane*. Allow the layers to separate for 10 minutes. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of *hexane*, and transfer the washings to the volumetric flask. Dilute the *hexane* extracts with *hexane* to volume, add 3 g of *anhydrous sodium sulphate*, shake, and allow to settle. Quantitatively transfer a volume of the solution, equivalent to 100 µg of beta carotene, to a 50-ml volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add *cyclohexane*. Add 2 ml of a freshly prepared 0.001 per cent w/v solution of *iodine* in *cyclohexane* and heat for 15 minutes in a water-bath maintained at 65°. Cool rapidly, and dilute with *cyclohexane* to volume.

Measure the absorbance of the resulting solution at 452 nm (2.4.7). Calculate the content of $C_{40}H_{56}$, taking 223 as specific absorbance at the maximum at 452 nm.

Storage. Store protected from light and moisture.

Labelling. The label states (1) the product is oil-soluble vitamins tablets; (2) the quantity of each vitamin in mg per tablet or µg per tablet and where necessary the chemical form in which it is present; (3) where the products contains vitamin E, indicates whether it is *RRR* or *all-rac* form.

NOTE— One vitamin A unit = 0.3 µg of all-trans-retinol (vitamin A alcohol) or 0.344 µg of all-trans-retinyl acetate (vitamin A acetate) or 0.55 µg of all-trans-retinyl palmitate (vitamin A palmitate) and 1 µg of retinol (3.3 vitamin A units)= 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-trans-beta carotene; 1 Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of all-rac-alpha-tocopherol = 1.1 Vitamin E Units; 1 mg of all-rac-alpha-tocopheryl acetate = 1 Vitamin E Unit; 1 mg of all-rac-alpha-tocopheryl acid succinate = 0.89 Vitamin E Units; 1 mg of RRR-alpha-tocopherol = 1.49 Vitamin E Units; 1 mg of RRR-alpha-tocopheryl acetate = 1.36 Vitamin E Units; and 1 mg of RRR-alpha-tocopheryl acid succinate = 1.21 Vitamin E Units. In terms of RRR-alpha-tocopherol equivalents, 1 mg of RRR-alpha-tocopheryl acetate = 0.91; 1 mg of RRR-alpha-tocopheryl acid succinate = 0.81; 1 mg of all-rac-alpha-tocopherol = 0.74; 1 mg of all-rac-alpha-tocopheryl acetate = 0.67; and 1 mg of all-rac-alpha-tocopheryl acid succinate = 0.60. 1 mg of Institute of Medicine (IOM) alpha-tocopherol equivalent = 1 mg of 2R-alpha-tocopherol = 1 mg of RRR-alpha-tocopherol = 2 mg of all-rac-alpha-tocopherol.

Water-Soluble Vitamins Capsules

Water-Soluble Vitamins Capsules contain two or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Dexpanthenol or Panthenol, Niacin or Niacinamide, Pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate. Capsules contain not less than 90.0 per cent and not more than 150.0 per cent of the labeled amounts of ascorbic acid ($C_6H_8O_6$), biotin ($C_{10}H_{16}N_2O_3S$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), dexpanthenol or panthenol ($C_9H_{19}NO_4$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3$,HCl), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate.

They do not contain any form of Beta Carotene or Vitamin A, D, E, or K. They do not contain any minerals for which nutritional value is claimed. They may contain other labeled added substances in quantities that are unobjectionable.

Tests

Microbial contamination (2.2.9). The total aerobic viable count is not more than 3000 cfu per g, the total combined molds and yeasts count is not more than 300 cfu per g. 1 g is free from *Escherichia coli*. 10 g is free from *salmonella*.

Other tests. Comply with the tests stated under Capsules.

Assay

Ascorbic Acid, Calcium Ascorbate, and Sodium Ascorbate. Weigh 20 capsules. Open the capsules without the loss of shell material and transfer the contents to a 100-ml beaker. Remove any content adhering to empty shell by washing, if necessary with several portions of ether. Discard the washing and dry the capsule shell with the aid of current of dry air until the odour of ether is no longer perceptible. By subtracting the weights of dry empty shell from weight of intact capsules, calculate the average net weight per capsules. Transfer a quantity of capsule contents containing 100 mg of ascorbic acid, to a 200-ml volumetric flask. Add 75 ml of *metaphosphoric-acetic acid solution* and shake by mechanical means for 30 minutes, dilute to volume with *water* and centrifuge. Transfer a 4 ml volume of the solution into a 50-ml conical flask, and add 5 ml of *metaphosphoric-acetic acid solution*

and titrate with *standard 2,6-dichlorophenol-indophenol solution*. [Prepared by dissolving 50 mg of *2,6-dichlorophenol-indophenol sodium* (stored in a desiccators over *soda lime*) in 50 ml of *water* containing 42 mg *sodium bicarbonate*, shake vigorously and when the dye is dissolved, add *water* to make 200 ml. Filter into an amber glass-stoppered bottle. Use within 3 days and standardize immediately before use.] until the rose-pink colour persists for at least 5 seconds. Carry out a blank titration (A mixture of 5.5 ml of *metaphosphoric-acetic acids solution* and 15 ml of *water*).

Calculate the percentage of ascorbic acid, $C_6H_8O_6$ by using following expression.

$$\text{Result} = \frac{(V_S - V_B) \times F}{W} \times 100$$

where, V_S = Titrant volume consumed by the test solution (ml);

V_B = Titrant volume consumed by the Blank;

F = Concentration of Titrant in terms of its equivalent of ascorbic acid (mg per ml);

W = nominal amount of ascorbic acid taken for analysis (mg);

Biotin. Determine by liquid chromatography (2.4.14).

NOTE— Use low-actinic glassware throughout this procedure.

Test solution. Weigh 20 capsules. Open the capsules without the loss of shell material and transfer the contents to a 100-ml beaker. Remove any content adhering to empty shell by washing, if necessary with several portions of ether. Discard the washing and dry the capsule shell with the aid of current of dry air until the odour of ether is no longer perceptible. By subtracting the weights of dry empty shell from weight of intact capsules, calculate the average net weight per capsules.

Transfer a quantity of capsule contents containing 1 mg of Biotin to a 200-ml volumetric flask, add 3 ml *dimethyl sulphoxide* and swirl to wet the contents. Place the flask in a water-bath at 60°to 70° for 5 minutes and with the aid of ultrasound for 5 minutes with intermittent shaking, dilute with *water* to volume and filter.

Reference solution. A 0.0333 per cent w/v solution of *biotin IPRS* in *dimethyl sulphoxide*. Dilute the solution to obtain a concentration of 0.0005 per cent w/v of *biotin IPRS* with *water*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed octylsilane bonded to porous silica (3 µm),
- mobile phase: a mixture of 85 ml of *acetonitrile*, 1 g of *sodium perchlorate* and 1 ml of *orthophosphoric acid* dilute to 1000 ml with *water*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 200 nm,
- injection volume: 100 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of biotin ($C_{10}H_{16}N_2O_3S$) in the capsules.

Cyanocobalamin. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Test solution. Weigh 30 capsules. Open the capsules without the loss of shell material and transfer the contents to a 100-ml beaker. Remove any content adhering to empty shell by washing, if necessary with several portions of ether. Discard the washing and dry the capsule shell with the aid of current of dry air until the odour of ether is no longer perceptible. By subtracting the weights of dry empty shell from weight of intact capsules, calculate the average net weight per capsules.

Transfer a quantity of capsule contents containing 0.1 mg of Cyanocobalamin, to a 250-ml volumetric flask and add 100.0 ml of *water*, and carefully extract for 2 minutes. Filter 10 ml of the extract, and use the filtrate.

Reference solution. A 0.0001 per cent w/v solution of *cyanocobalamin (crystalline) IPRS* in *water*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of *methanol* and 65 volume of *water*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 550 nm,
- injection volume: 200 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the capsules.

Folic acid. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Solution A. A 25 per cent solution of *tetrabutylammonium hydroxide* in *methanol*.

Solution B. Transfer 5.0 g of pentetic acid to a 50-ml volumetri flask. Using sonication if necessary, dissolve in and dilut with *1 M sodium hydroxide* to volume.

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Internal standard solution. Transfer 40 mg of *methylparaben* to a 1000-ml volumetric flask, and add 220 ml of *methanol* to dissolve. Dissolve 2.0 g of *monobasic potassium phosphate* in 300 ml of *water* in a separate beaker, quantitatively transfer this solution to the flask containing the *methylparaben* solution and add an additional 300 ml of *water*. Add 19 ml of solution A, 7 ml of 3 M *orthophosphoric acid* and 30 ml of solution B. Adjusted to pH 9.8 with *ammonia solution*, and bubble nitrogen through the solution for 30 minutes, dilute with *water* to volume and mix.

Test solution. Weigh 30 capsules. Open the capsules without the loss of shell material and transfer the contents to a 100-ml beaker. Remove any content adhering to empty shell by washing, if necessary with several portions of ether. Discard the washing and dry the capsule shell with the aid of current of dry air until the odour of ether is no longer perceptible. By subtracting the weights of dry empty shell from weight of intact capsules, calculate the average net weight per capsules.

Transfer a quantity of capsule contents containing 0.0016 per cent w/v solution of Folic acid in internal standard solution, shake by mechanical means for 10 minutes, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate

Reference solution. A 0.0016 per cent w/v solution of *folic acid IPRS* in internal standard solution.

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm, packed octadecylsilane bonded to porous silica (10 µm),
- mobile phase: dissolve 2 g of *monobasic potassium phosphate* in 650 ml of *water* in a 1000-ml volumetric flask, add 12.0 ml of solution A, 7.0 ml of *3 M orthophosphoric acid* and 240 ml of *methanol*. Cool to room temperature, adjusted to pH 7.0 with *orthophosphoric acid* or *ammonia solution*, dilute to volume with *water* (Recheck the pH before use by adding *water* or *methanol* to the prepared mobile phase to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation). *[NOTE—The methanol and water content may be varied (between 1 per cent and 3 per cent).]*
- flow rate: 1 ml per minute,
- spectrophotometer set at 280nm,
- injection volume: 15 µl.

The relative retention times with reference to methylparaben for folic acid is about 0.8.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of folic acid ($C_{19}H_{19}N_7O_6$) using ratio of the peak area of folic acid to that of peak area of the internal standard in the capsules.

Dexpanthenol or Panthenol

NOTE — The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Test solution. Weigh 30 capsules. Open the capsules without the loss of shell material and transfer the contents to a 100-ml beaker. Remove any content adhering to empty shell by washing, if necessary with several portions of ether. Discard the washing and dry the capsule shell with the aid of current of dry air until the odour of ether is no longer perceptible. By subtracting the weights of dry empty shells from weight of intact capsules, calculate the average net weight per capsules. Transfer a quantity of capsule contents containing 1.2 mg of Dexpanthenol or 2.4 mg of Panthenol in 100.0 ml of *water*. Dilute the solution with *water* to obtain a nominal concentration of 0.00012 per cent w/v of dexpanthenol or 0.00024 per cent w/v of panthenol.

Reference solution (a). A solution containing 0.08 per cent w/v of *dexpanthenol IPRS* or 0.16 per cent w/v of *racemic panthenol IPRS* in *water*. Store in a refrigerator protected from light, and use within 30 days.

Reference solution (b). On the day of the assay, prepare a dilution of 0.00012 per cent w/v of *dexpanthenol IPRS* or 0.00024 per cent w/v of *panthenol IPRS* from reference solution (a) diluted with *water*.

Acid-hydrolyzed casein solution. Dissolve 100 g of vitamin-free *casein* with 500 ml of *6 M hydrochloric acid* and reflux the mixture for 8 to 12 hours. Remove the *hydrochloric acid* from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 ml of *water*, adjusted to pH 3.5 with *1 M sodium hydroxide solution* and dilute with *water* to 1000 ml. Add 20 g of *activated charcoal*, stir for 1 hour, and filter. Repeat the treatment with *activated charcoal*. Store under *toluene* in a cool place at a temperature not less than 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution. Suspend 4.0 g of *L-cystine* in a solution of 1.0 g of *L-tryptophan* (or 2.0 g of *D,L-tryptophan*) in 700 to 800 ml of *water*, heat to 75 ± 5° and add *hydrochloric acid solution* (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool and dilute with *water* to 1000 ml. Store under *toluene* in a cool place at a temperature not less than 10°.

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Adenine-guanine-uracil solution. Dissolve 0.2 g each of *adenine sulphate*, *guanine hydrochloride*, and *uracil*, with the aid of heat, in 10 ml of *4 M hydrochloric acid*. Cool, and dilute with water to 200 ml. Store under *toluene* in a refrigerator.

Polysorbate 80 solution. A 10 per cent w/v solution of *polysorbate 80* in *ethanol*.

Riboflavin-thiamine hydrochloride-biotin solution. A solution containing 0.002 per cent w/v of riboflavin, 0.001 per cent w/v of thiamine hydrochloride and 0.000004 per cent w/v of biotin in *0.02 M acetic acid*. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution. A solution containing 0.001 per cent w/v of p-aminobenzoic acid, 0.005 per cent w/v of niacin and 0.004 per cent w/v of pyridoxine hydrochloride in neutral 25 per cent ethanol. Store in a refrigerator.

Salt solution A. A solution containing 5 per cent w/v, each of, *monobasic potassium phosphate* and *dibasic potassium phosphate* in *water*. Add 10 drops of *hydrochloric acid* per liter of solution. Store under *toluene*.

Salt solution B. A solution containing 2 per cent w/v of *magnesium sulphate*, 0.1 per cent w/v of *sodium chloride*, 0.1 per cent w/v of *ferrous sulphate*, and 0.1 per cent w/v of *manganese sulphate* in *water*. Add 10 drops of *hydrochloric acid* per liter of the solution. Store under *toluene*.

Pyridoxal-calcium pantothenate solution. A solution containing 0.02 per cent w/v of *pyridoxal hydrochloride* and 0.0001875 per cent w/v of *calcium pantothenate* in 10 per cent *ethanol*. Store in a refrigerator and use within 30 days.

Polysorbate 40-oleic acid solution. A solution containing 5 per cent w/v of *polysorbate 40* and 0.05 per cent w/v of *oleic acid* in 20 per cent *ethanol*. Store in a refrigerator and use within 30 days.

Modified pantothenate medium: Dissolve *anhydrous dextrose* and *sodium acetate* in the solutions previously mixed according to table and adjusted to pH 6.8 with *1 M sodium hydroxide*. Dilute with *water* to 250 ml.

Acid-hydrolyzed casein solution	25 ml
Cystine-tryptophan solution	25 ml
Polysorbate 80 solution	0.25 ml
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 ml
Riboflavin-thiamine hydrochloride–biotin solution	5 ml
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 ml
Salt solution A	5 ml
Salt solution B	5 ml
Pyridoxal-calcium pantothenate solution	5 ml
Polysorbate 40-oleic acid solution	5 ml

Double-strength modified pantothenate medium. Prepare as directed in modified pantothenate medium, but make the final dilution to 125 ml instead of 250 ml. Prepare fresh.

Stock culture of Pediococcus acidilactici. Dissolve 6.0 g of peptone, 4.0 g of pancreatic digest of *casein*, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of *dextrose*, and 15.0 g of agar in 800 ml of *water*, with the aid of heat. Adjusted to pH 6.5 to 6.6 with *0.1 M sodium hydroxide* or *0.1 M hydrochloric acid* and dilute with *water* to 1000 ml. Add 10 ml portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 minutes. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *pediococcus acidilactici* on a slant of this medium. Incubate at 35° for 20 to 24 hours, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum. Inoculate three 250 ml portions of sterile modified pantothenate medium from a stock culture slant and incubate at 35° for 20 to 24 hours. Centrifuge the suspension from the combined portions and wash the cells with sterile modified pantothenate medium. Resuspend the cells in sufficient modified pantothenate medium so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80 per cent light transmission at 530 nm. Transfer 1.2 ml portions of this stock suspension to sterile glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 ml of thawed culture with sterile saline to 150 ml.

NOTE —This dilution may be altered when necessary to obtain the desired test response.

Analysis. Prepare in triplicate a series of eight culture tubes by adding the following quantities of *water* to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 ml. To these same tubes and in the same order add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 ml of reference solution (b).

Prepare in duplicate a series of five culture tubes by adding the following quantities of *water* to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 ml. To these same tubes and in the same order add 1.0, 1.5, 2.0, 3.0, and 4.0 ml of the test solution.

Add 5.0 ml of double-strength modified pantothenate medium to each tube. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 minutes. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 ml of the Inoculum. Allow to incubate at 37° for 16 hours. Terminate growth by heating to a temperature not less than 80°, such as by steaming at atmospheric pressure in a sterilizer for 5 to10 minutes. Cool, and determine the percentage transmittance of

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the suspensions, in cells of equal path length, on a suitable spectrophotometer at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percentage of transmittance, for each set of tubes of the standard curve against the Standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency of each tube containing portions of the test solution. To obtain the individual responses, divide the potency of each tube by the amount of the test solution added to it. Calculate the mean response by averaging the individual responses that vary from their mean by not more than 15 per cent, using not less than half the total number of tubes. Calculate the potency of the portion of the material taken for assay by multiplying the mean response by the appropriate dilution factor.

Calculate the percentage of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$) in the portion of Capsules taken:

$$\text{Result} = \frac{P}{N} \times 100$$

where, P = potency of dexpanthenol or panthenol in the portion of Capsules taken (mg);

N = nominal amount of dexpanthenol or panthenol in the portion of Capsules taken (mg).

Calcium Pantothenate. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Internal standard solution. Dissolve 80 mg of *p-hydroxybenzoic acid* in 3 ml of *ethanol*, add 50 ml of *water*, 7.1 g of *dibasic sodium phosphate* and dilute with *water* to 1000 ml, adjusted to pH 6.7 with *orthophosphoric acid*.

Test solution. Weigh 20 capsules. Open the capsules without the loss of shell material and transfer the contents to a 100-ml beaker. Remove any content adhering to empty shell by washing, if necessary with several portions of ether. Discard the washing and dry the capsule shell with the aid of current of dry air until the odour of ether in no longer perceptible. By subtracting the weights of dry empty shell from weight of intact capsules, calculate the average net weight per capsules. Transfer a quantity of capsule contents containing 0.06 per cent w/v solution of Calcium Pantothenate in internal standard solution, to a centrifuge tube.

Reference solution. A 0.06 per cent w/v solution of *calcium pantothenate IPRS* in internal standard solution.

Chromatographic system

- a stainless steel column 15 cm x 3.9 mm, packed octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a 0.1 per cent v/v solution of *orthophosphoric acid* in *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

The relative retention times with reference to p-hydroxybenzoic acid for calcium pantothenate is about 0.5.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) using ratio of the peak area of calcium pantothenate to that of peak area of the internal standard in the capsules.

Niacin or Niacinamide, Pyridoxine Hydrochloride, Riboflavin, and Thiamine. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Solvent mixture. a mixture 5 volumes of *acetonitrile*, 1 volume of *glacial acetic acid and* 94 volumes of *water*.

Test solution. Weigh 20 capsules. Open the capsules without the loss of shell material and transfer the contents to a 100-ml beaker. Remove any content adhering to empty shell by washing, if necessary with several portions of ether. Discard the washing and dry the capsule shell with the aid of current of dry air until the odour of ether in no longer perceptible. By subtracting the weights of dry empty shell from weight of intact capsules, calculate the average net weight per capsules. Transfer a quantity of capsule contents containing 10 mg of Niacinamide and 2.5 mg each of, Pyridoxine hydrochloride, Riboflavin and Thiamine hydrochloride, to a 50-ml centrifuge tube. Add 25.0 ml of solvent mixture, and mix using a vortex mixer for 30 seconds to completely suspend the powder. Immerse the centrifuge tube in a hot water-bath maintained at 65°to70°, heat for 5 minutes, and mix using a vortex mixer for 30 seconds. Return the tube to the hot water bath, heat for another 5 minutes, and mix on a vortex mixer for 30 seconds. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. (Use the filtrate within 3 hours).

Reference solution. Transfer 80 mg of *niacinamide IPRS* and 20 mg each of *pyridoxine hydrochloride IPRS*, *riboflavin IPRS* and *thiamine hydrochloride IPRS* to a 200-ml volumetric flask and add 180 ml of the solvent mixture. Immerse the flask in a hot water bath maintained at 65° to 70° for 10 minutes with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10

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minutes to room temperature and dilute with solvent mixture to volume. *(NOTE — Use niacin IPRS in place of niacinamide IPRS for formulations containing niacin.)*

Chromatographic system
- a stainless steel column 30 cm x 3.9 mm, packed octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 73 volumes of *water*, 27 volumes of *methanol* and 1 volume of *glacial acetic acid* containing 0.14 per cent w/v solution of *sodium 1-hexanesulfonate*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

The relative retention times with reference to theamine for niacinamide, pyridoxine, riboflavin, are about 0.3, 0.5, 0.8, respectively.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3$,HCl), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS$,HCl) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the capsules.

1 mg of thiamine mononitrate $C_{12}H_{17}N_5O_4S$ is equivalent to 0.9706 mg of thiamine hydrochloride $C_{12}H_{17}ClN_4OS$,HCl.

Storage. Store protected from light and moisture.

Water-Soluble Vitamins Tablets

Water-Soluble Vitamins Tablets contain two or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, Pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate. Tablets contain not less than 90.0 per cent and not more than 150.0 per cent of the labeled amount of ascorbic acid ($C_6H_8O_6$), biotin ($C_{10}H_{16}N_2O_3S$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine ($C_8H_{11}NO_3$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine ion ($C_{12}H_{17}N_4OS^+$).

They do not contain any form of Beta Carotene or Vitamin A, D, E, or K. They do not contain any minerals for which nutritional value is claimed. They may contain other labeled added substances in quantities that are unobjectionable.

Identification

The retention times of the vitamin peaks of the test solutions correspond to those of the corresponding vitamin peaks of the reference solutions as obtained in the test solution.

Tests

Microbial contamination (2.2.9). The total aerobic viable count is not more than 3000 CFU per g, the total combined molds and yeasts count is not more than 300 CFU per g and 1 g is free from *Escherichia coli* and 10 g is free from *salmonella* species.

Other tests. Comply with the tests stated under Tablets.

Ascorbic Acid, Calcium Ascorbate, and Sodium Ascorbate. Weigh and powder 20 tablets. Weigh a quantity of the powder containing 100 mg of Ascorbic acid, to a 200-ml volumetric flask, add 75 ml of *metaphosphoric-acetic acids solution* and shake by mechanical means for 30 minutes. Dilute to volume with *water* and centrifuge until a clear supernatant is observed. Transfer a volume of the solution, equivalent to 2 mg of ascorbic acid into a 50-ml conical flask, add 5 ml of *metaphosphoric-acetic acids* and titrate with *standard 2,6-dichlorophenolindo-phenol solution* [Prepared by dissolving 50 mg of *2,6-dichlorophenolindo-phenol sodium* (stored in a desiccators over *soda lime*) in 50 ml of *water* containing 42 mg *sodium bicarbonate*, shake vigorously and when the dye is dissolved, add *water* to make 200 ml. Filter into an amber glass-stoppered bottle. Use within 3 days and standardize immediately before use.] until the rose-pink colour persists for at least 5 seconds. Repeat the operation with a mixture of 5.5 ml of *metaphosphoric acetic acid solution* and 15 ml of *water* omitting the preparation being examined. Correct for the volume of *standard 2,6-dichlorophenolindo-phenol solution* consumed by the blank.

Calculate the percentage of ascorbic acid, $C_6H_8O_6$ by using following expression.

$$\text{Result} = \frac{(V_S - V_B) \times F}{W} \times 100$$

where, V_S = Titrant volume consumed by the test solution;
V_B = Titrant volume consumed by the Blank;
F = Concentration of Titrant in terms of its equivalent of ascorbic acid;
W = nominal amount of ascorbic acid taken for analysis.

Biotin. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 1 mg of Biotin, to a 200-ml volumetric

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flask and add 3 ml *dimethyl sulphoxide,* and swirl to wet the contents. Place the flask in a water bath at 60°to 70° for 5 minutes and sonicate for 5 minutes, dilute with *water* to volume and filter.

Reference solution. A 0.0333 per cent w/v solution of *biotin IPRS* in *dimethyl sulphoxide.* Dilute the solution to obtain a concentration of 0.0005 per cent w/v of *biotin IPRS* with *water.*

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed octylsilane bonded to porous silica (3 µm),
- mobile phase: a mixture of 85 ml of *acetonitrile,* 1.0 g of *sodium perchlorate,* 1 ml of *orthophosphoric acid* and dilute to 1000 ml with *water,*
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 200 nm,
- injection volume: 100 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of biotin $C_{10}H_{16}N_2O_3S$ in the tablets.

Cyanocobalamin. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Test solution. Weigh and powder not less than 30 tablets. Disperse a quantity of the powder containing 0.1 mg of Cyanocobalamin, to a 250-ml volumetric flask and add 100.0 ml of *water* and carefully extract for 2 minutes. Filter 10 ml of the extract, and use the filtrate.

Reference solution. A 0.0001 per cent w/v solution of *cyanocobalamin IPRS* in *water.* [*NOTE* — Store in dark place and discard after 1 week].

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of *methanol* and 65 volumes of *water,*
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 550 nm,
- injection volume: 200 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of cyanocobalamin $C_{63}H_{88}CoN_{14}O_{14}P$ in the tablets.

Folic acid. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Solution A. A 25 per cent w/v solution of *tetrabutylammonium hydroxide* in *methanol.*

Solution B. Dissolve 5.0 g of pentetic acid in 50 ml of *1 M sodium hydroxide.*

Internal standard solution. Transfer 40 mg of *methylparaben* to a 1000-ml volumetric flask, and add 220 ml of *methanol* to dissolve. Dissolve 2.0 g of *monobasic potassium phosphate* in 300 ml of *water* in a separate beaker, quantitatively transfer this solution to the flask containing the *methylparaben solution,* and add an additional 300 ml of *water.* Add 19 ml of solution A, 7 ml of *3 M orthophosphoric acid,* and 30 ml of solution B. Adjusted to pH 9.8 with *ammonia,* and bubble nitrogen through the solution for 30 minutes, dilute with *water* to volume and mix.

Test solution. Weigh and powder 30 tablets. Disperse a quantity of the powder containing 0.4 mg of Folic acid, to a 50 ml amber-colored centrifuge tube and add 25.0 ml of the internal standard solution shake by mechanical means for 10 minutes, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Reference solution. A 0.0016 per cent w/v solution of *folic acid IPRS* in internal standard solution.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 2.0 g of *monobasic potassium phosphate* in 650 ml of *water* add 12.0 ml of solution A, 7.0 ml of *3 M orthophosphoric acid,* and 240 ml of *methanol.* Cool to room temperature, adjusted to pH 7.0 with *orthophosphoric acid* or *ammonia,* dilute to 1000 ml with *water,* (Recheck the pH before use by adding *water* or *methanol* to the prepared mobile phase to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation) *[NOTE: The methanol and water content may be varied 1 per cent to 3 per cent].*
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 15 µl.

The relative retention times with reference to methylparaben for folic acid is about 0.8.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

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Calculate the content of folic acid $C_{19}H_{19}N_7O_6$ in the tablets by using the peak area ratio of folic acid to that of peak area of the internal standard.

Calcium Pantothenate. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Internal standard solution. Dissolve 80 mg of *p-hydroxybenzoic acid* in 3 ml of *ethanol,* add 50 ml of *water,* 2.1 g of *dibasic sodium phosphate* and dilute with *water* to 1000 ml, adjusted to pH 6.7 with *orthophosphoric acid.*

Test solution. Weigh and powder not less than 30 tablets. Disperse a quantity of the powder containing 15 mg of Calcium Pantothenate, to a centrifuge tube, add 25.0 ml of the internal standard solution and shake vigorously for 10 minutes. Centrifuge filter, and use the clear filtrate.

Reference solution. A 0.06 per cent w/v solution of *calcium pantothenate IPRS* in internal standard solution.

Chromatographic system

- a stainless steel column 15 cm x 3.9 mm, packed octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 0.1 per cent v/v solution of *orthophosphoric acid* in *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

The relative retention times with reference to p-hydroxybenzoic acid for calcium pantothenate is about 0.5.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of calcium pantothenate $C_{18}H_{32}CaN_2O_{10}$ in the tablets by using the peak area ratio of calcium pantothenate to that of peak of the internal standard.

Niacin or Niacinamide, Pyridoxine Hydrochloride, Riboflavin, and Thiamine. Determine by liquid chromatography (2.4.14).

NOTE — Use low-actinic glassware throughout this procedure.

Solvent mixture. A mixture of 5 volumes of *acetonitrile,* 1 volume of *glacial acetic acid* and 94 volumes of *water.*

Test solution. Weigh and powder not less than 30 tablets. Disperse a quantity of the powder containing 10 mg of Niacinamide and 2.5 mg each of Pyridoxine hydrochloride, Riboflavin and Thiamine hydrochloride, to a 50-ml centrifuge tube. Add 25.0 ml of solvent mixture, and mix using a vortex mixer for 30 seconds to completely suspend the powder Immerse the centrifuge tube in a hot water bath maintained at 65° to70°, heat for 5 minutes, and mix using a vortex mixer for 30 seconds. Return the tube to the hot water bath, heat for another 5 minutes, and mix on a vortex mixer for 30 seconds. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. *(NOTE — Use the filterate with in 3 hour of filtration).*

Reference solution. (NOTE — Use Niacin IPRS in place of Niacinamide IPRS for formulations containing Niacin). Transfer 80 mg of *niacinamide IPRS* and 20 mg each of *pyridoxine hydrochloride IPRS, riboflavin IPRS* and *thiamine hydrochloride IPRS* in 180 ml of solvent mixture. Immerse the flask in a hot water bath maintained at 65° to 70° for 10 minutes with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water-bath for 10 minutes to room temperature and dilute to 200.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm, packed octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 73 volumes of *water* and 27 volume of *methanol* and 1 volume of *glacial acetic acid* containing 0.14 per cent w/v solution of *sodium 1-hexanesulphonate*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of vitamin B_3 as niacin $C_6H_5NO_2$ or niacinamide $C_6H_6N_2O$, vitamin B_6 as pyridoxine $C_8H_{11}NO_3$, vitamin B_2 as riboflavin $C_{17}H_{20}N_4O_6$, and vitamin B_1 as thiamine ion $C_{12}H_{17}N_4OS^+$ in the tablets.

Storage. Store protected from light and moisture.

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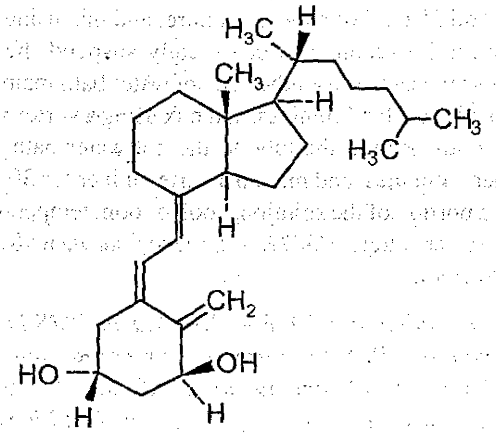
Alfacalcidol



$C_{27}H_{44}O_2$ Mol. Wt. 400.6

Alfacalcidol is (5*Z*,7*E*)-9,10-Secocholesta-5,7,10(19)-triene-1α,3β-diol.

Alfacalcidol contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{27}H_{44}O_2$.

Category. Antiosteoporotic.

Description. A white or off white crystals.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *alfacalcidol IPRS* or with the reference spectrum of alfacalcidol.

B. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with reference solution (a).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Carry out the test as rapidly as possible, avoiding exposure to actinic light and air.

Test solution. Dissolve 1 mg of the substance under examination in 10.0 ml of the mobile phase.

Reference solution (a). A 0.01 per cent w/v solution of *alfacalcidol IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Heat 2 ml of reference solution (a) in a water-bath at 80° under a reflux condenser for 2 hours and cool.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 0.1 volume of *ammonia*, 20 volumes of *water* and 80 volumes of *acetonitrile*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 100 µl.

Name	Relative retention time
Alfacalcidol impurity C[1]	0.4
Alfacalcidol impurity A[2]	0.9
Alfacalcidol	1.0
Alfacalcidol impurity B[3]	1.15
Prealfacalcidol	1.3

[1]triazoline adduct of pre-alfacalcidol,

[2]*trans*-alfacalcidol,

[3]1b-calcidol.

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to pre-alfacalcidol and alfacalcidol is not less than 4.0.

Inject reference solution (b) and the test solution. Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak due to alfacalcidol impurities A, B and C, each of, is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances, with the following modifications.

Inject reference solution (a) and (c). The test is not valid unless the resolution between the peaks due to pre-alfacalcidol and alfacalcidol is not less than 4.0 in the chromatogram obtain with reference solution (c) and the relative standard deviation for replicate injections is not more than 1.0 per cent in the chromatogram obtain with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{27}H_{44}O_2$.

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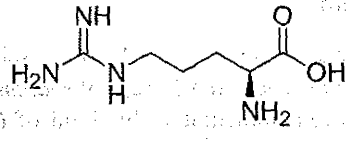
Storage. Store protected from light and moisture, under nitrogen at a temperature of 2° to 8°. The contents of an opened container are to be used immediately.

Arginine

L-Arginine



$C_6H_{14}N_4O_2$ Mol. Wt. 174.2

Arginine contains not less than 98.5 per cent and not more than 101.5 per cent of $C_6H_{14}N_4O_2$, calculated on the dried basis.

Category. α-amino acid.

Description. A white or almost white crystalline powder or colourless crystals.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *L-arginine IPRS* or with the reference spectrum of arginine.

Tests

Specific optical rotation (2.4.22). +26.3° to +27.7°, determined in an 8.0 per cent w/v solution in *6 M hydrochloric acid*.

Chlorides (2.3.12). 0.5 g complies with the limit test for chlorides (500 ppm).

Sulphates (2.3.17). 0.5 g complies with the limit test for sulphates (300 ppm).

Iron (2.3.14). 1.33 g complies with the limit test for iron (30 ppm).

Heavy metals (2.3.13). 1.33 g complies with the limit test for heavy metals Method A (15 ppm).

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 70 volumes of *isopropyl alcohol* and 30 volumes of *ammonia*.

Test solution. Weigh 1.0 g of the substance under examination and dissolve in sufficient *2 M hydrochloric acid* to produce 100.0 ml.

Reference solution (a). A 0.005 per cent w/v solution of *L-arginine IPRS* in *0.1 M hydrochloric acid*.

Reference solution (b). A solution containing 0.04 per cent w/v of each of *L-arginine IPRS* and *L-lysine hydrochloride IPRS* in *0.1 M hydrochloric acid*.

Apply to the plate 5 µl of each solution. After development, dry the plate at 100° until the ammonia disappears completely. Spray the plate with a 0.2 per cent w/v solution of *ninhydrin* in a mixture of 95 volumes of *butyl alcohol* and 5 volumes of *2 M acetic acid*. Heat the plate at 105° for 15 minutes. Cool and examine in daylight. Any secondary spot in the chromatogram obtained with the test solution is not larger or more intense than the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless in the chromatogram obtained with reference solution (b), two clearly separated spots are seen.

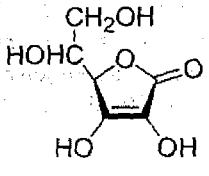
Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1 g by drying in an oven at 105°.

Assay. Dissolve 0.08 g in a mixture of 3 ml of *formic acid* and 50 ml of *glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.00871 g of $C_6H_{14}N_4O_2$.

Ascorbic Acid

Vitamin C; *L*-Ascorbic Acid



$C_6H_8O_6$ Mol. Wt. 176.1

Ascorbic Acid is (*R*)-5-[(*S*)-1,2-dihydroxyethyl)-3,4-dihydroxy-5(*H*)-furan-2-one.

Ascorbic Acid contains not less than 99.0 per cent and not more than 100.5 per cent of $C_6H_8O_6$.

Category. Vitamin (antiscorbutic) and pharmaceutical aid (antioxidant).

Description. A white to almost white, crystalline powder or colourless crystals, becoming discoloured on exposure to air and moisture.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests C and D may be omitted if tests A and B are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ascorbic acid IPRS* or with the reference spectrum of ascorbic acid.

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B. Add 2 ml of a 2 per cent w/v solution to a few ml of *2,6-dichlorophenolindophenol solution*; the solution is decolourised.

C. Dilute 1 ml of a 2 per cent w/v solution with 5 ml of *water* and add 1 drop of a freshly prepared 5 per cent w/v solution of *sodium nitroprusside* and 2 ml of *dilute sodium hydroxide solution*. Add 0.6 ml of *hydrochloric acid* dropwise and stir; the yellow colour turns blue.

D. To 2 ml of a 2 per cent w/v solution add 2 ml of *water*, 0.1 g of *sodium bicarbonate* and about 20 mg of *ferrous sulphate*, shake and allow to stand; a deep violet colour is produced. Add 5 ml of *1 M sulphuric acid*; the colour disappears.

Tests

Appearance of solution. A 5.0 per cent w/v solution in *water* is clear (2.4.1), and not more intensely coloured than reference solution BYS7 (2.4.1).

pH (2.4.24). 2.2 to 2.5, determined in a 5.0 per cent w/v solution.

Specific optical rotation (2.4.22). +20.5° to +21.5°, determined in a 10.0 per cent w/v solution.

Light absorption. Absorbance of a 0.001 per cent w/v solution in *0.01 M hydrochloric acid* at the maximum at about 244 nm (2.4.7) is about 0.56.

Oxalic acid. Dissolve 0.25 g in 5 ml of *water* and neutralise to *litmus paper* with *2 M sodium hydroxide*. Add 1 ml of *2 M acetic acid* and 0.5 ml of *0.5 M calcium chloride*. Any opalescence, after 60 minutes, is not more intense than that produced by treating 5 ml of a solution prepared by dissolving 75 mg of *oxalic acid* in 500 ml of *water* in a similar manner (0.3 per cent).

Heavy metals (2.3.13). 1.0 g dissolved in 25 ml of *water* complies with the limit test for heavy metals, Method A (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Dissolve 0.1 g in a mixture of 100 ml of freshly boiled and cooled *water* and 25 ml of *1 M sulphuric acid*. Immediately titrate with *0.05 M iodine*, using *starch solution* as indicator until a persistent blue-violet colour is obtained.

1 ml of *0.05 M iodine* is equivalent to 0.008806 g of $C_6H_8O_6$.

Storage. Store protected from light and moisture avoiding contact with metals. It undergoes rapid decomposition in solutions in contact with air.

Ascorbic Acid Injection

Vitamin C Injection; L-Ascorbic Acid Injection

Ascorbic Acid Injection is a sterile solution of Sodium Ascorbate or of Ascorbic Acid prepared with the aid of Sodium Hydroxide or Sodium Carbonate or Sodium Bicarbonate in Water for Injections.

Ascorbic Acid Injection contains not less than 95.0 per cent and not more than 115.0 per cent of the stated amount of ascorbic acid, $C_6H_8O_6$.

Usual strength. 500 mg in 2 ml.

Description. A clear, colourless liquid.

Identification

A. To a volume containing 5 mg of Ascorbic Acid add 0.5 ml of *0.1 M hydrochloric acid* and 3 drops of *sodium nitroprusside solution* followed immediately by 1 ml of *0.1 M sodium hydroxide*; a transient blue colour is produced.

B. To a volume containing 40 mg of Ascorbic Acid add 4 ml of *0.1 M hydrochloric acid* and 4 drops of *methylene blue solution* and warm to 40°; the deep blue colour becomes appreciably lighter or is completely discharged within 3 minutes.

C. The solution responds to the flame test for sodium salts (2.3.1), when exposed to non-luminous flame; the injection imparts an intense yellow colour.

Tests

pH (2.4.24). 5.5 to 7.0.

Oxalic acid. Dilute a volume containing 0.25 g of Ascorbic Acid in 5 ml of *water* and neutralise to *litmus paper* with *2 M sodium hydroxide*. Add 1 ml of *2 M acetic acid* and 0.5 ml of *0.5 M calcium chloride*. Any opalescence, after 60 minutes, is not more intense than that produced by treating 5 ml of a solution prepared by dissolving 75 mg of *oxalic acid* in 500 ml of *water* in a similar manner (0.3 per cent).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Bacterial endotoxins (2.2.3). Not more than 1.2 Endotoxin Units per mg of ascorbic acid.

Assay. Measure a volume containing 0.2 g of Ascorbic acid, add 5 ml of *2 M sulphuric acid* and titrate, with *0.05 M iodine*, using *starch solution* as indicator until a persistent blue-violet colour obtained.

1 ml of *0.05 M iodine* is equivalent to 0.008806 g of $C_6H_8O_6$.

Storage. Store protected from light, in a single dose container.

Ascorbic Acid Tablets

Vitamin C Tablets; L-Ascorbic Acid Tablets

Ascorbic Acid Tablets contain not less than 95.0 per cent and not more than 115.0 per cent of the stated amount of ascorbic

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acid, $C_6H_8O_6$. The tablets may contain permitted flavouring agents.

Usual strengths. 50 mg; 100 mg; 500 mg.

Identification

A. Shake a quantity of the powdered tablets with sufficient *water* to make approximately the equivalent of a 2 per cent w/v solution of Ascorbic Acid and filter. The filtrate (solution A) is acid to *litmus solution*.

B. To solution A add a few ml of *2,6-dichlorophenolindophenol solution*; the solution is decolourised.

C. To 1 ml of solution A, add about 0.1 ml of *2 M nitric acid* and 0.05 ml of *silver nitrate solution*; a grey precipitate is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle).

Medium. 900 ml of *water*.

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Solvent mixture. Dissolve 0.56 g of *disodium edetate dihydrate* and 2.04 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*.

Test solution. use the filtrate, dilute if necessary, with the solvent mixture.

Reference solution. A 0.025 per cent w/v solution of *ascorbic acid IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a buffer solution prepared by dissolving 2.04 g of *monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 3.0 with *orthophosphoric acid*.
- flow rate: 1 ml per minute,
- spectrophotometer set at 245 nm,
- injection volume: 5 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_6H_8O_6$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_6H_8O_6$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14), as described under Dissolution with the following modification.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 25 mg of Ascorbic Acid in 60 ml of the solvent mixture, shake mechanically for 15 minutes and dilute to 100.0 ml with the solvent mixture, filter.

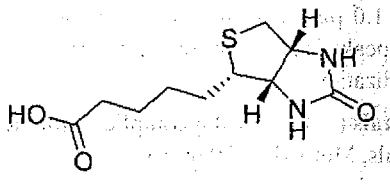
Inject the reference solution and the test solution.

Calculate the content of $C_6H_8O_6$ in the tablets.

Storage. Store protected from light and moisture avoiding contact with metals.

Labelling. For tablets containing 500 mg or more of Ascorbic Acid the label states, where applicable, that the tablets should be chewed before swallowing.

Biotin



$C_{10}H_{16}N_2O_3S$ Mol. Wt. 244.3

Biotin is 1*H*-Thieno[3,4-*d*]imidazole-4-pentanoic acid, hexahydro-2-oxo-, [3a*S*-(3aα,4β,6aα)]; (3a*S*,4*S*,6a*R*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-valeric acid.

Biotin contains not less than 97.5 per cent and not more than 102.0 per cent of $C_{10}H_{16}N_2O_3S$.

Category. Vitamin.

Description. A white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *biotin IPRS* or with the reference spectrum of biotin.

B. Specific optical rotation (see Test).

C. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). +89° to +93°, determined in 2.0 per cent w/v solution in *0.1 M sodium hydroxide*.

Related substances. Determine by liquid chromatography (2.4.14).

Solvent mixture. 20 volumes of *acetonitrile* and 80 volumes of *water*.

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Test solution. Dissolve 0.1 g of the substance under examination in the solvent mixture and dilute to 100.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *biotin IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octylsilane bonded to porous silica (3 µm),
- mobile phase: a mixture of 91.5 volumes of a buffer solution prepared by dissolving 1 g of *sodium perchlorate monohydrate* in 1000 ml of *water*, containing 1 ml of *orthophosphoric acid* and 8.5 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 200 nm,
- injection volume 50 µl.

Inject the test solution. The area of any secondary peak is not more than 1.0 per cent and the sum of the areas of all the secondary peaks is not more than 2.0 per cent, calculated by area normalization.

Heavy metals (2.3.13): 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances.

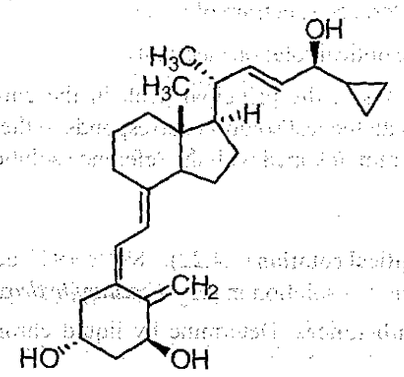
Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{10}H_{16}N_2O_3S$.

Storage. Store protected from moisture.

Calcipotriol Anhydrous



$C_{27}H_{40}O_3$ Mol. Wt. 412.6

Calcipotriol is (5*Z*,7*E*,22*E*)-(1*S*,3*R*,24*S*)-26,27-Cyclo-9,10-secocholesta-5,7,10(19),22-tetraene-1,3,24-triol.

Calcipotriol contains not less than 95.5 per cent and not more than 102.0 per cent, calculated on the dried basis.

Category. Antipsoriasis.

Description. A white or almost white, crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *anhydrous calcipotriol IPRS* or with the reference spectrum of anhydrous calcipotriol.

Tests

Related substances. A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel F254*.

Solution A. To 1 ml of *triethylamine* add 9 ml of *chloroform*.

Mobile phase: A mixture of 20 volumes of *isobutyl alcohol* and 80 volumes of *dichloromethane*.

Test solution. Dissolve 1.0 mg of the substance under examination in 100 µl of solution A.

Reference solution (a). To 10 µl of the test solution add 990 µl of solution A.

Reference solution (b). To 250 µl of reference solution (a) add 750 µl of solution A.

Reference solution (c). To 100 µl of reference solution (a) add 900 µl of solution A.

Reference solution (d). Place 2.0 mg of the substance under examination in a vial and dissolve in 200 µl of solution A. Close the vial and keep it in a water-bath at 60° for 2 hours.

Name	Relative retention factor
Calcipotriol impurity G[1]	0.4
Calcipotriol impurity H[2]	0.4
Pre-calcipotriol	0.9
Calcipotriol (Retention factor: about 0.4)	1.0
Calcipotriol impurity A[3]	1.2

[1]24,24'-oxybis[(5*Z*,7*E*,22*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19), 22- tetraene-1α,3β-diol,

[2](5*Z*,7*E*,22*E*,24*R*)-24-cyclopropyl-24-[[(5*Z*,7*E*,22*E*,24*S*)-24-cyclopropyl-1α,3β- dihydroxy-9,10-secochola-5,7,10(19),22-tetraen-24-yl]oxy]-9,10-secochola-5,7,10 (19),22-tetraene-1α,3β -diol,

[3](5*Z*,7*E*,22*E*)-24-cyclopropyl-1α3β -dihydroxy-9,10-secochola-5,7,10 (19), 22-tetraen-24-one.

Apply to the plate 10 µl of reference solutions (b), (c), (d) and the test solution. Allow the mobile phase to rise 15 cm. Dry the plate in air, then at 140° for 10 minutes and spray the hot plate

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with 20 per cent *ethanolic sulphuric acid*. Dry the plate at 140° for not more than 1 minute and examine under ultraviolet light at 365 nm.

Any secondary spot due to calcipotriol impurity A in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent), any secondary spot due to calcipotriol impurities G or H in the chromatogram obtained with the test solution is not more intense than the chromatogram obtained with reference solution (b) (0.25 per cent for the sum), and any other secondary spot is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows a secondary spot due to pre-calcipotriol.

B. Determine by liquid chromatography (2.4.14).

NOTE—Use freshly prepared solutions and protected from light and air.

Solution A. Dissolve 1.32 g of *ammonium phosphate* in *water* and dilute to 10.0 ml with *water*.

Solvent mixture. 3 volumes of solution A, 297 volumes of *water* and 700 volumes of *methanol*.

Test solution (a). Dissolve 2.0 mg of the substance under examination in the solvent mixture and dilute to 5.0 ml with the solvent mixture.

Test solution (b). Dissolve 2.0 mg of the substance under examination in the solvent mixture and dilute to 20.0 ml with the solvent mixture.

Reference solution (a). Dilute 1.0 ml of test solution (a) to 100.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the solvent mixture.

Reference solution (c). Dissolve 2.0 mg of *anhydrous calcipotriol IPRS* in the solvent mixture and dilute to 20.0 ml with the solvent mixture.

Chromatographic system
- a stainless steel column 10 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- mobile phase: a mixture of 30 volumes of *water* and 70 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 264 nm,
- injection volume: 20 µl.

Name	Relative retention time
Calcipotriol impurity B[1]	0.86
Calcipotriol impurity C[2]	0.92
Calcipotriol (Retention time: about 13.5 minutes)	1.0
Calcipotriol impurity D[3]	1.3

[1](5*Z*,7*Z*,22*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α3β, 24-triol,

[2](5*E*,7*E*,22*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α3β, 24-triol,

[3](5*Z*,7*E*,22*E*,24*R*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α3β,24-triol.

Inject reference solutions (a), (b) and test solution (a). Run the chromatogram twice the retention time of the principal peak. In the chromatogram obtained with test solution (a) the area of any peak due to calcipotriol impurity B is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent), the area of any peak due to calcipotriol impurities C and D is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.4.19). Not more than 1.0 per cent determined on 5 mg, by thermogravimetry (2.4.31), heated to 105° at a rate of 10° per minute and maintain at 105° for 60 minutes.

Assay. Determine by liquid chromatography (2.4.14), as described under related substances.

Inject reference solution (c) and test solution (b).

Calculate the content of $C_{27}H_{40}O_3$.

Storage. Store protected from light and moisture at a temperature -20° or below.

Calcipotriol Ointment

Calcipotriol Ointment contains anhydrous Calcipotriol in suitable ointment base.

Calcipotriol Ointment contains not less than 90.0 per cent and not more than 120.0 per cent w/w of the stated amount of calcipotriol, $C_{27}H_{40}O_3$.

Usual strength. 0.005 per cent w/w.

Description. A white viscous ointment.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

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Tests

Related substances. Determine by liquid chromatography (2.4.14) as described under Assay with the following modifications.

Inject the test solution. The area of any secondary peak is not more than 2.5 per cent and the sum of areas of all the secondary peaks is not more than 5.0 per cent, calculated by area normalization.

Microbial contamination (2.2.9). The total aerobic viable count is not more than 100 CFU per g. 1 g is free from *staphylococcus aureus* and *pseudomonas aeruginosa.*

Other tests. Comply with the tests stated under Ointments.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. 30 volumes of buffer solution prepared by dissolving 1.32 g of *ammonium dihydrogen phosphate* in 1000 ml of *water* and 70 volumes of *methanol.*

Test solution. Disperse a quantity of ointment containing 75 µg of Calcipotriol with 5 ml of *tetrahydrofuran.* Add 35 ml of the solvent mixture and sonicate for 45 minutes with intermittent shaking and dilute to 50.0 ml with the solvent mixture, centrifuge and filter.

Reference solution. A 0.0025 per cent w/v solution of *calcipotriol IPRS* in the solvent mixture. To 3.0 ml of the solution, add 5.0 ml of *tetrahydrofuran* and dilute to 50.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 50°,
- mobile phase: A. a mixture of 30 volumes of *water* and 70 volumes of *methanol*,
  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 264 nm,
- injection volume: 100 µl.

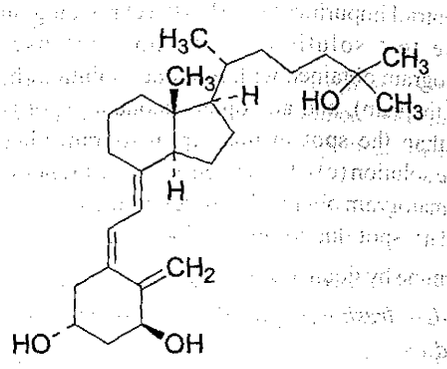
Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
18	100	0
22	80	20
35	80	20
37	100	0
40	100	0

Inject the reference solution. The test is not valid unless the column efficiency is not more than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{40}O_3$ in the ointment.

Calcitriol



$C_{27}H_{44}O_3$ Mol. Wt. 416.6

Calcitriol is (5*Z*,7*E*)-9,10-secocholesta-5,7,10(19)-triene-1α,3β,25-triol.

Calcitriol contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{27}H_{44}O_3$.

Category. Vitamin D analogue.

Description. A white or almost white crystals.

A reversible isomerisation to pre-calcitriol takes place in solution, depending on temperature and time. The activity is due to both compounds.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *calcitriol IPRS* or with the reference specturm of calcitriol.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Carry out the test as described under Assay.

In the chromatogram obtained with the test solution, the area of any secondary peak, apart from pre-calcitriol, eluted within twice the retention time of calcitriol, is not more than 0.5 times the area of the principal peak obtained with reference solution (b) (0.5 per cent) and the sum of areas of all the secondary peaks is not more than the area of the principal peaks obtained

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with reference solution (b) (1.0 per cent). Ignore any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Assay. Determine by liquid chromatography (2.4.14).

NOTE—Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.

Test solution. Dissolve 1.0 mg of the substance under examination in 10.0 ml of the mobile phase.

Reference solution (a). A 0.01 per cent w/v solution of *calcitriol IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Keep 2.0 ml of reference solution (a) at 80° for 30 minutes.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 45 volumes of a solution containing 0.1 per cent w/v of *tris(hydroxymethyl) aminomethane*, adjusted to pH 7.3 with *orthophosphoric acid* and 55 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 50 µl.

Inject reference solution (c). Run the chromatogram for twice the retention time of calcitriol. The relative retention times with reference to calcitriol for precalcitriol is about 0.9 and the resolution between the peaks due to calcitriol and pre-calcitriol is not less than 3.5. Inject reference solution (a) in replicate. The relative standard deviation is not more than 2.0 per cent and the column efficiency for calcitriol peak is not less than 10000 theoretical plates.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{27}H_{44}O_3$.

Storage. Store under nitrogen, protected from light and moisture, in a refrigerator (2° to 8°). The contents of an opened container are to be used immediately.

Calcitriol Capsules

Calcitriol Capsules contain a solution of Calcitriol in a suitable fixed oil.

Calcitriol Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of calcitriol, $C_{27}H_{44}O_3$.

Usual strength. 0.25 µg.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

NOTE—Carry out the test in subdued light.

Test solution. For capsules containing 0.25 µg or less calcitriol use the mixed contents of 10 capsules as test solution. For capsules containing more than 0.25 µg of calcitriol, dilute a quantity of mixed contents of 10 capsules with the mobile phase to obtain a concentration of 1.5 µg of calcitriol per ml.

Reference solution. A 0.00015 per cent w/v solution of *calcitriol IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with silica (5 µm) (Such as Lichrosorb Si60),
- mobile phase: a mixture of 1 volume of *propanol*, 2 volumes of *methanol*, 40 volumes of *hexane* and 60 volumes of *ethyl acetate*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 265 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{27}H_{44}O_3$ in the capsules.

Calcium Carbonate

Precipitated Chalk

$CaCO_3$ Mol. Wt. 100.1

Calcium Carbonate contains not less than 98.0 per cent and not more than 100.5 per cent of $CaCO_3$, calculated on the dried basis.

Category. Antacid; Pharmaceutical aid (excipient).

Description. A fine, white, microcrystalline powder.

Identification

A. Dissolve 5.0 g in 80 ml of *2 M acetic acid*. When effervescence ceases, boil the solution for 2 minutes, allow to

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cool, dilute to 100 ml with *2 M acetic acid* and filter, if necessary, through a sintered-glass filter reserving any residue for the test for Substances insoluble in *acetic acid*; the filtrate (solution A) gives reactions A and B of calcium salts (2.3.1).

B. It gives reaction (A) of carbonates (2.3.1).

Tests

Substances insoluble in acetic acid. Wash any residue obtained in Identification test A with four quantities, each of 5 ml of hot *water* and dry at 100° for 1 hour; the residue weighs not more than 10 mg (0.2 per cent).

Arsenic (2.3.10). Dissolve 2.5 g in 15 ml of *brominated hydrochloric acid* and 45 ml of *water* and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (4 ppm).

Heavy metals (2.3.13). To 1.0 g add 5 ml of *water*, and 8 ml of *dilute hydrochloric acid*, the latter being added slowly, shake and evaporate to dryness on a water-bath. Dissolve the residue in 20 ml of *water*, filter, add to the filtrate 3 ml of *dilute acetic acid* and *water* to make 25 ml. The solution complies with the limit test for heavy metals, Method A (20 ppm).

Barium. Dissolve 0.6 g in 10 ml of *2 M acetic acid* by boiling, cool and add 10 ml of *calcium sulphate solution*; the solution remains clear for not less than 15 minutes.

Iron (2.3.14). Dissolve 0.2 g in 5 ml *water* and 0.5 ml of *iron-free hydrochloric acid*, boil and dilute to 40 ml with *water*, the solution complies with the limit test for iron (200 ppm).

Magnesium and alkali metals. Dissolve 1.0 g in 10 ml of *dilute hydrochloric acid*, neutralise the solution by adding *dilute ammonia solution*, heat the solution to boiling and add 50 ml of hot *ammonium oxalate solution*. Cool, dilute to 100 ml with *water* and filter. To 50 ml of the filtrate add 1.5 ml of *dilute sulphuric acid*, evaporate to dryness on a water-bath, heat the residue to redness, allow to cool and weigh. The residue weighs not more than 5 mg (1.0 per cent).

Chlorides (2.3.12). 1.0 g dissolved in *water* by the addition of 3 ml of *nitric acid* complies with the limit test for chlorides (250 ppm).

Sulphates (2.3.17). Suspend 50.0 mg in 5 ml of *water* and add dropwise sufficient *dilute hydrochloric acid* to effect solution. Add 2 ml of *dilute hydrochloric acid*; the resulting solution complies with the limit test for sulphates (0.3 per cent).

Loss on drying (2.4.19). Not more than 2.0 per cent, determined on 1.0 g by drying in an oven at 200°.

Assay. Weigh accurately about 0.1 g and dissolve in 3 ml of *dilute hydrochloric acid* and 10 ml of *water*. Boil for 10 minutes, cool and dilute to 50 ml with *water*. Titrate with *0.05 M disodium edetate* to within a few ml of the expected end-point, add 8 ml of *sodium hydroxide solution* and 0.1 g of *calcon mixture* and continue the titration until the colour of the solution changes from pink to a full blue colour.

1 ml of *0.05 M disodium edetate* is equivalent to 0.005004 g of $CaCO_3$.

Calcium Carbonate Tablets

Calcium Carbonate Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of calcium, Ca.

Usual strengths. 250 mg; 400 mg; 500 mg; 625 mg; 1000 mg; 1250 mg.

Identification

A. It gives the reaction (A) of calcium salts (2.3.1).

B. It gives the reaction (A) of carbonates (2.3.1).

Tests

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 0.25 g of Calcium; dissolve in 50 ml of *water* and 5 ml of *hydrochloric acid*. Heat the dispersion gently to boiling and continue to boil for about 2 minutes. Allow to cool, add sufficient *water* to produce 250 ml and filter, if necessary. To 50 ml of the solution, add 50 ml of *0.05 M disodium edetate*. Neutralise the solution using *2 M sodium hydroxide* and add 10 ml of *ammonia buffer pH 10.9* and 50 ml of *water*. Titrate the excess of *disodium edetate* with *0.05 M zinc chloride* using *mordant black II solution* as indicator. Carry out a blank titration.

1 ml of *0.05 M disodium edetate* is equivalent to 0.002004 g of Ca.

Storage. Store protected from moisture.

Labelling. The label states (1) the quantity of the active ingredient in terms of the equivalent amount of calcium; (2) that the tablets should be chewed before swallowing.

Calcium Chloride

Calcium Chloride Dihydrate

$CaCl_2,2H_2O$ Mol. Wt. 147.0

Calcium Chloride contains not less than 97.0 per cent and not more than 103.0 per cent of $CaCl_2,2H_2O$.

Category. Calcium replenisher.

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Description. A white, crystalline powder or fragments or granules; hygroscopic.

Identification

A. It gives reactions (A) and (B) of calcium salts (2.3.1).

B. A 10 per cent w/v solution in *carbon dioxide-free water* prepared from *distilled water* (solution A) gives reaction A of chlorides (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1) and not more intensely coloured than reference solution YS6 (2.4.1).

Acidity or alkalinity. To 10 ml of a freshly prepared 10 per cent w/v solution add 2 drops of *phenolphthalein solution*. Titrate with *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide*; not more than 0.2 ml is required.

Arsenic (2.3.10). Dissolve 3.33 g in 15 ml of *brominated hydrochloric acid* and 45 ml of *water* and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (3 ppm).

Aluminium and phosphate. To 10 ml of a 5.0 per cent w/v solution, add 2 drops of *dilute hydrochloric acid* and 1 drop of *phenolphthalein solution*. Add *ammonium chloride-ammonium hydroxide solution* dropwise until the solution is faintly pink, add a few drops in excess and heat the liquid to boiling; no turbidity or precipitate is produced.

Barium. To 10 ml of solution A add 1 ml of *calcium sulphate solution*. After not less than 15 minutes the solution is not more opalescent than a mixture of 10 ml of solution A and 1 ml of *distilled water*.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method A (10 ppm).

Iron (2.3.14). Dissolve 2.0 g in 0.5 ml of *hydrochloric acid* and 25 ml of *water*; the resulting solution complies with the limit test for iron (20 ppm).

Magnesium and alkali salts. Dissolve 1.0 g in 50 ml of *water*, add 0.5 g of *ammonium chloride* heat the solution to boiling and add 50 ml of hot *ammonium oxalate solution*. Cool, dilute to 100 ml with *water* and filter. To 50 ml of the filtrate add 1.5 ml of *dilute sulphuric acid*, evaporate to dryness on a water-bath, heat the residue to redness, allow to cool and weigh. The residue weighs not more than 5 mg (1.0 per cent).

Sulphates (2.3.17). 0.5 g dissolved in 15 ml of *distilled water* complies with the limit test for sulphates (300 ppm).

Assay. Weigh accurately about 0.15 g and dissolve in 50 ml of *water*. Titrate with *0.05 M disodium edetate* to within a few ml of the expected end-point, add 8 ml of *sodium hydroxide solution* and 0.1 g of *calcon mixture* and continue the titration until the colour of the solution changes from pink to a full blue colour.

1 ml of *0.05 M disodium edetate* is equivalent to 0.007351 g of $CaCl_2,2H_2O$.

Storage. Store protected from moisture.

Calcium Chloride Injection

Calcium Chloride Dihydrate Injection

Calcium Chloride Injection is a sterile solution of Calcium Chloride Dihydrate in Water for Injections.

Calcium Chloride Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of calcium chloride dihydrate, $CaCl_2, 2H_2O$.

Usual strengths. Each gram of calcium chloride dihydrate represents approximately 6.8 mmol (13.6 mEq) calcium and 13.6 mmol (13.6 mEq) chloride. Each ml of the 10 ml ampoule contains 0.68 mmol (1.36 mEq) calcium.

Identification

A. It gives reaction (A) of calcium salts (2.3.1).

B. Dilute 1 volume of the injection to 50 ml with *water*. Gives the reactions of chlorides (2.3.1).

Tests

pH (2.4.24). 5.0 to 8.0.

Appearance of solution. The injection is not more intensely coloured than reference solution BYS6 (2.4.1).

Bacterial endotoxins (2.2.3). Not more than 0.2 Endotoxin Unit per mg of calcium chloride.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Measure a volume of the injection containing about 0.3 g of Calcium Chloride Dihydrate, add 300 ml of *water*, 6.0 ml of *sodium hydroxide solution* (40 per cent w/v) and 0.1 g of *calcon mixture*. Titrate with *0.1 M disodium edetate* until the colour changes from violet to blue.

1 ml of *0.1 M disodium edetate* is equivalent to 0.0147 g of $CaCl_2,2H_2O$.

Labelling. The label states (1) the percentage w/v of Calcium Chloride Dihydrate; (2) the concentration of calcium ion as millimoles in a suitable volume; (3) the concentration of chloride ion as millimoles in a suitable volume; (4) that the injection should be used in accordance with the manufacturer's instructions; (5) that solutions containing visible solid particles must not be used.

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cool, dilute to 100 ml with *2 M acetic acid* and filter, if necessary, through a sintered-glass filter reserving any residue for the test for Substances insoluble in *acetic acid*; the filtrate (solution A) gives reactions A and B of calcium salts (2.3.1).

B. It gives reaction (A) of carbonates (2.3.1).

Tests

Substances insoluble in acetic acid. Wash any residue obtained in Identification test A with four quantities, each of 5 ml of hot *water* and dry at 100° for 1 hour; the residue weighs not more than 10 mg (0.2 per cent).

Arsenic (2.3.10). Dissolve 2.5 g in 15 ml of *brominated hydrochloric acid* and 45 ml of *water* and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (4 ppm).

Heavy metals (2.3.13). To 1.0 g add 5 ml of *water*, and 8 ml of *dilute hydrochloric acid*, the latter being added slowly, shake and evaporate to dryness on a water-bath. Dissolve the residue in 20 ml of *water*, filter, add to the filtrate 3 ml of *dilute acetic acid* and *water* to make 25 ml. The solution complies with the limit test for heavy metals, Method A (20 ppm).

Barium. Dissolve 0.6 g in 10 ml of *2 M acetic acid* by boiling, cool and add 10 ml of *calcium sulphate solution*; the solution remains clear for not less than 15 minutes.

Iron (2.3.14). Dissolve 0.2 g in 5 ml *water* and 0.5 ml of *iron-free hydrochloric acid*, boil and dilute to 40 ml with *water*, the solution complies with the limit test for iron (200 ppm).

Magnesium and alkali metals. Dissolve 1.0 g in 10 ml of *dilute hydrochloric acid*, neutralise the solution by adding *dilute ammonia solution*, heat the solution to boiling and add 50 ml of hot *ammonium oxalate solution*. Cool, dilute to 100 ml with *water* and filter. To 50 ml of the filtrate add 1.5 ml of *dilute sulphuric acid*, evaporate to dryness on a water-bath, heat the residue to redness, allow to cool and weigh. The residue weighs not more than 5 mg (1.0 per cent).

Chlorides (2.3.12). 1.0 g dissolved in *water* by the addition of 3 ml of *nitric acid* complies with the limit test for chlorides (250 ppm).

Sulphates (2.3.17). Suspend 50.0 mg in 5 ml of *water* and add dropwise sufficient *dilute hydrochloric acid* to effect solution. Add 2 ml of *dilute hydrochloric acid*; the resulting solution complies with the limit test for sulphates (0.3 per cent).

Loss on drying (2.4.19). Not more than 2.0 per cent, determined on 1.0 g by drying in an oven at 200°.

Assay. Weigh accurately about 0.1 g and dissolve in 3 ml of *dilute hydrochloric acid* and 10 ml of *water*. Boil for 10 minutes, cool and dilute to 50 ml with *water*. Titrate with *0.05 M disodium edetate* to within a few ml of the expected end-point, add 8 ml of *sodium hydroxide solution* and 0.1 g of *calcon mixture* and continue the titration until the colour of the solution changes from pink to a full blue colour.

1 ml of *0.05 M disodium edetate* is equivalent to 0.005004 g of $CaCO_3$.

Calcium Carbonate Tablets

Calcium Carbonate Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of calcium, Ca.

Usual strengths. 250 mg; 400 mg; 500 mg; 625 mg; 1000 mg; 1250 mg.

Identification

A. It gives the reaction (A) of calcium salts (2.3.1).

B. It gives the reaction (A) of carbonates (2.3.1).

Tests

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 0.25 g of Calcium; dissolve in 50 ml of *water* and 5 ml of *hydrochloric acid*. Heat the dispersion gently to boiling and continue to boil for about 2 minutes. Allow to cool, add sufficient *water* to produce 250 ml and filter, if necessary. To 50 ml of the solution, add 50 ml of *0.05 M disodium edetate*. Neutralise the solution using *2 M sodium hydroxide* and add 10 ml of *ammonia buffer pH 10.9* and 50 ml of *water*. Titrate the excess of *disodium edetate* with *0.05 M zinc chloride* using *mordant black II solution* as indicator. Carry out a blank titration.

1 ml of *0.05 M disodium edetate* is equivalent to 0.002004 g of Ca.

Storage. Store protected from moisture.

Labelling. The label states (1) the quantity of the active ingredient in terms of the equivalent amount of calcium, (2) that the tablets should be chewed before swallowing.

Calcium Chloride

Calcium Chloride Dihydrate

$CaCl_2,2H_2O$ Mol. Wt. 147.0

Calcium Chloride contains not less than 97.0 per cent and not more than 103.0 per cent of $CaCl_2,2H_2O$.

Category. Calcium replenisher.

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Description. A white, crystalline powder or fragments or granules; hygroscopic.

Identification

A. It gives reactions (A) and (B) of calcium salts (2.3.1).

B. A 10 per cent w/v solution in *carbon dioxide-free water* prepared from *distilled water* (solution A) gives reaction A of chlorides (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1) and not more intensely coloured than reference solution YS6 (2.4.1).

Acidity or alkalinity. To 10 ml of a freshly prepared 10 per cent w/v solution add 2 drops of *phenolphthalein solution*. Titrate with *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide*; not more than 0.2 ml is required.

Arsenic (2.3.10). Dissolve 3.33 g in 15 ml of *brominated hydrochloric acid* and 45 ml of *water* and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (3 ppm).

Aluminium and phosphate. To 10 ml of a 5.0 per cent w/v solution, add 2 drops of *dilute hydrochloric acid* and 1 drop of *phenolphthalein solution*. Add *ammonium chloride-ammonium hydroxide solution* dropwise until the solution is faintly pink, add a few drops in excess and heat the liquid to boiling; no turbidity or precipitate is produced.

Barium. To 10 ml of solution A add 1 ml of *calcium sulphate solution*. After not less than 15 minutes the solution is not more opalescent than a mixture of 10 ml of solution A and 1 ml of *distilled water*.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method A (10 ppm).

Iron (2.3.14). Dissolve 2.0 g in 0.5 ml of *hydrochloric acid* and 25 ml of *water*; the resulting solution complies with the limit test for iron (20 ppm).

Magnesium and alkali salts. Dissolve 1.0 g in 50 ml of *water*, add 0.5 g of *ammonium chloride* heat the solution to boiling and add 50 ml of hot *ammonium oxalate solution*. Cool, dilute to 100 ml with *water* and filter. To 50 ml of the filtrate add 1.5 ml of *dilute sulphuric acid*, evaporate to dryness on a water-bath, heat the residue to redness, allow to cool and weigh. The residue weighs not more than 5 mg (1.0 per cent).

Sulphates (2.3.17). 0.5 g dissolved in 15 ml of *distilled water* complies with the limit test for sulphates (300 ppm).

Assay. Weigh accurately about 0.15 g and dissolve in 50 ml of *water*. Titrate with *0.05 M disodium edetate* to within a few ml of the expected end-point, add 8 ml of *sodium hydroxide solution* and 0.1 g of *calcon mixture* and continue the titration until the colour of the solution changes from pink to a full blue colour.

1 ml of *0.05 M disodium edetate* is equivalent to 0.007351 g of $CaCl_2,2H_2O$.

Storage. Store protected from moisture.

Calcium Chloride Injection

Calcium Chloride Dihydrate Injection

Calcium Chloride Injection is a sterile solution of Calcium Chloride Dihydrate in Water for Injections.

Calcium Chloride Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of calcium chloride dihydrate, $CaCl_2, 2H_2O$.

Usual strengths. Each gram of calcium chloride dihydrate represents approximately 6.8 mmol (13.6 mEq) calcium and 13.6 mmol (13.6 mEq) chloride. Each ml of the 10 ml ampoule contains 0.68 mmol (1.36 mEq) calcium.

Identification

A. It gives reaction (A) of calcium salts (2.3.1).

B. Dilute 1 volume of the injection to 50 ml with *water*. Gives the reactions of chlorides (2.3.1).

Tests

pH (2.4.24). 5.0 to 8.0.

Appearance of solution. The injection is not more intensely coloured than reference solution BYS6 (2.4.1).

Bacterial endotoxins (2.2.3). Not more than 0.2 Endotoxin Unit per mg of calcium chloride.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Measure a volume of the injection containing about 0.3 g of Calcium Chloride Dihydrate, add 300 ml of *water*, 6.0 ml of *sodium hydroxide solution* (40 per cent w/v) and 0.1 g of *calcon mixture*. Titrate with *0.1 M disodium edetate* until the colour changes from violet to blue.

1 ml of *0.1 M disodium edetate* is equivalent to 0.0147 g of $CaCl_2,2H_2O$.

Labelling. The label states (1) the percentage w/v of Calcium Chloride Dihydrate; (2) the concentration of calcium ion as millimoles in a suitable volume; (3) the concentration of chloride ion as millimoles in a suitable volume; (4) that the injection should be used in accordance with the manufacturer's instructions; (5) that solutions containing visible solid particles must not be used.

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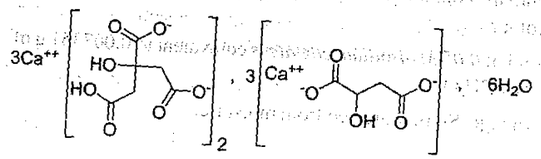
Calcium Citrate Malate



$Ca_3(C_6H_5O_7)_2,3CaC_4H_4O_5,6H_2O$ Mol. Wt.1123.0

Calcium Citrate Malate is 1,2,3-Propanetricarboxylic acid, 2-hydroxy- calcium salt mixture with 2-hydroxybutanedioic acid calcium salt (2:3:6), hexahydrate.

Calcium Citrate Malate is a complex of calcium, citrate and malate.

Calcium Citrate Malate contains not less than 23.0 per cent and not more than 24.2 per cent of calcium, calculated on the dried basis. The sum of citrate and malate is not less than 73.0 per cent, calculated on the dried basis.

Category. Calcium supplement.

Description. A white or almost white powder.

Identification

A. Ignite completely 0.5 g at very low temperature, cool and dissolve the residue in *dilute glacial acetic acid*, filter. Add 10 ml of *ammonium oxalate* to the filtrate, a voluminous white precipitate is formed that is soluble in *hydrochloric acid*.

B. In the test for content of citrate, malate, and fumarate, the peaks due to citric acid and malic acid in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (d).

Tests

pH (2.4.24). 4.0 to 8.0, determined in 3.0 per cent w/v solution (*NOTE — The solution is slurry*).

Arsenic (2.3.10). Dissolve 3.3 g in 15 ml of *brominated hydrochloric acid* and 45 ml of *water* and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (3 ppm).

Lead (2.3.15). Not more than 0.0001 per cent.

Fluoride. Not more than 30 ppm.

NOTE — Prepare and store all solutions in plastic containers.

Buffer solution. A 29.4 per cent w/v solution of *sodium citrate dihydrate*.

Test solution. To 1.0 g of the substance under examination, add 10 ml of *water*, place on a hot plate stir with a magnetic stirrer. While stirring, add 10 ml of *1M hydrochloric acid* to dissolve. When dissolved, boil rapidly for 1 minute transfer to a 250-ml conical flask. Cool rapidly at room temperature in an ice bath, add 15 ml of buffer solution and 10 ml of *0.2 M EDTA* adjusted to pH 5.5 with *1 M hydrochloric acid* or *1 M sodium hydroxide*. Transfer the solution to a 100-ml volumetric flask and dilute to volume with *water*. Pour into 250-ml beaker for titration.

Reference solution. A 0.11052 per cent w/v solution of *sodium fluoride* in *water*. Dilute 2.0 ml of the solution to 1000.0 ml with *water* (1µg per ml of fluoride ion).

Reference solution response line. Transfer 5.0 ml, 25.0 ml and 50.0 ml of the reference solution to three beakers containing plastic coated stirring bar, add 10 ml of *1 M hydrochloric acid*, 15 ml of buffer solution and *0.2 M EDTA* to each beaker, adjusted to pH 5.5 with *1M hydrochloric acid* or *1M sodium hydroxide*. Transfer the solution to individual 100-ml volumetric flask, dilute to volume with *water*. Pour the solutions back to 250-ml individual beakers for titration. Insert the electrode into solution and allow to stand for 15 minutes with constant stirring, determining the end point potentiometricaly (2.4.25), using a suitable electrode system such as silver chloride reference electrode and fluoride specific ion indicating electrode. Plot the logarithms of the fluoride ion concentration (0.05, 0.25 and 0.5 ppm) versus potential in milivolts. Rinse and dry the electrodes, insert them into the test solution, stir for 5 minutes and read the potential in milivolts.

Determine the concentration (in ppm) of fluoride ions in the test solution by measuring potential and the reference solution response line.

Content of Citrate, Malate, and Fumarate. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.5 g of the substance under examination in 100.0 ml of *0.2M hydrochloric acid*.

Reference solution (a). A 0.18 per cent w/v solution of *citric acid IPRS* in *0.2M hydrochloric acid*.

Reference solution (b). A 0.18 per cent w/v solution of *malic acid IPRS* in *0.2M hydrochloric acid*.

Reference solution (c). A 0.002 per cent w/v solution of *fumaric acid IPRS* in *0.2M hydrochloric acid*.

Reference solution (d). A 0.5 per cent w/v solution of *calcium citrate malate IPRS* in *0.2M hydrochloric acid*.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: a *0.05 M monobasic potassium phosphate* in *water*, adjusted to pH 2.5 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,

- spectrophotometer set at 226 nm,
- injection volume: 10µl.

Name	Relative retention time
Malic acid	0.6
Citric acid	1.0
Fumaric acid	1.4

Inject reference solution (a), (b), (c) and (d). The test is not valid unless the resolution between the peaks due to malic acid and citric acid is not less than 8.0 in the chromatogram obtained with reference solution (d), the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatograms obtained with reference solution (a), (b) and (c).

Inject reference solution (a), (b) (c) and the test solution.

Calculate the content of citric acid, malic acid and fumaric acid. Multiply by 0.98 to convert molecular weight of the acid form to the salt form.

The sum of citrate and malate is not less than 73.0 per cent and fumarate is not more than 0.1 per cent, calculated on the dried basis.

Loss on drying (2.4.19). 8.0 per cent to 11.0 per cent, by drying in an oven at 150° for 4 hours.

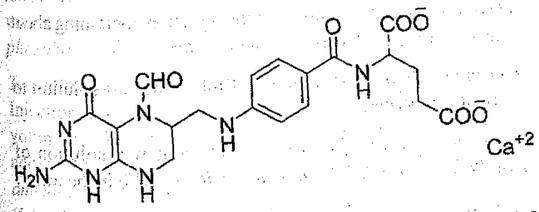
Assay. Dissolve 0.35 g of the substance under examination in 10 ml of *water* and 3 ml of *3M hydrochloric acid* with swirling, add 90 ml of *water* and stir using a magnetic stirrer. While stirring, add 30.0 ml of *0.05 M disodium edetate* from the titration burette and 25 ml of *1 M sodium hydroxide*, and continue the titration with *0.05 M disodium edetate* using *hydroxyl naphthol blue* as indicator and titrate to as blue end-point. Carry out a blank titration.

1 ml of *0.05 M disodium edetate* is equivalent to 0.002004 g of calcium.

Storage. Store protected from moisture.

Calcium Folinate

Leucovorin Calcium



$C_{20}H_{21}CaN_7O_7$ Mol. Wt. 511.5

Calcium Folinate is calcium *N*-[4-(2-amino-5-formyl-1,4,5,6,7,8-hexahydro-4-oxo-6-pteridinyl)methylaminobenzoyl]-L-glutamate.

Calcium Folinate contains not less than 95.0 per cent and not more than 105.0 per cent of $C_{20}H_{21}CaN_7O_7$, calculated on the anhydrous basis.

Category. Antidote to folate antagonists.

Description. A yellowish white or yellow powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *calcium folinate IPRS* or with the reference spectrum of calcium folinate.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

NOTE— Protect the solutions from light.

Test solution. Dissolve 10.0 mg of the substance under examination to 10.0 ml with *water*.

Reference solution (a). A 0.1 per cent w/v solution of *calcium folinate IPRS* in *water*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *water*.

Reference solution (c). A 0.01 per cent w/v solution of *formylfolic acid IPRS (folinate impurity A IPRS)* in the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with *water*.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 10.0 ml with *water*.

Reference solution (e). Dilute 5.0 ml of reference solution (c) to 10.0 ml with reference solution (b).

Chromatographic system
- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 22 volumes of *methanol* and 78 volumes of a solution containing 2.0 ml of a 40 per cent w/v solution of *tetrabutylammonium hydroxide solution* and 2.2 g of *disodium hydrogen phosphate*, previously adjusted to pH 7.8 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Inject reference solution (e). The test is not valid unless the resolution between the peaks due to calcium folinate and calcium folinate impurity A is not less than 2.2.

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Inject reference solution (b), (c), (d) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak. The area of the peak due to folinate impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and the sum of areas of all the secondary peaks other than folinate impurity A is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

Heavy metals (2.3.13). 0.4 g complies with the limit test for heavy metals, Method B (50 ppm).

Water (2.3.43). Not more than 17.0 per cent determined on 0.5 g.

Assay. *Use only freshly deionised water wherever water is specified throughout this procedure. Protect the solutions from unnecessary exposure to light and complete the Assay without prolonged interruption.*

Determine by liquid chromatography (2.4.14).

Solvent mixture. Add 15 ml of a 25 per cent w/v solution of *tetrabutylammonium hydroxide* in *methanol* to 900 ml of *water*, adjusted to pH 7.5 ± 0.1 with *0.67 M sodium dihydrogen phosphate* and dilute with *water* to 1000 ml.

Test solution. A 0.02 per cent w/v solution of the substance under examination in the solvent mixture.

Reference solution. A solution containing 0.0175 per cent w/v each of *calcium folinate IPRS* and *folic acid IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 30 cm x 4 mm, packed with octadecylsilane bonded to porous silica (3 to 10 µm),
- mobile phase: a mixture of 15 ml of a 25 per cent w/v solution of *tetrabutylammonium hydroxide*, 825 ml of *water* and 125 ml of *acetonitrile*, adjusted to pH 7.5 ± 0.1 with *0.67 M sodium dihydrogen phosphate*, diluted with *water* to 1000 ml,
- flow rate: 1 to 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The relative retention times for calcium folinate and folic acid are 1.0 and about 1.6 respectively. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.6 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{21}CaN_7O_7$.

Calcium Folinate intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.5 Endotoxin Unit per mg of calcium folinate.

Storage. Store protected from light and moisture.

Calcium Folinate Injection

Leucovorin Calcium Injection

Calcium Folinate Injection is a sterile solution of Calcium Folinate in Water for Injection.

Calcium Folinate Injection contains not less than 90.0 per cent and not more than 120.0 per cent of the stated amount of folinic acid, $C_{20}H_{23}N_7O_7$.

Usual strength. The equivalent of 3 mg of folinic acid per ml. (3.25 mg of calcium folinate is approximately equivalent to 3 mg of folinic acid).

Description. A clear, yellowish solution.

Identification

Transfer a volume containing about 6 mg of folinic acid to a glass-stoppered, 50-ml centrifuge tube, add about 40 ml of *acetone*, mix, centrifuge for a few minutes and decant the liquid phase. Repeat the washing with an additional 40 ml of *acetone*. Dry the precipitate obtained with a stream of dry nitrogen. The precipitate complies with the following test.

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *calcium folinate IPRS* or with the reference spectrum of calcium folinate.

Tests

pH (2.4.24). 6.5 to 8.5.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE—Protect the solutions from light.

Test solution. Dilute a volume of the injection containing about 10 mg of folinic acid to 10.0 ml with *water*.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *water*.

Reference solution (b). A 0.001 per cent w/v solution of *formylfolic acid IPRS (folinate impurity A IPRS)* in the *water*.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 10.0 ml with *water*.

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Reference solution (d). A mixture of 10.0 ml of reference solution (a) and 5.0 ml of reference solution (b).

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm) (Such as Hypersil ODS),
- column temperature: 40°,
- mobile phase: a mixture of 220 volumes of *methanol* and 780 volumes of a solution containing 2.0 ml of *tetrabutylammonium hydroxide solution* and 2.2 g of *disodium hydrogen orthophosphate*, previously adjusted to pH 7.5 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Inject reference solution (d). The test is not valid unless the resolution between the peaks due to calcium folinate and formylfolic acid is not less than 2.2.

Inject reference solution (a), (b), (c) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak. The area of the peak due to formylfolic acid is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent) and the sum of areas of all the secondary peaks, other than folinate impurity A is not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent). Ignore any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *Use only freshly deionised water wherever water is specified throughout this procedure. Protect the solutions from unnecessary exposure to light and complete the Assay without prolonged interruption.*

Determine by liquid chromatography (2.4.14).

Solvent mixture. Add 15 ml of a 25 per cent w/v solution of *tetrabutylammonium hydroxide* in *methanol to 900 ml of water*, adjusted to pH 7.5 ± 0.1 with *0.67 M sodium dihydrogen phosphate* and dilute with *water* to 1000 ml.

Test solution. Transfer an accurately measured volume of the Injection containing about 9 mg of folinic acid to a 50-ml volumetric flask, dilute to volume with a solution prepared by adding 15 ml of a 25 per cent w/v solution of *tetrabutylammonium hydroxide* in *methanol* to 900 ml of *water*, adjusted to pH 7.5 ± 0.1 with *0.67 M sodium dihydrogen phosphate* and diluting with *water* to 1000 ml. Transfer 25.0 ml of the solution into a 60-ml separator, add 25 ml of *dichloromethane*, shake the mixture, allow the layers to separate and discard the dichloromethane extract. Repeat the extraction with two more quantities, each of 25ml, of *dichloromethane*, discarding the dichloromethane extracts. Filter the aqueous layer, discarding the first 5 ml of the filtrate, and collect the remaining filtrate in a glass-stoppered conical flask.

Reference solution. A solution containing 0.0175 per cent w/v each of *calcium folinate IPRS* and *folic acid IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 30 cm x 4 mm, packed with octadecylsilane bonded to porous silica (3 to 10 µm),
- mobile phase: a mixture of 15 ml of a 25 per cent w/v solution of *tetrabutylammonium hydroxide*, 825 ml of *water* and 125 ml of *acetonitrile*, previously adjusted to pH 7.5 ± 0.1 with *0.67 M sodium dihydrogen phosphate*, diluted with *water* to 1000 ml,
- flow rate: 1 to 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention times for calcium folinate and folic acid are 1.0 and about 1.6 respectively.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.6 per cent.

Inject the reference solution and the test solution.

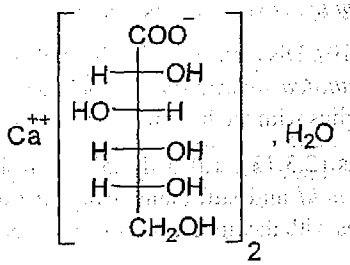
Calculate the content of $C_{20}H_{23}N_7O_7$ in the injection.

1 mg calcium folinate is approximately equivalent to 0.93 mg of folinic acid.

Storage. Store in single dose containers preferably of type I glass, protected from light.

Labelling. The label states the strength in terms of the equivalent amount of folinic acid.

Calcium Gluconate



$C_{12}H_{22}O_{14}Ca,H_2O$ Mol. Wt. 448.4

Calcium Gluconate is calcium D-gluconate monohydrate.

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Calcium Gluconate contains not less than 98.5 per cent and not more than 102.0 per cent of $C_{12}H_{22}O_{14}Ca,H_2O$.

Category. Calcium replenisher.

Description. A white, crystalline powder or granules.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 50 volumes of *ethanol (95 per cent)*, 30 volumes of *water*, 10 volumes of *strong ammonia solution* and 10 volumes of *ethyl acetate*.

Test solution. A 2.0 per cent w/v solution of the substance under examination in *water*, heating if necessary, to 60° in a water-bath to effect solution.

Reference solution. A 2.0 per cent w/v solution of *calcium gluconate IPRS* in *water*, heating if necessary, to 60° in a water-bath to effect solution.

Apply to the plate 5 µl of each solution. After development, dry the plate at 100° for 20 minutes, cool and spray with a 5 per cent w/v solution of *potassium dichromate* in a 40 per cent w/w solution of *sulphuric acid*. After 5 minutes the principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. To 1 ml of a 3 per cent w/v solution in *water* at 60°, add 0.05 ml of *ferric chloride test solution*; a yellow colour is produced.

C. A 2.0 per cent w/v solution in *water* at 60° gives reactions A and B of calcium salts (2.3.1).

Tests

Appearance of solution. A 2.0 per cent w/v solution at 60° is not more intensely coloured than reference solution YS6 (2.4.1). On cooling to room temperature the solution is not more opalescent than opalescence standard OS2 (2.4.1).

Acidity and alkalinity. Dissolve 0.5 g in 20 ml of *water* at 60°, add 0.1 ml of *0.01 M hydrochloric acid* and 0.1 ml of *phenolphthalein solution*; no colour is produced. Add 0.3 ml of *0.01 M sodium hydroxide*; a pink colour is produced.

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water* at 60° and 12 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). 1.0 g dissolved in 4 ml of *dilute hydrochloric acid* and sufficient *water* at 60° to produce 25 ml complies with the limit test for heavy metals, Method A (20 ppm).

Chlorides (2.3.12). 1.0 g complies with the limit test for chlorides (250 ppm).

Sulphates (2.3.17). 1.0 g complies with the limit test for sulphates (150 ppm). (if necessary heat at 60° do dissolve).

Sucrose and reducing sugars. To 10 ml of 5 per cent w/v solution in hot *water* add 2 ml of *dilute hydrochloric acid* and boil for 2 minutes. Cool, add 15 ml of *sodium carbonate solution*, allow to stand for 5 minutes and filter. Add 5 ml of the clear filtrate to 2 ml of *potassium cupri-tartrate solution* and boil for 2 minutes; no red precipitate is formed.

Assay. Weigh 0.5 g and dissolve in 50 ml of warm *water*, cool, add 5.0 ml of *0.05 M magnesium sulphate* and 10 ml of *strong ammonia solution* and titrate with *0.05 M disodium edetate* using *mordant black II mixture* as indicator. Carry out a blank titration.

1 ml of *0.05 M disodium edetate* is equivalent to 0.02242 g of $C_{12}H_{22}O_{14}Ca,H_2O$.

Calcium Gluconate Injection

Calcium Gluconate Injection is a sterile solution of Calcium Gluconate in Water for Injections. Not more than 5.0 per cent of the Calcium Gluconate may be replaced with a suitable calcium salt as a stabilising agent.

Calcium Gluconate Injection contains a quantity of calcium equivalent to not less than 8.5 per cent and not more than 9.4 per cent of the stated amount of calcium gluconate, $C_{12}H_{22}O_{14}Ca, H_2O$.

Usual strengths. The equivalent of 500 mg and 1 g of calcium gluconate in 5 ml; the equivalent of 1 g of calcium gluconate in 10 ml. (A 10 per cent w/v solution of Calcium Gluconate contains approximately 0.45mEq of Ca^{++} per ml).

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 50 volumes of *ethanol (95 per cent)*, 30 volumes of *water*, 10 volumes of *strong ammonia solution* and 10 volumes of *ethyl acetate*.

Test solution. Dilute a suitable volume of the substance under examination in *water* to obtain a solution containing 2.0 per cent w/v of Calcium Gluconate.

Reference solution. A 2.0 per cent w/v solution of *calcium gluconate IPRS* in *water*, heating if necessary, to 60° in a water-bath to effect solution.

Apply to the plate 5 µl of each solution. After development, dry the plate at 100° for 20 minutes, cool and spray with a 5 per

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cent w/v solution of *potassium dichromate* in a 40 per cent w/w solution of *sulphuric acid*. After 5 minutes the principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. To 1 ml add 0.05 ml of *ferric chloride test solution*; an intense yellow colour is produced.

C. It gives the reactions of calcium salts (2.3.1).

Tests

pH (2.4.24). 6.0 to 8.2.

Bacterial endotoxins (2.2.3). Not more than 0.17 Endotoxin Unit per mg of calcium gluconate.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. To a volume containing 0.5 g of Calcium Gluconate add 50 ml of *water*; cool, add 5.0 ml of *0.05 M magnesium sulphate* and 10 ml of *strong ammonia solution* and titrate with *0.05 M disodium edetate* using *mordant black II mixture* as indicator. Carry out a blank titration.

1 ml of *0.05 M disodium edetate* is equivalent to 0.002004 g of Ca.

Labelling. The label states (1) the strength as a percentage w/v of calcium gluconate equivalent to the total amount of calcium present; (2) that solutions containing visible solid particles must not be used; (3) the percentage of any added stabilising agent.

Calcium Gluconate Tablets

Calcium Gluconate Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of calcium gluconate, $C_{12}H_{22}O_{14}Ca,H_2O$.

Usual strengths. 325 mg; 500 mg; 650 mg; 1 g.

Identification

A warm filtered solution of the powdered tablets equivalent to a 10 per cent w/v solution of Calcium Gluconate complies with the following tests.

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 50 volumes of *ethanol (95 per cent)*, 30 volumes of *water*, 10 volumes of *strong ammonia solution* and 10 volumes of *ethyl acetate*.

Test solution. A 2.0 per cent w/v solution of the substance under examination in *water*, heating if necessary, to 60° in a water-bath to effect solution.

Reference solution. A 2.0 per cent w/v solution of *calcium gluconate IPRS* in *water*, heating if necessary, to 60° in a water-bath to effect solution.

Apply to the plate 5 µl of each solution. After development, dry the plate at 100° for 20 minutes, cool and spray with a 5 per cent w/v solution of *potassium dichromate* in a 40 per cent w/w solution of *sulphuric acid*. After 5 minutes the principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. To 1 ml of a 3.0 per cent w/v solution add 0.05 ml of *ferric chloride test solution*; a yellow colour is produced.

C. A 2.0 per cent w/v solution gives reactions A and B of calcium salts (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter through a membrane filter. Measure the absorbance of the filtrate, diluted if necessary at 422.7 nm by atomic absorption spectrophotometry (2.4.2) using a calcium hollow-cathode lamp as the radiation source, an air-acetylene flame. Calculate the content of calcium in the medium from the absorbance obtained from solution of known concentration of *calcium solution AAS*, suitably diluted with *water*.

Calculate the content of calcium gluconate.

1 mg of calcium is equivalent to 11.21 mg of $C_{12}H_{22}O_{14}Ca,H_2O$.

Q. Not less than 70 per cent of the stated amount of $C_{12}H_{22}O_{14}Ca,H_2O$.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 0.5 g of Calcium Gluconate and ignite, gently at first, until free from carbon. Cool, add 10 ml of *water* and sufficient *dilute hydrochloric acid*, dropwise, to effect complete solution of the residue. Neutralise with *dilute ammonia solution*, add 5.0 ml of *0.05 M magnesium sulphate* and 10 ml of *strong ammonia solution* and titrate with *0.05 M disodium edetate* using *mordant black II mixture* as indicator. Carry out a blank titration.

1 ml of *0.05 M disodium edetate* is equivalent to 0.02242 g of $C_{12}H_{22}O_{14}Ca,H_2O$.

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Calcium Pantothenate contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{18}H_{32}CaN_2O_{10}$, calculated on the dried basis.

Category. B-group vitamin (enzyme co-factor).

Description. A white powder; slightly hygroscopic.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *calcium pantothenate IPRS* or with the reference spectrum of calcium pantothenate.

B. Specific optical rotation. (see Test)

C. Gives reaction (A) of calcium salts (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution in *carbon dioxide-free water* is clear, (2.4.1) and colourless (2.4.1).

pH (2.4.24). 6.8 to 8.0, determined in a 5.0 per cent w/v solution.

Specific optical rotation (2.4.22). +25.0° to +27.5°, determined at 20° in a 5.0 per cent w/v solution.

β-Alanine. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 65 volumes of *ethanol* and 35 volumes of *water*.

Test solution (a). A 4.0 per cent w/v solution of the substance under examination in *water*.

Test solution (b). A 0.4 per cent w/v solution of the substance under examination in *water*.

Reference solution (a). A 0.4 per cent w/v solution of *calcium pantothenate IPRS* in *water*.

Reference solution (b). A 0.02 per cent w/v solution of b-*alanine* in *water*.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in a current of air, spray with *ethanolic ninhydrin solution* and heat at 110° for 10 minutes. Any spot corresponding to β-alanine in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b).

Heavy metals (2.3.13). 1.0 g dissolved in 25 ml of *water* complies with the limit test for heavy metals, Method A (20 ppm).

Loss on drying (2.4.19). Not more than 3.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh accurately about 0.18 g and dissolve in 50 ml of *anhydrous glacial acetic* acid. Titrate *0.1 M perchloric acid*, determining the end point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02383 g of $C_{18}H_{32}CaN_2O_{10}$.

Storage. Store protected from moisture.

Calcium Pantothenate Tablets

Calcium Pantothenate Tablets contain not less than 95.0 per cent and not more than 115.0 per cent of the stated amount of the dextrorotatory isomer of calcium pantothenate, $C_{18}H_{32}CaN_2O_{10}$.

Usual strengths. 50 mg; 100 mg.

Identification

A. Disperse a quantity of powder containing 150 mg of Calcium Pantothenate in 15 ml of *1 M sodium hydroxide* and filter. The resulting solution gives reactions of calcium (2.3.1).

B. To 5 ml of the filtrate obtained in test A, add 5 ml of *1 M hydrochloric acid* and 2 drops of *ferric chloride TS*; a strong yellow colour is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. A solution of *calcium pantothenate IPRS* with the dissolution medium to obtain a solution of the same concentration as the test solution.

Chromatographic system
- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 1 volume of *orthophosphoric acid* and 1000 volumes of *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

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Calculate the content of $C_{18}H_{32}CaN_2O_{10}$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{18}H_{32}CaN_2O_{10}$.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 50.0 mg of Calcium Pantothenate in 5 ml of *methanol* and dilute to 100.0 ml with *water*, filter.

Reference solution (a). A solution containing 0.05 per cent w/v of *calcium pantothenate IPRS* and 0.01 per cent w/v of *racemic panthenol IPRS* in *water*.

Reference solution (b). A 0.05 per cent w/v solution of *calcium pantothenate IPRS* in *water*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 50°,
- mobile phase: a mixture of 10 volumes of *methanol* and 90 volumes of buffer solution prepared by dissolving 10.0 g of *monobasic potassium phosphate* in 2000 ml of *water*, adjusted to pH 3.5 with *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 25 µl.

The relative retention time for panthenol with reference to pantothenate is about 1.1.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to pantothenate and panthenol is not less than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent for the principal peak.

Inject reference solution (b) and the test solution.

Calculate the content of calcium pantothenate, $C_{18}H_{32}CaN_2O_{10}$ in the tablets.

Calcium — 7.9 per cent to 9.7 per cent of the weight of $C_{18}H_{32}CaN_2O_{10}$ in the tablets, using a quantity of powdered tablets containing 0.5 g of Calcium Pantothenate.

Transfer a quantity of powder to a suitable crucible. Ignite, gently at first, until free from carbon. Cool the crucible. Add 10 ml of *water*, and dissolve the residue by adding sufficient *3 M hydrochloric acid*, dropwise, to achieve complete solution. Transfer the solution to a suitable container, and dilute with *water* to 150 ml. Add 15 ml of *1 M sodium hydroxide*, then add 300 mg of *hydroxy naphthol blue*. Titrate with *0.05 M disodium edetate* to a distinct blue endpoint. Carry out a blank titration.

1 ml of *0.05 M disodium edetate* is equivalent to 0.002004 g of Calcium.

Storage. Store protected from moisture.

Labelling. The label states the strength in terms of amount of dextrorotatory calcium pantothenate.

Dibasic Calcium Phosphate

Calcium Hydrogen Phosphate

$CaHPO_4$ Mol. Wt. 136.1 (anhydrous)

$CaHPO_4,2H_2O$ Mol. Wt. 172.1 (dihydrate)

Dibasic Calcium Phosphate is anhydrous or contains two molecules of water of hydration.

Dibasic Calcium Phosphate contains not less than 98.0 per cent and not more than 105.0 per cent of $CaHPO_4$ (for anhydrous material) or of $CaHPO_4,2H_2O$ (for the dihydrate).

Category. Calcium supplement; pharmaceutical aid (excipient).

Description. A white, crystalline powder.

Identification

A. Gives reaction A of calcium salts (2.3.1).

B. Dissolve 0.1 g in a mixture of 5 ml of *2 M nitric acid* and 5 ml of *water*; the solution gives reaction C of phosphates (2.3.1).

Tests

Acid-insoluble substances. Heat 5.0 g with a mixture of 40 ml of *water* and 10 ml of *hydrochloric acid* and dilute to 100 ml with *water*. Filter, wash with hot *water* until the last washing is free from chloride and dry the residue at 105° for 1 hour (0.1 per cent).

Arsenic (2.3.10). Dissolve 1.0 g in 15 ml of *brominated hydrochloric acid*, add 45 ml of *water* and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (10 ppm).

Heavy metals (2.3.13). Dissolve 2.5 g in 20 ml of *2 M hydrochloric acid*, filter if necessary, and add *6 M ammonia* until a precipitate is formed. Add *2 M hydrochloric acid* just enough to dissolve the precipitate and dilute to 50 ml with *distilled water* (solution A). 10 ml of the solution complies with the limit test for heavy metals, Method A (40 ppm).

Barium. To 10 ml of solution A add 0.5 ml of *1 M sulphuric acid*, mix and set aside for 15 minutes. The solution is not more opalescent than a mixture of 10 ml of solution A and 0.5 ml of *distilled water* treated in the same manner.

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Iron (2.3.14). 2.0 ml of solution A diluted to 10 ml with *water* complies with the limit test for Iron (400 ppm).

Carbonate. Suspend 1 g in 5 ml of *water* and add 2 ml of *hydrochloric acid*; no effervescence is produced.

Chlorides (2.3.12). Dissolve 0.2 g in *water* by the addition of 2 ml of *nitric acid*. The solution complies with the limit test for chlorides (0.125 per cent).

Sulphates (2.3.17). Dissolve 30.0 mg in 25 ml of *water* by the addition of 2 ml of *hydrochloric acid*. The solution complies with the limit test for sulphates (2.3.17) (0.5 per cent).

Nitrate. To 0.1 g add 10 ml of *water*, 10 ml of *nitrogen-free sulphuric acid* and 1 ml of *indigo carmine solution* and heat to boiling; the blue colour does not disappear.

Reducing substances. Shake 1.0 g with 5 ml *of water* and 5 ml of *3 M sulphuric acid* for 1 minute. Add 0.1 ml of *0.005 M potassium permanganate* and shake for 20 seconds. The slight pink colour is not less intense than that produced by treating 1 g of *calcium carbonate* in the same manner.

Proteinous impurities. Heat 0.5 g gently in a dry test-tube; no change in colour is observed and no unpleasant odour is emitted.

Monocalcium and tricalcium phosphates. Dissolve 2.0 g in 30.0 ml of *1 M hydrochloric acid*, add 20 ml of *water* and 0.05 ml of *methyl orange solution* and titrate the excess of acid with *1 M sodium hydroxide*. Not less than 14.0 ml and not more than 15.5 ml of *1 M sodium hydroxide* (for anhydrous material) and not less than 11.0 ml and not more than 12.5 ml of *1 M sodium hydroxide* (for the dihydrate) is required.

Loss on ignition (2.4.20). 6.5 to 8.5 per cent (for anhydrous material) and 24.5 to 26.5 per cent (for the dihydrate), determined on 1.0 g by igniting at 800° to 825°.

Assay. Weigh accurately about 0.3 g and dissolve in a mixture of 5 ml of *water* and 1 ml of *7 M hydrochloric acid*, add 25.0 ml of *0.1 M disodium edetate* and dilute to 200 ml with *water*. Neutralise with *strong ammonia solution*, add 10 ml of *ammonia buffer pH 10.0* and 50 mg of *mordant black 11 mixture* and titrate the excess of disodium edetate with *0.1 M zinc sulphate*. Carry out the blank titration.

1 ml of *0.1 M disodium edetate* is equivalent to 0.01361 g of $CaHPO_4$ or 0.01721 g of $CaHPO_4,2H_2O$.

Tribasic Calcium Phosphate

Calcium Hydroxide Phosphate; Calcium Phosphate

Tribasic Calcium Phosphate consists mainly of tricalcium diorthophosphate, $Ca_3(PO_4)_2$, together with calcium phosphates of more acidic or basic character.

Tribasic Calcium Phosphate contains not less than 90.0 per cent and not more than 100.5 per cent of calcium phosphates, calculated as $Ca_3(PO_4)_2$.

Category. Pharmaceutical aid (excipient).

Description. A white, amorphous powder.

Identification

A. Gives reaction (A) of calcium salts (2.3.1).

B. Dissolve 0.1 g in a mixture of 5 ml of *2 M nitric acid* and 5 ml of *water*; the solution gives reaction C of phosphates (2.3.1).

Tests

Acid-insoluble substances. Heat 5.0 g with a mixture of 40 ml of *water* and 10 ml of *hydrochloric acid* and dilute to 100 ml with *water*. Filter, wash with hot *water* until the last washing is free from chloride and dry the residue at 105° for 1 hour (0.3 per cent).

Water-soluble substances. Digest 2.0 g with 100 ml of *water* for 30 minutes on a water-bath, cool, add sufficient *water* to restore the original volume, stir well and filter. Evaporate 50 ml of the filtrate to dryness and dry the residue at 105° to constant weight (0.5 per cent).

Arsenic (2.3.10). Dissolve 2.0 g in a mixture of 15 ml of *brominated hydrochloric acid*, add 45 ml of *water* and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (5 ppm).

Heavy metals (2.3.13). Warm 1.0 g with 4 ml of *dilute hydrochloric acid*, add sufficient *water* to produce 50 ml and filter. 25 ml of the solution complies with the limit test for heavy metals, Method A (40 ppm).

Iron (2.3.14). Dissolve 0.2 g in a mixture of 5 ml of *water* and 0.5 ml of *iron-free hydrochloric acid* with the addition of 1 g of citric acid. Dilute the solution to 40 ml with *water*. The solution complies with the limit test for iron (200 ppm).

Carbonate. Suspend 1 g in 10 ml of *water* and add 2 ml of *hydrochloric acid*; no effervescence is produced.

Chlorides (2.3.12). Dissolve 0.25 g in 25 ml of *water* by the addition of 1 ml of *nitric acid*. The solution complies with the limit test for chlorides (0.1 per cent).

Sulphates (2.3.17). Dissolve 100.0 mg in *water* with the aid of 3 ml of *1 M hydrochloric acid* and dilute to 60 ml with *water*. 15 ml of the resulting solution complies with the limit test for sulphates (0.6 per cent).

Proteinous impurities. Heat 0.5 g gently in a dry test-tube; no change in colour is observed and no unpleasant odour is emitted.

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Loss on ignition (2.4.20). Not more than 8.0 per cent, determined on 1.0 g by igniting at 800° for 30 minutes.

Water (2.3.43). Not more than 2.5 per cent, determined on 1.0 g.

Assay. Weigh accurately about 1.0 g and dissolve in 10 ml of *hydrochloric acid* by heating on a water-bath, add 50 ml of *water*, cool and dilute to 250.0 ml with *water*. To 25.0 ml of the resulting solution add 30.0 ml of *0.05 M disodium edetate*, 10.0 ml of *ammonia buffer pH 10.9* and 100 ml of *water* and titrate the excess of disodium edetate with *0.05 M zinc chloride* using *mordant black II solution* as indicator. Carry out the blank titration.

1 ml of *0.05 M disodium edetate* is equivalent to 0.00517 g of $Ca_3(PO_4)_2$.

Calcium and Vitamin D_3 Tablets

Calcium and Vitamin D_3 Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of calcium (Ca), derived from substances generally recognized as safe, and not less than 90.0 per cent of the stated amount of Vitamin D_3 ($C_{27}H_{44}O$).

Usual strengths. Calcium, 250 mg and Vitamin D_3, 200 IU; Calcium, 500 mg and Vitamin D_3, 200 IU; Calcium, 500 mg and Vitamin D_3, 250 IU; Calcium, 500 mg and Vitamin D_3, 500 IU.

Identification

A. In the Assay, the retention time of principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Disperse a quantity of powdered tablets containing 10 mg of calcium in 5 ml of *water* and add 5 ml of *5 M acetic acid* or 1 ml of *glacial acetic acid* and filter. It gives the reaction (A) of calcium salts (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *0.1 M hydrochloric acid*,

Speed and time. 75 rpm and 30 minutes.

Withdraw a suitable volume of the medium and filter the solution through a filter paper (Whatman No 1), collect the filtrate. Transfer 20 ml of the solution in to 250-ml volumetric flask, add 150 ml of *water*, add 3 ml of *1 M sodium hydroxide* and 10 ml of *ammonia solution*. Titrate with *0.05 M disodium edetate*, using *hydroxy napthol blue* as an indicator. Carry out a blank titration.

1 ml of *0.05 M disodium edetate* is equivalent to 0.002004 g of Ca.

Q. Not less than 70 per cent of the stated amount of Ca.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay.

Test solution. Disperse one tablet in 2.5 ml of *water* by shaking for 5 minutes. Add about 15 ml of *methanol* and sonicate for 20 minutes with intermittent shaking and dilute to 25.0 ml with *methanol*, mix and centrifuge the solution at 4000 rpm for 5 minutes and filter. Dilute the filtrate, if necessary, with *methanol*.

Reference solution. A 0.000025 per cent w/v solution of *cholecalciferol IPRS* in *methanol (90 per cent, v/v)*.

Use chromatographic system as described under Assay.

Calculate the content of $C_{27}H_{44}O$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. *For Calcium* — Weigh and powder 20 tablets. Disperse a quantity of the powder containing equivalent to 50.0 mg of calcium add 50.0 ml of *water* and 5 ml of *hydrochloric acid*. Heat the dispersion gently to boiling and continue to boil for about 2 minutes. Allow to cool and add 50 ml of *0.05M disodium edetate*. Neutralise the solution using *2M sodium hydroxide*, add 10 ml of *ammonia buffer pH 10.9* and 50.0 ml of *water*. Titrate the excess of *disodium edetate* with *0.05M zinc chloride* using *mordant black II solution* as indicator. Carry out a blank titration.

1 ml of *0.05 M disodium edetate* is equivalent to 0.002004 g of Ca.

Calculate the content of Ca in the Tablets.

For Vitamin D_3 (Cholecalciferol) —

NOTE — Use low-actinic glassware throughout the procedure.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder equivalent to 0.1 mg of vitamin D_3 in 20 ml of *water* by shaking for 5 minutes. Add about 120 ml of *methanol* and sonicate for 20 minutes with intermittent shaking and dilute to 200.0 ml with *methanol*, mix and centrifuge the solution at 4000 rpm for 5 minutes and filter. Use the filtrate.

Reference solution. A 0.00005 per cent w/v solution of *cholecalciferol IPRS* in *methanol (90 per cent, v/v)*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 3 volumes of *water* and 97 volumes of *methanol*,

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- flow rate: 1 ml per minute,
- spectrophotometer set at 264 nm,
- injection volume: 50 µl.

Inject the reference solution and the test solution.

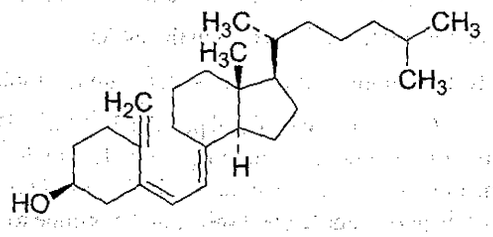
Calculate the content of $C_{27}H_{44}O$ in the Tablets.

Storage. Store protected from light and moisture.

Labelling. The label states (1) the quantity of the active ingredient in terms of the equivalent amount of calcium; (2) the equivalent number of IU (units) of vitamin D_3 per tablet.

Cholecalciferol

Vitamin D_3; Colecalciferol



$C_{27}H_{44}O$ Mol Wt. 384.6

Cholecalciferol is (5*Z*,7*E*)-(3*S*)-9,10-secocholesta-5,7,10(19)-triene-3-ol.

Cholecalciferol contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{27}H_{44}O$.

Category. Vitamin D (antirachitic).

Description. White or almost white crystals. It is sensitive to air, heat and light. A reversible isomerisation to precholecalciferol may occur in solution, depending on temperature and time.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B, C and D may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *cholecalciferol IPRS* or with the reference spectrum of cholecalciferol.

B. Dissolve 1 mg in 1 ml of *1,2-dichloroethane* and 4 ml of *antimony trichloride solution*; a yellowish-orange colour is produced.

C. In the test for 7-Dehydrocholesterol, the principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (b).

D. To a solution of about 0.5 mg in 5 ml of *chloroform* add 0.3 ml of *acetic anhydride* and 0.1 ml *sulphuric acid* and shake vigorously; a bright red colour is produced which rapidly changes through violet and blue to green.

Tests

Specific optical rotation (2.4.22). + 105° to + 112°, determined within 30 minutes of preparation, in a solution prepared by dissolving 0.2 g rapidly and without heating in sufficient *aldehyde-free ethanol (95 per cent)* to produce 25.0 ml.

Light absorption. Dissolve 10 mg, rapidly and without heating, in sufficient *aldehyde-free ethanol (95 per cent)* to produce 100.0 ml. Dilute 5.0 ml of the solution to 50.0 ml with *aldehyde-free ethanol (95 per cent)*. Absorbance of the resulting solution at the maximum at about 265 nm, measured within 30 minutes of preparation, 0.46 to 0.50 (2.4.7).

7-Dehydrocholesterol. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A 0.01 per cent w/v solution of *butylated hydroxytoluene* in a mixture of equal volumes of *cyclohexane* and *peroxide-free ether*.

NOTE—Prepare the following solutions immediately before use.

Test solution. Dissolve 0.25 g of the substance under examination in sufficient of *1,2-dichloroethane* containing 1 per cent w/v of *squalane* and 0.01 per cent w/v of *butylated hydroxytoluene* (solvent A) to produce 5.0 ml.

Reference solution (a). A solution containing 0.005 per cent w/v of *7-dehydrocholesterol IPRS* in solvent A.

Reference solution (b). A solution containing 2.5 per cent w/v of *cholecalciferol IPRS* in solvent A.

Reference solution (c). Mix equal volumes of reference solutions (a) and (b).

Apply to the plate 10 µl of each solution. Develop the chromatograms immediately, protected from light. After development, dry the plate in air and spray three times with *antimony trichloride reagent*. Examine the chromatograms for not more than 4 minutes after spraying. The principal spot in the chromatogram obtained with the test solution is initially orange-yellow but becomes brown later. In the chromatogram obtained with the test solution any violet spot with an R_f value slightly lower than that of the principal spot (due to 7-dehydrocholesterol and appearing slowly) is not more intense than the spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

Assay. Determine by liquid chromatography (2.4.14).

NOTE—Carry out the following procedure as rapidly as possible in subdued light and protected from air.

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Test solution. Weigh accurately about 50.0 mg of the substance under examination, dissolve in 10 ml of *toluene* without heating and dilute to 100.0 ml with the mobile phase; dilute 5.0 ml of the solution to 50.0 ml with the mobile phase; further dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *cholecalciferol IPRS* in 10 ml of toluene without heating and dilute to 100.0 ml with the mobile phase; dilute 5.0 ml of the solution to 50.0 ml with the mobile phase (Solution A); further dilute 5.0 ml of solution A to 50.0 ml with the mobile phase.

Reference solution (b). Reflux 5.0 ml of solution A, under nitrogen, on a water-bath for 60 minutes to obtain a solution of cholecalciferol, precholecalciferol and *trans*-cholecalciferol.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with porous silica particles (5 µm) (Such as Nucleosil 50-S),
- mobile phase: a mixture of 997 volumes of *hexane* and 3 volumes of *1-pentanol*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (b) and record the chromatogram adjusting the sensitivity so that the height of the peak due to cholecalciferol is more than 50 per cent of full-scale deflection. The approximate relative retention times calculated with reference to cholecalciferol are 0.4 for precholecalciferol and 0.5 for *trans*-cholecalciferol. The resolution between precholecalciferol and *trans*-cholecalciferol should be not less than 1.0; if necessary adjust the proportions of the constituents and flow rate of the mobile phase to obtain the required resolution.

Inject reference solution (a) and record the chromatogram adjusting the sensitivity so that the height of the peak due to cholecalciferol is more than 50 per cent of full-scale deflection.

Inject the test solution. Measure the areas for the major peaks.

Calculate the content of $C_{27}H_{44}O$.

Storage. Store protected from light in hermetically sealed containers under nitrogen in a refrigerator. The contents of an opened container should be used immediately.

Cholecalciferol Injection

Colecalciferol Injection

Cholecalciferol Injection is a sterile solution of Cholecalciferol in Ethyl Oleate.

Cholecalciferol Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of cholecalciferol, $C_{27}H_{44}O$.

Usual strengths. 3,00,000 IU per ml; 6,00,000 IU per ml.

Description. A clear, colourless to pale yellow liquid.

Identification

To 1 ml of a 0.2 per cent v/v solution of the injection in *ethanol-free chloroform*, add 9 ml of *antimony trichloride solution*, the light absorption of the resulting solution exhibits a maximum at 500 nm (2.4.7).

Tests

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *Carry out the following procedure in subdued light.*

Dilute 0.1 g of the injection to 50 ml with dry *1,2-dichloroethane* that has been purified by passing it through a column of *silica gel*. To 1 ml of the solution add rapidly 9 ml of *antimony trichloride* in *dichloroethane solution* and measure the absorbance of the solution at 500 and 550 nm (2.4.7), within 90 to 120 seconds after adding the reagent. Repeat the operation using 1 ml of a 0.002 per cent w/v solution of *cholecalciferol IPRS* in the dry, purified *1,2- dichloroethane*, beginning at the words 'add rapidly 9 ml of ....'. Calculate the result of the assay from the difference between the absorbances at 500 and 550 nm.

Calculate the content taking 0.87 g as the value of the weight per ml (2.4.29) of the injection.

Storage. Store protected from light.

Labelling. The label states (1) that the preparation is for intramuscular use only; (2) the equivalent number of IU (units) of antirachitic activity (vitamin D) in 1 ml.

Each µg of cholecalciferol is equivalent to 40 IU of antirachitic activity (vitamin D).

Cholecalciferol Tablets

Colecalciferol Tablets

Cholecalciferol Tablets contain not less than 90.0 per cent and not more than 125.0 per cent of the stated amount of cholecalciferol, $C_{27}H_{44}O$.

Usual strengths. 0.25 mg; 1 mg.

Identification

A. In the test for Uniformity of content, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

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B. Extract one tablet, in powder, with 5 ml of *ethanol-free chloroform*, filter. To 1 ml of the filtrate, add 9 ml of *antimony trichloride solution*. A brownish red colour is produced.

Tests

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay using the following test solution.

Test solution. For tablets containing less than 250 µg, add 2 ml of *water* to one tablet in an amber-coloured flask and disperse with the aid of ultrasound. Add 3 to 6 ml (As appropriate depending upon strength) of *dimethyl sulphoxide*, mix, extract with *hexane* by shaking for 30 minutes to get final concentration same as reference solution (a), centrifuge the hexane layer and use the clear supernatant liquid. For tablets containing more than 250 µg, prepare the solution in the same manner but using 4 ml of *water*, 12 to 24 ml (As appropriate depending upon strength) of *dimethyl sulphoxide* and quantity of *hexane* to obtain a final concentration same as reference solution (a).

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

NOTE—Carry out the following procedure as rapidly as possible in subdued light and protected from air.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 250 µg of Calciferol in 4 ml of *water*. Add 6 to12 ml (As appropriate depending upon strength) of *dimethyl sulphoxide*, mix, extract with quantity of *hexane* by shaking for 30 minutes, to get final concentration same as reference solution (a), centrifuge the hexane layer and use the clear supernatant liquid.

Reference solution (a). A 0.001 per cent w/v solution of *cholecalciferol IPRS* or *ergocalciferol IPRS*, as appropriate.

Reference solution (b). Dissolve 50 mg of *cholecalciferol IPRS* or *ergocalciferol IPRS* as appropriate in 10 ml of *toluene* without heating and dilute with the mobile phase to 100.0 ml. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase. Reflux 5.0 ml of the solution, under *nitrogen*, using a water-bath, for 60 minutes to obtain a solution of cholecalciferol, precholecalciferol and *trans*-cholecalciferol. Cool and dilute the refluxed solution to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with porous silica or ceramic microparticles (3 to 10 µm) (Such as Nucleosil 50-S 5 µm),
- mobile phase: a mixture of 997 volumes of *hexane* and 3 volumes of *1-pentanol*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 or 20 µl.

Inject a suitable volume of reference solution (b). Adjust the sensitivity so that the height of the peak due to cholecalciferol is more than 50 per cent of the full-scale deflection. Record the chromatograms after five more injections. The approximate relative retention times calculated with reference to cholecalciferol are 0.4 for precholecalciferol and 0.5 for *trans*-cholecalciferol. The resolution between precholecalciferol and *trans*-cholecalciferol should be not less than 1.0; if necessary adjust the proportions of the constituents and flow rate of the mobile phase to obtain the required resolution.

Inject reference solution (a). Adjust the sensitivity so that the height of the peak due to cholecalciferol or ergocalciferol is more than 50 per cent of the full-scale deflection.

Inject reference solution (a) and the test solution.

Calculate the content of cholecalciferol, $C_{27}H_{44}O$, or ergocalciferol, $C_{28}H_{44}O$ in the tablets.

Labelling. The label states the equivalent number of IU (units) of antirachitic activity (vitamin D) per tablet.

Each µg of Cholecalciferol is equivalent to 40 IU of antirachitic activity (vitamin D).

Cholecalciferol Concentrate (Powder Form)

Powder concentrate obtained by dispersing an oily solution of Cholecalciferol in an appropriate matrix, which is usually based on a combination of gelatin and carbohydrates of suitable quality. It may contain suitable stabilizers such as antioxidants.

Cholecalciferol contains not less than 90.0 per cent and not more than 110.0 per cent of $C_{27}H_{44}O$, which is not less than 100,000 IU per g.

Category. Vitamin D (antirachitic).

Description. A white or yellowish white small particles.

Identification

Test B may be omitted if tests A and C are carried out. Tests C may be omitted if test A and B are carried out.

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A 0.01 per cent w/v solution of *butylated hydroxytoluene* in a mixture of equal volumes of *cyclohexane* and *peroxide-free ether*.

Solution A. Ethylene chloride containing 1 per cent, w/v solution of *squalane* and 0.01 per cent, w/v solution of *butylated hydroxytoluene*.

NOTE—Prepare the following solutions immediately before use. Protect from light.

Test solution. Take 10 ml of the test solution prepared for the Assay in a suitable flask and evaporate to dryness under reduced pressure by swirling in a water-bath at 40°. Cool under running water and restore atmospheric pressure with *nitrogen*. Dissolve the residue immediately in 0.4 ml of solution A.

Reference solution (a). Dissolve 10 mg of *cholecalciferol IPRS* in 4 ml of solution A.

Reference solution (b). Dissolve 10 mg of *ergocalciferol IPRS* in 4 ml of solution A.

Apply to the plate 20 µl of each solution. Develop the plate immediately and allow the mobile phase rise to 15 cm, protected from light. After development, dry the plate in air and spray with *sulphuric acid*. The principal spot in the chromatogram obtained with the test solution is initially bright-yellow which becomes orange-brown later, then gradually greenish-grey, remaining so for 10 minutes. This spot is similar in position, colour and size to the spot obtained with reference solution (a). The spot obtained with reference solution (b) shows immediately at the same level an orange principal spot, which gradually becomes reddish-brown and remains so for 10 minutes.

B. When examined in the range 250 nm to 300 nm (2.4.7), a solution prepared by, taking 5.0 ml of the test solution prepared for the Assay in a suitable flask and evaporate to dryness under reduced pressure by swirling in a water-bath at 40°. Cool under running water and restore atmospheric pressure with *nitrogen*. Dissolve the residue immediately in 50.0 ml of *cyclohexane*. Exhibits the maxima at about 265 nm.

C. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Assay. Determine by liquid chromatography (2.4.14).

NOTE—Carry out the following procedure as rapidly as possible in subdued light and protected from air.

Test solution. Weigh accurately containing 100,000 IU of the substance under examination in a saponification flask. Add 5 ml of *water*, 20.0 ml of *anhydrous ethanol*, 1.0 ml of 17.5 per cent w/v solution of *sodium ascorbate* in *1 M sodium hydroxide solution* and 3.0 ml of freshly prepared 50 per cent w/w solution of *potassium hydroxide*. Heat in a water-bath under a reflux condenser for 30 minutes. Cool rapidly under running water. Transfer the liquid to a separating funnel with the aid of 2 quantities, each of 15.0 ml of *water*, 1 quantity of 10.0 ml of *ethanol (95 per cent)* and 2 quantities, each of 50.0 ml of *pentane*. Shake vigorously for 30 seconds. Allow to stand until the 2 layer are clear. Transfer the lower aqueous-alcoholic layer to a second separating funnel and shake with a mixture of 10.0 ml *ethanol (95 per cent)* and 50.0 ml of *pentane*. After separation, transfer the aqueous-alcoholic layer to a third separating funnel and the pentane layer to the first separating funnel, washing the second separating funnel with 2 quantities, each of 10.0 ml of *pentane* and adding the washings to the first separating funnel. Shake the aqueous-alcoholic layer with 50.0 ml of pentane and add the pentane layer to the first funnel. Wash the pentane layer with 2 quantities, each of 50.0 ml of freshly prepared 3.0 per cent w/v solution of *potassium hydroxide* in *ethanol* (10 per cent v/v). shaking vigorously, then wash with successive quantities, each of 50.0 ml of *water* until the washings are neutral, using a pH indicator strip. Transfer the washed pentane extract to a ground-glass-stoppered flask. Evaporate the contents of the flask to dryness under reduced pressure by swirling in a water-bath at 40°. Cool under running water and restore atmospheric pressure with *nitrogen*. Dissolve the residue immediately in 5.0 ml of *toluene* and add 20.0 ml of the mobile phase to obtain a solution containing about 4000 IU per ml.

Reference solution (a). Dissolve 10.0 mg of *cholecalciferol IPRS* in 10.0 ml of *toluene* without heating and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 5.0 ml with the mobile phase. Heat in a water-bath at 90° under a reflux condenser for 45 minutes and cool.

Reference solution (c). Dissolve 100.0 mg of *cholecalciferol IPRS* without heating, in *toluene* and dilute to 100.0 ml with the same solvent.

Reference solution (d). Dilute 5.0 ml of reference solution (c) to 50.0 ml with the mobile phase. Keep the solution in iced water.

Reference solution (e). Take 5.0 ml of reference solution (c) in a volumetric flask add 10 mg of *butylhydroxytoluene* under *nitrogen*, heat in a water-bath at 90° under reflux condenser for 45 minutes. Cool and dilute to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with porous silica particles (5 µm) (Such as Nucleosil 50-S),
- mobile phase: a mixture of 3 volumes of *1-pentanol* and 997 volumes of *hexane*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (b) and record the chromatogram. The approximate relative retention times calculated with reference to cholecalciferol are about 0.4 for *pre*-cholecalciferol

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and 0.5 for *trans*-cholecalciferol. The resolution between *pre*-cholecalciferol and *trans*-cholecalciferol should not less than 1.0, if necessary adjust the proportions of the constituents and flow rate of the mobile phase to obtain the required resolution.

Inject reference solutions (a), (d), (e) and the test solution. Measure the areas for the major peaks.

Calculate the conversion factor (*f*) using the following expression:

$$f = \frac{K - L}{M}$$

where, K = area of the peak due to cholecalciferol in the chromatogram obtained with reference solution (d);

L = area of the peak due to cholecalciferol in the chromatogram obtained with reference solution (e);

M = area of the peak due to pre-cholecalciferol in the chromatogram obtained with reference solution (e).

The value of *f* determined in duplicate on different days may be used during the entire procedure.

Calculate the content of $C_{27}H_{44}O$ in International Units per gram using the following expression:

$$\frac{m'}{v'} \times \frac{v}{m} \times \frac{S_d + (f \times S_p)}{S'_d} \times 40000 \times 1000$$

where, m = weight of the substances under examination, in mg;

m' = weight of *cholecalciferol IPRS* in reference solution (a), in mg;

v = volume of the test solution (25 ml);

v' = volume of reference solution (a) (100 ml);

S_d = area of the peak due to cholecalciferol in the chromatogram obtained with the test solution;

S'_d = area of the peak due to cholecalciferol in the chromatogram obtained with reference solution (a);

S_p = area of the peak due to pre-cholecalciferol in the chromatogram obtained with the test solution;

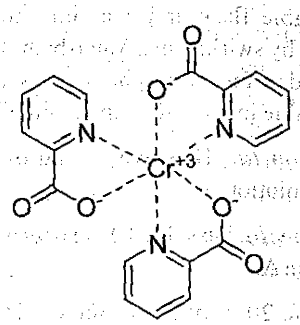
f = conversion factor.

Storage. Store protected from light, in well-filled containers. The contents of an opened container should be used as soon as possible. Any unused part protected by an atmosphere of nitrogen.

Labelling. The label states the number of International Units per g.

Chromium Picolinate

Chromium Tripicolinate



$C_{18}H_{12}N_3O_6Cr$ Mol. Wt. 418.3

Chromium Picolinate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{18}H_{12}N_3O_6Cr$, calculated on the dried basis.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *chromium picolinate IPRS* in mineral oil or with the reference spectrum of chromium picolinate.

B. Dissolve 20 mg of the substance under examination in 5 ml of *water*, add 1 ml of *5M sodium hydroxide* and 0.5 ml of *hydrogen peroxide (30 per cent)*, and heat gently for 2 minutes. A yellow colour is produced.

Tests

Chlorides (2.3.12). Dissolve 0.42 g of the substance under examination in 35 ml of *water* and heat at 70°, cool overnight and filter. The filtrate complies with the limit test for chlorides (600 ppm).

Sulphates (2.3.17). Dissolve 75 mg of the substance under examination in 35 ml of *water* and heat at 90°, cool overnight and filter. The filtrate complies with the limit test for sulphates (2000 ppm).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 4.0 per cent, by drying in an oven at 105° for 4 hours.

Assay. Determine by atomic absorption spectrophotometry (2.4.2), measuring at 357.9 nm using a reducing air acetylene flame and chromium hollow-cathode lamp.

Test solution. Dissolve 0.2 g of the substance under examination in 25 ml of *water*, add 10 ml of *nitric acid* and boil for 10 minutes with continuous swirling. Cool the solution,

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quantitatively transfer to a 500-ml volumetric flask and dilute to volume with *water* and filter. To 5.0 ml of the solution, add 1 ml of *nitric acid* and dilute to 100.0 ml with *water*.

Reference solution (a). A 0.01 per cent w/v solution of chromium prepared by dissolving 0.283 g of *potassium dichromate* (previously dried at 120° for 4 hours) in *water* and dilute to 1000 ml with *water*.

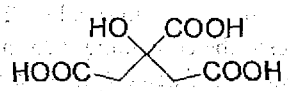
Reference solution (b). Transfer 1.0 ml and 2.0 ml of reference solution (a) to separate 100-ml volumetric flasks and 1.5 ml and 2.0 ml to separate 50-ml volumetric flask, add 1.0 ml of *nitric acid* to each volumetric flask and dilute to volume with *water* to obtain a 1.0, 2.0, 3.0 and 4.0 ppm of chromium.

Determine the absorbance of reference solution (b) and the test solution. Plot the absorbance of reference solution (b) versus the chromium concentration and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the chromium concentration in the test solution.

Calculate the content of $C_{18}H_{12}N_3O_6Cr$.

Storage. Store protected from moisture.

Citric Acid



$C_6H_8O_7$ Mol. Wt. 192.1

Citric Acid is 2-hydroxypropane-1,2,3-tricarboxylic acid.

Citric Acid contains not less than 99.0 per cent and not more than 101.0 per cent of $C_6H_8O_7$, calculated on the anhydrous basis.

Category. Pharmaceutical aid; anticoagulant for storage of whole blood (along with sodium citrate).

Description. Colourless crystals or a white powder; slightly hygroscopic in moist air.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Dry the substances and the reference substances at 105° for 2 hours. Compare the spectrum with that obtained with *citric acid IPRS* or with the reference spectrum of citric acid.

B. It gives reaction A of citrates (2.3.1).

C. A 10 per cent w/v solution is strongly acidic (2.4.46).

Tests

Appearance of solution. Dissolve 2.0 g in sufficient *water* to produce 10 ml. The solution is clear (2.4.1), and not more intensely coloured than reference solution YS7, BYS7 or GYS7 (2.4.1).

Arsenic (2.3.10). Dissolve 10.0 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid*; the resulting solution complies with the limit test for arsenic (1 ppm).

Barium. Dissolve 5.0 g in several portions in 39 ml of *2 M sodium hydroxide* and dilute to 50 ml with *distilled water* (solution A). To 5 ml of solution A add 5 ml of *1 M sulphuric acid* and allow to stand for 1 hour. Any opalescence produced is not more intense than that of a mixture of 5 ml of solution A and 5 ml of *distilled water*.

Calcium. To 0.2 ml of *ethanolic calcium standard solution (100 ppm Ca)* add 1 ml of a 4 per cent w/v solution of *ammonium oxalate*. After 1 minute add 1 ml of *2 M acetic acid* and 5 ml of solution A diluted to 10 ml with *distilled water* and shake. After 15 minutes any opalescence produced is not more intense than that of a standard prepared in the same manner using a mixture of 10 ml of *calcium standard solution (10 ppm Ca)* and 5 ml of *water* in place of solution A (200 ppm).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method A (10 ppm).

Iron (2.3.14). 8 ml of solution A diluted to 10 ml with *water* complies with the limit test for iron (50 ppm).

Chlorides (2.3.12). Dissolve 5.0 gm in 10 ml of *water*, add 1 ml of *2 M nitric acid* and dilute to 15 ml with *water*. The resulting solution complies with the limit test for chlorides (50 ppm).

Sulphates (2.3.17). Dissolve 1.0 g in sufficient *distilled water* to produce 15 ml. The resulting solution complies with the limit test for sulphates (150 ppm).

Oxalic acid. Dissolve 0.8 g in 4 ml of *water*, add 2 ml of *hydrochloric acid* and 1 g of *granulated zinc* and heat in a water-bath for 1 minute. Allow to stand for 2 minutes, decant the liquid into a test-tube containing 0.25 ml of a 1 per cent w/v solution of *phenylhydrazine hydrochloride* and heat to boiling. Cool rapidly, transfer to a graduated measuring cylinder, add an equal volume of *hydrochloric acid* and 0.25 ml of a 5 per cent solution of *potassium ferricyanide*, shake and allow to stand for 30 minutes. Any pink colour produced is not more intense than that produced by carrying out the test using 0.2 mg of *oxalic acid* dissolved in 4 ml of *water*.

Readily carbonisable substances. Heat 0.75 g in powder, with 10 ml of *sulphuric acid* (containing 94.5 to 95.5 per cent w/w of H_2SO_4) in a water-bath at 90° ± 1°. Shake after one minute, continue the heating for a total of 1 hour and cool rapidly and immediately. Any colour produced is not more intense than that of a mixture of 1.0 ml of CCS and 9.0 ml of FCS (2.4.1).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

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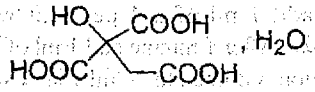
Water (2.3.43). Not more than 1.0 per cent, determined on 2.0 g.

Assay. Weigh accurately about 2 g and dissolve in 100 ml of *water*. Titrate with *1 M sodium hydroxide* using 0.5 ml of *phenolphthalein solution* as indicator.

1 ml of *1 M sodium hydroxide* is equivalent to 0.06403 g of $C_6H_8O_7$.

Storage. Store protected from moisture.

Citric Acid Monohydrate



$C_6H_8O_7,H_2O$ Mol. Wt. 210.1

Citric Acid Monohydrate is 2-hydroxypropane-1,2,3-tricarboxylic acid monohydrate.

Citric Acid Monohydrate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_6H_8O_7$, calculated on the anhydrous basis.

Category. Pharmaceutical aid; anticoagulant for storage of whole blood (along with sodium citrate).

Description. Colourless crystals or a white, crystalline powder; slightly efflorescent in warm, dry air.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Dry the substances and the reference substances at 105° for 2 hours. Compare the spectrum with that obtained with *citric acid monohydrate IPRS* or with the reference spectrum of citric acid monohydrate.

B. It gives reaction (A) of citrates (2.3.1).

C. A 10.0 per cent w/v solution is strongly acidic (2.4.46).

Tests

Appearance of solution. Dissolve 2.0 g in sufficient *water* to produce 10 ml. The solution is clear (2.4.1), and not more intensely coloured than reference solution YS7, BYS7 or GYS7 (2.4.1).

Arsenic (2.3.10). Dissolve 10 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid*; the resulting solution complies with the limit test for arsenic (1 ppm).

Barium. Dissolve 5.0 g in several portions in 39 ml of *2 M sodium hydroxide* and dilute to 50 ml with *distilled water* (solution A). To 5 ml of solution A, add 5 ml of *1 M sulphuric acid* and allow to stand for 1 hour. Any opalescence produced is not more intense than that of a mixture of 5 ml of solution A and 5 ml of *distilled water*.

Calcium. To 0.2 ml of *ethanolic calcium standard solution (100 ppm Ca)* add 1 ml of a 4 per cent w/v solution of *ammonium oxalate*. After 1 minute add 1 ml of *2 M acetic acid* and 5 ml of solution A diluted to 15 ml with *distilled water* and shake. After 15 minutes any opalescence produced is not more intense than that of a standard prepared in the same manner using a mixture of 10 ml of *calcium standard solution (10 ppm Ca)* and 5 ml of *water* in place of solution A (200 ppm).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method A (10 ppm).

Iron (2.3.14). 8 ml of solution A diluted to 10 ml with *water* complies with the limit test for iron (50 ppm).

Chlorides (2.3.12). Dissolve 5.0 g in 10 ml of *water*, add 1 ml of *2 M nitric acid* and dilute to 15 ml with *water*. The resulting solution complies with the limit test for chlorides (50 ppm).

Sulphates (2.3.17). Dissolve 1.0 g in sufficient *distilled water* to produce 15 ml. The resulting solution complies with the limit test for sulphates (150 ppm).

Oxalic acid. Dissolve 0.8 g in 4 ml of *water*, add 2 ml of *hydrochloric acid* and 1 g of *granulated zinc* and heat in a water-bath for 1 minute. Allow to stand for 2 minutes, decant the liquid into a test-tube containing 0.25 ml of a 1 per cent w/v solution of *phenylhydrazine hydrochloride* and heat to boiling. Cool rapidly, transfer to a graduated measuring cylinder, add an equal volume of *hydrochloric acid* and 0.25 ml of a 5 per cent w/v solution of *potassium ferricyanide*, shake and allow to stand for 30 minutes. Any pink colour produced is not more intense than that produced by carrying out the test using 0.2 mg of *oxalic acid* dissolved in 4 ml of *water*.

Readily carbonisable substances. Heat 0.75 g with 10 ml of *sulphuric acid* (containing between 94.5 and 95.5 per cent w/w of H_2SO_4) in a water-bath maintained at 90° ± 1°. Shake after 1 minute, continue the heating for a total of one hour and cool rapidly and immediately. Any colour produced is not more intense than that of a mixture of 1.0 ml of CCS and 9.0 ml of FCS (2.4.1).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). 7.5 to 9.0 per cent, determined on 0.5 g.

Assay. Weigh 2 g and dissolve in 50 ml of *water*. Titrate with *1 M sodium hydroxide* using 0.5 ml of *phenolphthalein solution* as indicator.

1 ml of *1 M sodium hydroxide* is equivalent to 0.06403 g of $C_6H_8O_7$.

Storage. Store protected from moisture.

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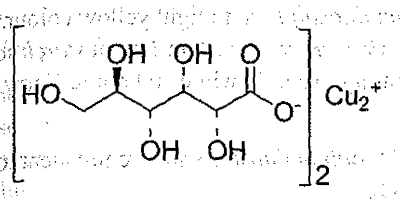
Copper Gluconate



$C_{12}H_{22}CuO_{14}$ Mol. Wt. 453.8

Copper Gluconate is copper, bis(D-gluconato-O^1,O^2).

Copper Gluconate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{12}H_{22}CuO_{14}$.

Description. A light blue to bluish-green colour powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with silica gel G.

Spray reagent. Dissolve 2.5 g of *ammonium molybdate* in 50 ml of *1M sulphuric acid*, add 1.0 g of *ceric sulphate* and dilute to 100 ml with *1M sulphuric acid*.

Mobile phase. A mixture of 50 volumes of *ethanol*, 30 volumes of *water*, 10 volumes of *ammonium hydroxide* and 10 volumes of *ethyl acetate*.

Test solution. Dissolve 0.1 g of the substance under examination in *water* by heating in a water-bath at 60° and dilute to 10.0 ml with *water*.

Reference solution. A 1.0 per cent w/v solution of *potassium gluconate IPRS* in *water*.

Apply to the plate 5 µl of each solution. Allow the mobile phase to raise three fourth length of the plate. Dry the plate at 110° for 20 minutes. Allow to cool, spray with spraying reagent. Dry the plate at 110° for 10 minutes. The principal spot in the chromatogram obtained with the test solution corresponds to the spot in the chromatogram obtained with the reference solution.

B. An excess of *6 M ammonium hydroxide*, when added to 5 ml of 5 per cent w/v solution, produces first a bluish precipitate and then a deep blue colour solution.

Tests

Arsenic (2.3.10). Dissolve 3.3 g in 35 ml of *water*. The resulting solution complies with the limit test for arsenic (3 ppm).

Lead. Not more than 25 ppm.

Determined by atomic absorption spectrophotometry (2.4.2).

NOTE — For the preparation of all aqueous solution and for the rinsing of glassware before use, use water that has been passed through a strong acid, strong base, mixed-bed ion exchange resin. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in container of borosilicate. Cleanse glassware before use by soaking in warm 8M Nitric acid for 30 minutes and by rinsing with deionized water.

Solution A. A 1.2 per cent v/v solution of *nitric acid* in *water*.

Reference solution (a). Dissolve 159.8 mg of *lead nitrate* in 100 ml of *water*, add 1 ml of *nitric acid* and dilute to 1000.0 ml with *water*. Transfer 10.0 ml of the solution to a 100-ml volumetric flask, add 40 ml of *water* and 5 ml of *nitric acid* and dilute to volume with *water*.

Reference solution (b). Transfer 0.4 ml reference solution (a) to a 100-ml volumetric flask. Add 50 ml *water* and 1 ml of *nitric acid* and dilute to volume with *water*. This solution contains 0.04 ppm of lead.

Test solution (a). Dissolve 4.0 g of the substance under examination in 50 ml of *water* and 5 ml of *nitric acid*, with the aid of ultrasound and dilute to 100.0 ml with *water*. Transfer 4.0 ml of the solution to a 100-ml volumetric flask, add 50 ml of *water* and 1 ml of *nitric acid* and dilute to volume with *water*.

Test solution (b). To 10.0 ml of test solution (a), add 10.0 ml of solution A. This solution contains 0.00 ppm of added lead from reference solution (b).

Test solution (c). To 10.0 ml of test solution (a), add 4.0 ml of reference solution (b) and 6.0 ml of solution A. This solution contains 0.008 ppm of added lead from reference solution (b).

Test solution (d). To 10.0 ml of test solution (a), add 7.0 ml of reference solution (b) and 3.0 ml of solution A. This solution contains 0.014 ppm of added lead from reference solution (b).

Test solution (e). To 10.0 ml of test solution (a), add 10.0 ml of reference solution (b). This solution contains 0.020 ppm of added lead from reference solution (b).

Chromatographic system

- lamp: lead hallow-cathode,
- wavelength: 283.3,
- graphite tubes temperature:

Temperature (°)	Time (seconds)
70	10
90	60
120	15
250 (no gas flow)	5
250	10
250 (no gas flow)	2
2000	3.2

- argon flow rate: 3 litre per minute,
- injection volume: 20 µl.

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Inject solution A, test solution (b), (c), (d) and (e). The graphite tube is temperature programmed to reach 2000° in about 2 minutes. When the temperature reaches 2000°, determine the absorbance at 283.3 nm, corrected for background absorption. Inject solution A (blank) and the test solution, determine the absorbance. Correct the absorbance value from the test solutions by subtracting from each the absorbance value from the solution (blank). Plot the corrected absorbance of the test solution versus their added lead concentrations in ppm. Intercept, determine the concentration, C, in ppm of lead in test solution (a).

Calculate the content of lead in copper gluconate using following expression

$$\text{Lead (ppm)} = \frac{CV}{W}$$

where,

C = Concentration of lead in test solution (b) ppm, determined from the intercept of linear regression line;

V = Volume of solvent taken to prepare test solution (b);

W = Weight of calcium gluconate used to prepare test solution (b) (g).

Chlorides (2.3.12). 0.36 g complies with the limit test for chlorides (700 ppm).

Sulphates (2.3.17). 0.3 g complies with the limit test for sulphates (500 ppm).

Reducing substances. Not more than 1.0 per cent.

Transfer 1g of the substance under examination to a 250-ml conical flask, add 10 ml of *water* to dissolve the sample then add 25 ml of *alkaline cupric citrate*, cover the flask and boil for 5 minutes, accurately timed and cool rapidly to room temperature. Add 25 ml of *0.6M acetic acid*, 10 ml of *0.05M iodine* and 10 ml of *3M hydrochloric acid* and titrate with *0.1 M sodium thiosulphate*, using 3 ml of *starch solution*, as indicator. Carry out a blank titration.

Calculate the percentage of reducing sugar (as dextrose), using following expression.

$$\text{Reducing sugar} = \frac{(V_B - V_A) \times M \times F \times 100}{W}$$

where,

V_B = Volume of *0.1M sodium thiosulphate* consumed by the blank;

V_A = Volume of *0.1M sodium thiosulphate* consumed by the sample;

M = Molarity of 0.1 M sodium thiosulphate;

F = Equivalency factor, 27 mg/mEq;

W = Weight of sample (mg).

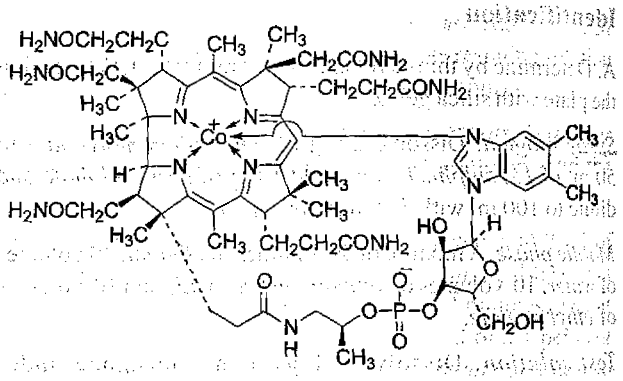
Assay. Dissolve 1.5 g in 100 ml of *water*, add 2 ml of *glacial acetic acid* and 5 g of *potassium iodide*, mix and titrate with *0.1 M sodium thiosulphate* to light yellow colour. Add 2 g of *ammonium thiocyanate*, mix. Add 3 ml of *starch solution*, and continue titrating to a milk white end point. Carry out a blank titration.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.04538 g of $C_{12}H_{22}CuO_{14}$.

Storage. Store protected from moisture.

Cyanocobalamin

Vitamin B_{12}



$C_{63}H_{88}CoN_{14}O_{14}P$ Mol. Wt. 1355.4

Cyanocobalamin is *Coα*-[α-(5,6-dimethylbenzimidazolyl)]-*Co*β-cyanocobamide.

Cyanocobalamin contains not less than 96.0 per cent and not more than 102.0 per cent of $C_{63}H_{88}CoN_{14}O_{14}P$, calculated on the dried basis.

Category. B-group Vitamin; haematopoietic.

Description. A dark red, crystalline powder; very hygroscopic.

Identification

A. When examined in the range 260 nm to 610 nm, a 0.0025 per cent w/v solution shows absorption maxima, at about 278 nm, 361 nm and 547 nm to 559 nm. The ratio of the absorbance at about 361 nm to that at about 547nm to 559 nm is 3.15 to 3.45 and the ratio of the absorbance at about 361 nm to that at about 278 nm is 1.70 to 1.90 (2.4.7).

B. Determine by thin-layer chromatography (2.4.17), protected form light and coating the plate with *silica gel G*.

Mobile phase. A mixture of 60 volumes of *chloroform*, 40 volumes of *methanol* and 12 volumes of *6 M ammonia*. Use an unlined tank.

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Test solution. Dissolve 20 mg of the substance under examination in 10.0 ml of *ethanol (50 per cent)*.

Reference solution. A 0.2 per cent w/v solution of *cyanocobalamin IPRS* in *ethanol (50 per cent)*.

Apply to the plate 10 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate in air and examine in day light. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

C. Mix about 1 mg with 10 mg of *potassium sulphate* and 0.1 ml of *0.5 M sulphuric acid* and heat carefully to redness. Allow to cool, add to the residue 0.1 ml of *water*, 0.5 ml of saturated solution of *ammonium thiocyanate* and 0.5 ml of *benzyl alcohol* and shake; a blue colour is formed and is taken into the benzyl alcohol layer.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

The following solutions should be used within 1 hour of preparation.

Test solution. Dissolve 10 mg of the substance under examination in 10.0 ml of the mobile phase.

Reference solution (a). A 0.003 per cent w/v solution of the substance under examination in the mobile phase.

Reference solution (b). A 0.0001 per cent w/v solution of the substance under examination in the mobile phase.

Reference solution (c). Dissolve 5 mg of the substance under examination in 2 ml of *water*, warming if necessary, allow to cool, add 1 ml of a 0.1 per cent w/v solution of *chloramine T* and 0.1 ml of *0.05 M hydrochloric acid*, dilute to 5 ml with *water*, shake and allow to stand for 5 minutes. Dilute 1 ml of the solution to 10 ml with the mobile phase. Use immediately.

Chromatographic system
- a stainless steel column 25 cm x 4 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 147 volumes of a 1.0 per cent w/v solution of *disodium hydrogen phosphate* and 53 volumes of *methanol* adjusted to pH 3.5 with *orthophosphoric acid* (to be used within 2 days of preparation),
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 361 nm,
- injection volume: 20 µl.

Inject reference solution (c). The test is not valid unless the resolution between two principal peaks is not less than 2.5.

Inject reference solutions (a), (b) and the test solution. Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution the sum of the areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a). Ignore any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 20.0 mg by drying in an oven at 105° at a pressure of 1.5 to 2.5 kPa for 2 hours.

Assay. Weigh accurately about 25 mg and dissolve in sufficient *water* to produce 1000.0 ml. Measure the absorbance of the solution at the maximum at about 361 nm (2.4.7). Calculate the content of $C_{63}H_{88}CoN_{14}O_{14}P$ taking 207 as the specific absorbance at 361 nm.

Storage. Store protected from light and moisture.

Cyanocobalamin Injection

Vitamin B_{12} Injection

Cyanocobalamin Injection is a sterile solution of Cyanocobalamin in Water for Injections containing sufficient *acetic acid* or *hydrochloric acid* to adjusted to pH about 4. It may contain suitable buffering agents.

Cyanocobalamin Injection contains not less than 95.0 per cent and not more than 110.0 per cent of the stated amount of anhydrous cyanocobalamin, $C_{63}H_{88}CoN_{14}O_{14}P$.

Usual strength. The equivalent of 500 µg and 1 mg of anhydrous cyanocobalamin per ml.

Identification

When examined in the range 260 nm to 610 nm, a 0.0025 per cent w/v solution shows absorption maxima at about 278 nm, 361 nm and 547 nm to 559 nm. The ratio of the absorbance at about 361 nm to that at about 547 nm to 559 nm is 3.15 to 3.45 and the ratio of the absorbance at about 361 nm to that at about 278 nm is 1.70 to 1.90 (2.4.7).

Tests

pH (2.4.24). 3.8 to 5.5.

Related substances. Determine by liquid chromatography (2.4.14).

NOTE— The following solutions should be used within 1 hour of preparation.

Test solution (a). Dilute a suitable volume of the injection, if necessary, with the mobile phase to produce a solution containing 0.0001 per cent w/v of Cyanocobalamin.

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Test solution (b). Dilute a suitable volume of the injection, if necessary, with the mobile phase to produce a solution containing 0.05 per cent w/v of Cyanocobalamin.

Reference solution (a). Dilute a suitable volume of the injection, if necessary, with the mobile phase to produce a solution containing 0.003 per cent w/v of Cyanocobalamin.

Reference solution (b). Add 1 ml of a 0.1 per cent w/v solution of *chloramine T* and 0.1 ml of *0.05 M hydrochloric acid* to a volume containing 5 mg of Cyanocobalamin, dilute to 10 ml with *water*, shake and allow to stand for 5 minutes. Dilute 2 ml of the solution to 10 ml with the mobile phase and use immediately.

Chromatographic system

- a stainless steel column 25 cm x 4 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 147 volumes of a 1.0 per cent w/v solution of *disodium hydrogen phosphate* and 53 volumes of *methanol*, adjusted to pH 3.5 with *orthophosphoric acid* (to be used within 2 days of preparation),
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 361 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the resolution between two principal peaks is not less than 2.5.

Inject reference solution (a), test solutions (a) and (b). Run the chromatogram 3 times the retention time of the principal peak. In the chromatogram obtained with test solution (b), the sum of the areas of all the secondary peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (a). Ignore any peak with an area less than that of the principal peak in the chromatogram obtained with test solution (a).

Bacterial endotoxins (2.2.3). Not more than 0.4 Endotoxin Unit per µg of cyanocobalamin.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

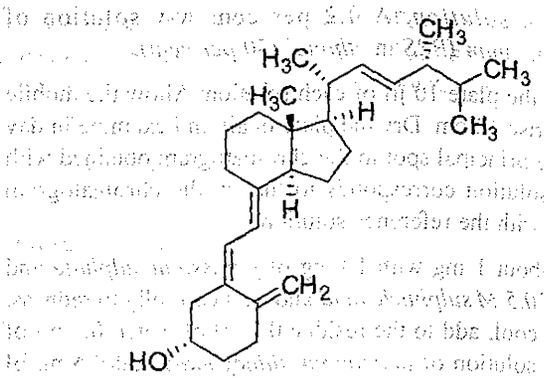
Assay. *Carry out the following procedure protected from light.*

Dilute the injection, if necessary, with *water* to produce a solution containing not more than the equivalent of 0.0025 per cent w/v of anhydrous cyanocobalamin and measure the absorbance at the maximum at about 361 nm (2.4.7). Calculate the content of $C_{63}H_{88}CoN_{14}O_{14}P$ taking 207 as the specific absorbance at 361 nm.

Storage. Store protected from light in single dose or multiple dose containers.

Labelling. The label states the strength in terms of the equivalent amount of anhydrous cyanocobalamin in a suitable dose-volume.

Ergocalciferol



$C_{28}H_{44}O$ Mol. Wt. 396.7

Ergocalciferol is (3β,5*Z*,7*E*,22*E*)-9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol.

Category. Vitamin.

When vitamin D_2 is prescribed or demanded, Ergocalciferol shall be dispensed or supplied. When calciferol or vitamin D is prescribed or demanded, Ergocalciferol or Cholecalciferol shall be dispensed or supplied.

Ergocalciferol contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{28}H_{44}O$.

1 mg of ergocalciferol is equivalent to 40 000 IU of antirachitic activity (vitamin D) in rats.

Description. A white or slightly yellowish, crystalline powder or white or almost white crystals.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *ergocalciferol IPRS* or with the reference spectrum of ergocalciferol.

Tests

Specific optical rotation (2.4.22). + 103.0° to + 107.0°, determined in a 0.8 per cent w/v solution in *aldehyde free ethanol* determined within 30 minutes of preparing the solution.

Reducing substances. Dissolve 0.1 g in *aldehyde-free ethanol* and dilute to 10.0 ml with the same solvent. Add 0.5 ml of a 0.5 per cent w/v solution of *tetrazolium blue* in *aldehyde-free ethanol* and 0.5 ml of *tetramethylammonium hydroxide solution (10 per cent)*. Allow to stand for 5 minutes and add 1.0 ml of *glacial acetic acid*. Prepare a reference solution at the same time and in the same manner using 10.0 ml of a solution containing 0.2 µg/ml of *hydroquinone* in *aldehyde-free ethanol*. Measure the absorbance of the two solutions at

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525 nm (2.4.7) using as the compensation liquid 10.0 ml of *aldehyde-free ethanol* treated in the same manner. The absorbance of the test solution is not more than that of the reference solution (20 ppm).

Ergosterol. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

NOTE—Carry out the procedure protected from light.

Solvent mixture. A solution containing 1.0 per cent w/v of *squalane* and 0.01 per cent w/v of *butylhydroxytoluene* in *ethylene chloride*.

Mobile phase. Equal volumes of *cyclohexane* and *peroxide-free ether*.

Test solution. Dissolve 0.25 g of the substance under examination in 5.0 ml of the solvent mixture.

Reference solution (a). Dissolve 0.1 g of *ergocalciferol IPRS* in 2.0 ml of the solvent mixture.

Reference solution (b). Dissolve 5 mg of *ergosterol IPRS* in 50.0 ml of the solvent mixture.

Reference solution (c). Mix equal volumes of reference solution (a) and reference solution (b).

Apply to the plate 10 µl of each solution. Immediately allow the mobile phase to rise 15 cm protected from light. Dry the plate in air and spray three times with *antimony trichloride solution*. Examine the chromatograms for 4 minutes after spraying. The principal spot in the chromatogram obtained with the test solution is initially orange-yellow and then becomes brown. In the chromatogram obtained with the test solution, any slowly appearing violet spot (corresponding to ergosterol) immediately below the principal spot is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). There is no spot in the chromatogram obtained with the test solution that does not correspond to one of the spots in the chromatograms obtained with reference solutions (a) and (b). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Assay. Determine by liquid chromatography (2.4.14).

NOTE—Carry out the procedure protected from light.

Test solution. Dissolve 10 mg of the substance under examination without heating in 10.0 ml of *toluene* and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 10 mg of *ergocalciferol IPRS* without heating in 10.0 ml of *toluene* and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of *cholecalciferol for system suitability IPRS* to 5.0 ml with the mobile phase. Heat in a water-bath at 90° under a reflux condenser for 45 minutes and cool.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with silica (5 µm),
- mobile phase: a mixture of 3 volumes of *pentanol* and 997 volumes of *hexane*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention time with reference to cholecalciferol for pre-cholecalciferol is 0.4 and for *trans*-cholecalciferol is 0.5.

Inject reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. The test is not valid unless the relative standard deviation for replicate injections is not more than 1.0 per cent and the resolution between the peaks due to pre-cholecalciferol and *trans*-cholecalciferol is not less than 1.0.

Inject reference solution (a) and the test solution.

Calculate the content of ergocalciferol from the expression:

$$\frac{m'}{m} \times \frac{S_D}{S'_D} \times 100$$

where,

m is mass of the substance under examination in the test solution, in mg;

m' is mass of *ergocalciferol IPRS* in reference solution (a) in mg;

S_D is area (or height) of the peak due to ergocalciferol in the chromatogram obtained with the test solution;

S'_D is area (or height) of the peak due to ergocalciferol in the chromatogram obtained with reference solution (a).

Storage. Store protected from light and moisture, under nitrogen, at a temperature between 2° to 8°.

Ergocalciferol Tablets

Ergocalciferol Tablets contain not less than 90.0 per cent and not more than 125.0 per cent of the stated amount of ergocalciferol, $C_{28}H_{44}O$.

Usual strength. 1.25 mg (50,000 IU).

Identification

A. In the test of Uniformity of content, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

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B. Extract one tablet, with 5 ml of *dichloromethane* and filter. To 1 ml of the filtrate, add 9 ml of *antimony trichloride solution*. A brownish red colour is produced.

Tests

Uniformity of content. Complies with the test stated under Tablets.

NOTE—Carry out the procedure protected from light.

Determine by liquid chromatography (2.4.14).

For tablets containing more than 0.25 mg of ergocalciferol—

Test solution. Disperse one tablet in 4 ml of *water* in amber flask with the aid of ultrasound. Add 12 ml of *dimethyl sulphoxide*, mix, extract with 100 ml of *hexane* by shaking for 30 minutes, centrifuge the hexane layer and use the clear supernatant liquid.

For tablets containing 0.25 mg or less of ergocalciferol—

Test solution. Add 2 ml of *water* to one tablet in an amber flask and disperse with the aid of ultrasound. Add 6 ml of *dimethyl sulphoxide*, mix, extract with 25 ml of *hexane* by shaking for 30 minutes, centrifuge the hexane layer and use the clear supernatant liquid.

Reference solution (a). A 0.001 per cent w/v solution of *ergocalciferol IPRS* in *hexane*.

Reference solution (b). Dissolve 0.5 g of *cholecalciferol for performance test IPRS* in 2 ml of *toluene* and dilute to 10.0 ml with the mobile phase, heat under a reflux condenser in a water-bath at 90° for 45 minutes and cool.

Chromatographic system

- a stainless steel column 20 cm × 4.6 mm, packed with silica (5 µm) (Such as Partisil),
- mobile phase: a mixture of 8 volumes of *pentan-1-ol* and 992 volumes of *hexane*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

The relative retention time with reference to cholecalciferol for precholecalciferol is about 0.4 and for *trans*-cholecalciferol is about 0.5.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to precholecalciferol and *trans*-cholecalciferol is not less than 1.0.

Inject reference solution (a) and the test solution.

Calculate the content of ergocalciferol, $C_{28}H_{44}O$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

NOTE—Carry out the procedure protected from light.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 1.25 mg of Ergocalciferol in 4 ml of *water* in amber flask with the aid of ultrasound. Add 12 ml of *dimethyl sulphoxide*, mix, extract with 100 ml of *hexane* by shaking for 30 minutes, centrifuge the hexane layer and use the clear supernatant liquid.

Reference solution (a). A 0.001 per cent w/v solution of *ergocalciferol IPRS* in *hexane*.

Reference solution (b). Dissolve 0.5 g of *cholecalciferol for performance test IPRS* in 2 ml of *toluene* and dilute to 10.0 ml with the mobile phase, heat under a reflux condenser in a water-bath at 90° for 45 minutes and cool.

Use Chromatographic system as described under test for Uniformity of content.

The relative retention time with reference to cholecalciferol for precholecalciferol is about 0.4 and for *trans*-cholecalciferol is about 0.5.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to precholecalciferol and *trans*-cholecalciferol is not less than 1.0.

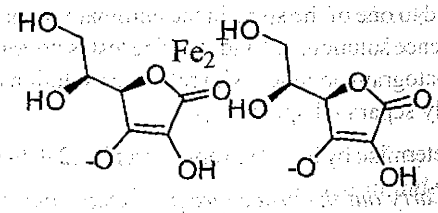
Inject reference solution (a) and the test solution.

Calculate the content of ergocalciferol, $C_{28}H_{44}O$ in the tablet.

Labelling. The label states the equivalent number of IU (units) of antirachitic activity (vitamin D) per tablet.

Each µg of ergocalciferol is equivalent to 40 IU of antirachitic activity (vitamin D).

Ferrous Ascorbate



$C_{12}H_{14}FeO_{12}$ Mol. Wt. 406.1

Ferrous Ascorbate is (2R)-2-[(1S)-1,2-dihydroxyethyl]-4,5dihydroxyfuran-3-one;iron (2+).

Ferrous Ascorbate contains not less than 13.5 per cent and not more than 15.5 per cent of ferrous iron and not less than 78.0 per cent and not more than 87.0 per cent of ascorbic acid, calculated on the dried basis.

Category. Haematinic.

Description. A fine, dark violet coloured dried powder.

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Identification

A. Dissolve 100 mg of the substance under examination in 10 ml of *water*, add 1 ml of *0.05 M sulphuric acid*, 1 ml of *2,6-dichlorophenolindophenol solution* and 1 ml of *dinitrophenyl hydrazine solution*. Mix the solutions and keep in boiling water-bath for 10 minutes. The solution gives orange-red colour.

B. It gives reaction (A) of ferrous salts (2.3.1).

Tests

Appearance of solution. A 1.0 per cent w/v solution in *1 M hydrochloric acid* is clear (2.4.1) and not more intensely coloured than reference solution BYS5 (2.4.1).

pH (2.4.24). 5.5 to 7.0, determined in a 1.0 per cent w/v solution.

Sulphated ash (2.3.18). 19.0 per cent to 25.0 per cent.

Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay.

Ferrous Iron —

Test solution. Dissolve 0.2 g of the substance under examination in 40 ml of *water*, add 8 ml of *glacial acetic acid* and dilute to 100.0 ml with *water*. Dilute 10.0 ml of the solution to 100.0 ml with *water*.

Reference solution. Dissolve 0.2 g of *ferrous ammonium sulphate* in 40 ml of *water*, add 8 ml of *glacial acetic acid* and dilute to 100.0 ml with *water*. Dilute 10.0 ml of the solution to 100.0 ml with *water*.

Transfer 5.0 ml of the reference solution and the test solution separately into 100-ml volumetric flask, add 20 ml of *water* and 3.0 ml of 0.15 per cent w/v of *1,10-phenanthroline*. Mix well and dilute to volume with *water*. Measure the absorbance after 1 hour at 515 nm (2.4.7). Calculate the content of ferrous iron.

Ascorbic acid —

Weigh 0.15 g of the substance under examination, disperse in 25.0 ml of *0.2 M disodium edetate solution*, 1.0 ml of 20 per cent *w/v citric acid solution*, and add 0.5 ml *starch solution* as indicator. Titrate immediately with *0.05 M iodine* until a persistent blue colour is obtained.

1 ml of *0.05 M iodine* is equivalent to 0.008806 g of $C_6H_8O_6$.

Storage. Store protected from light and moisture.

Ferrous Ascorbate and Folic Acid Suspension

Ferrous Ascorbate and Folic Acid Suspension contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amounts of ferrous ascorbate, $C_{12}H_{16}FeO_{12}$, equivalent to elemental iron and not less than 90.0 per cent of folic acid, $C_{19}H_{19}N_7O_6$.

Usual strength. Ferrous ascorbate equivalent to 30 mg of elemental iron and 550 mcg folic acid per 5 ml.

Identification

A. In the Assay of folic acid, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Dissolve 100 mg of the suspension in 10 ml of *water*, add 1 ml of *0.05 M sulphuric acid*, 1 ml of *2,6-dichlorophenolindophenol solution* and 1 ml of *dinitrophenylhydrazine solution*. Mix the solutions and keep in boiling water-bath for 10 minutes. The solution gives orange-red colour.

C. It gives reaction (A) of ferrous salts (2.3.1).

Tests

pH (2.4.24). 3.5 to 7.0.

Other tests. Comply with the tests stated under Oral Liquids.

Assay.

For Ferrous Iron —

Test solution. Weigh a quantity of the suspension containing 30 mg of elemental iron and disperse in sufficient amount of *water*, add 8 ml of *acetic acid* and dilute to 100.0 ml with *water*. Dilute 10.0 ml of the solution to 100.0 ml with *water*.

Reference solution. Dissolve 250 mg of *ferrous ammonium sulphate* in sufficient amount of *water*, add 8 ml of *acetic acid* and dilute to 100.0 ml with *water*. Dilute 10.0 ml of the solution to 100.0 ml with *water*.

Transfer 5.0 ml of the reference solution and the test solution separately into 100-ml volumetric flask, add 20 ml of *water* and 3.0 ml of 0.15 per cent w/v solution of *1,10-phenanthroline*. Mix well and dilute to volume with *water*. Measure the absorbance after 1 hour at 515 nm (2.4.7). Calculate the content of ferrous iron.

For Folic Acid —

Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of 1.36 per cent w/v solution of *sodium acetate trihydrate*, adjusted to pH 6.5 with *glacial acetic acid* and 20 volumes of *acetonitrile*.

Buffer solution. A solution prepared by dissolving 13.6 g of *sodium acetate trihydrate* in 1000 ml of *water*, adjusted to pH 6.5 with *glacial acetic acid*.

Test solution. Dissolve a quantity of oral suspension containing 1.0 mg of Folic acid in 20 ml of *0.1 M sodium*

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hydroxide with the aid of ultrasound and dilute to 200.0 ml with the solvent mixture.

Reference solution. Dissolve 30.0 mg of *folic acid IPRS* in 10 ml of *0.1 M sodium hydroxide* with the aid of ultrasound and dilute to 100.0 ml with the solvent mixture. Dilute 2.0 ml of the solution and 10 ml of *0.1 M sodium hydroxide* to 100.0 ml with the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm), (Such as Inertsil ODS)
- sample temperature: 5°,
- mobile phase: A. the buffer solution,
  B. a mixture of 75 volumes of *acetonitrile* and 25 volumes of the buffer solution,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 283 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	100	0
15	70	30
20	30	70
25	100	0
30	100	0

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0.

Inject the reference solution and the test solution.

Determine the weight per ml of the oral suspension (2.4.29) and calculate the content of $C_{19}H_{19}N_7O_6$.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 100 CFU per ml and total fungal count is not more than 10 CFU per ml. 1 ml is free from *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. 10 ml is free from *Salmonella*.

Storage. Store at a temperature not exceeding 30°.

Labelling. The label states, the strength in terms of ferrous ascorbate equivalent to elemental iron and folic acid.

Ferrous Ascorbate and Folic Acid Tablets

Ferrous Ascorbate and Folic Acid Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ferrous ascorbate, $C_{12}H_{16}FeO_{12}$, equivalent to elemental iron and not less than 90.0 per cent of folic acid, $C_{19}H_{19}N_7O_6$.

Usual strength. Ferrous ascorbate equivalent to 100 mg of elemental iron and 1.5 mg folic acid.

Identification

A. In the Assay of folic acid, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Dissolve 100 mg of powdered tablets in 10 ml of *water*, add 1 ml of *0.05 M sulphuric acid*, 1 ml of *2,6- dichlorophenolindophenol solution* and 1 ml of *dinitrophenylhydrazine solution*. Mix the solutions and keep in boiling water-bath for 10 minutes. The solution gives orange-red colour.

C. It gives reaction (A) of ferrous salts (2.3.1).

Tests

Uniformity of Content. Complies with the test stated under Tablets.

For Folic Acid —

Determine by liquid chromatography (2.4.14) as described under Assay with the following modification.

Test solution. Disperse one tablet in solvent mixture 1 and dilute suitably to obtain a solution containing 0.0004 per cent w/v of Folic acid in solvent mixture 2 and filter.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{19}N_7O_6$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay.

For Ferrous Iron —

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing 75 mg of elemental iron in sufficient amount of *water* with the aid of ultrasound for about 20 minutes, add 8 ml of *acetic acid* and dilute to 250.0 ml with *water*. Dilute 10.0 ml the solution to 100.0 ml with *water*.

Reference solution. Dissolve 250 mg of *ferrous ammonium sulphate* in sufficient amount of *water* with the aid of ultrasound for about 20 minutes, add 8 ml of *acetic acid* and dilute to 100.0 ml with *water*. Dilute 10.0 ml of the solution to 100.0 ml with *water*.

Transfer 5.0 ml of the reference solution and the test solution separately into 100-ml volumetric flask, add 20 ml of *water* and 3.0 ml of 0.15 per cent w/v solution of *1,10-phenanthroline*. Mix well and dilute to volume with *water*. Measure the absorbance after 1 hour at 515 nm (2.4.7). Calculate the content of ferrous iron.

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For Folic Acid —

Solvent mixture 1. 80 volumes of 0.57 per cent w/v of *dipotassium hydrogen orthophosphate* and 13.5 volumes of *methanol*.

Solvent mixture 2. 90 volumes of a solution containing 1.075 per cent w/v of *sodium perchlorate monohydrate* and 0.075 per cent w/v of *dipotassium hydrogen orthophosphate* and 10 volumes of *methanol*.

Test solution. Weigh and powder 20 tablets. Weigh a quantity of the powder containing 4 mg of folic acid, disperse in 180 ml of solvent mixture 1 with the aid of ultrasound and dilute to 250.0 ml with the same solvent. Centrifuge the solution at 3000 rpm. Dilute 5.0 ml of the supernatant solution to 20.0 ml with solvent mixture 2.

Reference solution. A 0.0175 per cent w/v solution of *folic acid IPRS* in solvent mixture 1. Dilute 5.0 ml of the solution to 50.0 ml with solvent mixture 1. Further dilute 5.0 ml of the solution to 20.0 ml with solvent mixture 2.

Chromatographic system

- stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as YMC triart),
- sample temperature: 5°,
- mobile phase: a mixture of 93 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen orthophosphate* in 1000 ml of *water* and 7 volumes of *acetonitrile*, adjusted to pH 6.0 with *5 M sodium hydroxide*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 283 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the column efficiency is not less than 2000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injection is not more than 2.0 per cent.

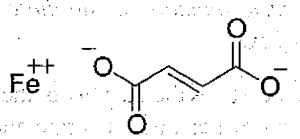
Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{19}N_7O_6$ in the tablet.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. The label states, the strength in terms of ferrous ascorbate equivalent to elemental iron and folic acid.

Ferrous Fumarate



$C_4H_2FeO_4$ Mol. Wt. 169.9

Ferrous Fumarate contains not less than 93.0 per cent and not more than 101.0 per cent of $C_4H_2FeO_4$, calculated on the dried basis.

Category. Haematinic.

Description. A reddish orange to reddish brown, fine powder; may contain soft lumps that produce a yellow streak when crushed.

Identification

A. Heat 1 g with 25 ml of a mixture of equal volumes of *hydrochloric acid* and *water* on a water-bath for 15 minutes, cool and filter. Reserve the precipitate for test B; the filtrate gives reaction A of ferrous salts (2.3.1).

B. Wash the precipitate reserved in test A with a mixture of 1 volume of *dilute hydrochloric acid* and 9 volumes of *water* and dry at 105°. Suspend 0.1 g of the residue in 2 ml of *sodium carbonate solution* and add *dilute potassium permanganate solution* dropwise; the permanganate is decolorised and a brownish solution is obtained.

C. Mix 0.5 g with 1 g of *resorcinol*. To 0.5 g of the mixture in a crucible, add a few drops of *sulphuric acid* and heat gently; a deep red semi-solid mass is formed. Add the mass to a large volume of *water*; an orange-yellow solution without any fluorescence is obtained.

Tests

Arsenic (2.3.10). Mix 2.0 g with 1.5 g of *anhydrous sodium carbonate*, add 10 ml *bromine water* and mix thoroughly. Evaporate to dryness on a water-bath, ignite gently and dissolve the cooled residue in 20 ml of *brominated hydrochloric acid* and 10 ml of *water*. Transfer to a small flask, add sufficient *stannous chloride solution AsT* to remove the yellow colour, connect to a condenser and distill 22 ml. The distillate complies with the limit test for arsenic (5 ppm).

Heavy metals. Not more than 20 ppm, determined by the following method. Ignite 1.0 g gently until free from carbon, dissolve in 5 ml of *hydrochloric acid* by heating on a water-bath and evaporate to dryness. Dissolve the residue in a mixture of 15 ml of *hydrochloric acid*, 4 ml of *nitric acid* and 6 ml of *water*. Boil gently for 1 minute, cool and extract with three quantities, each of 20 ml, of *ether*. If the aqueous layer is more than slightly yellow, extract with a fourth quantity of 20 ml of *ether* and reject the ether extracts, heat the aqueous solution gently to remove the dissolved ether, add 1 g of *citric acid*, make alkaline with *5 M ammonia* and add 1 ml of *potassium cyanide solution*. Dilute to 50 ml with *water* and add 0.1 ml of *sodium sulphide solution*. Any brown colour produced is not more intense than that produced by treating 1.0 ml of *lead standard solution (20 ppm Pb)* in a similar manner.

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Sulphates (2.3.17). Boil 0.15 g with 10 ml of *2 M hydrochloric acid* and 20 ml of freshly boiled and cooled *water*, cool in ice and filter; the filtrate complies with the limit test for sulphates (0.1 per cent).

Ferric iron. Not more than 2.0 per cent, determined by the following method. Weigh 3.0 g and dissolve in a mixture of 200 ml of *water* and 20 ml of *hydrochloric acid* by heating rapidly to boiling point. Boil for 15 seconds, cool rapidly, add 3 g of *potassium iodide*, close the flask, allow to stand in the dark for 15 minutes and titrate the liberated iodine with *0.1 M sodium thiosulphate* using *starch solution*, added towards the end of the titration, as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of iodine liberated by the ferric iron.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.005585 g of ferric iron.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Weigh 0.3 g and dissolve in 15 ml of *dilute sulphuric acid* with the aid of gentle heat. Cool, add 50 ml of *water* and immediately titrate with *0.1 M ceric ammonium sulphate* using *ferroin sulphate solution* as indicator.

1 ml of *0.1 M ceric ammonium sulphate* is equivalent to 0.01699 g of $C_4H_2FeO_4$.

Ferrous Fumarate Tablets

Ferrous Fumarate Tablets contain not less than 90.0 per cent and not more than 105.0 per cent of the stated amount of ferrous fumarate, $C_4H_2FeO_4$.

Usual strength. 200 mg, (200 mg of ferrous fumarate is approximately equivalent to 65 mg of ferrous iron).

Identification

The powdered tablets comply with the following tests.

A. Heat 1 g with 25 ml of a mixture of equal volumes of *hydrochloric acid* and *water* on a water-bath for 15 minutes, cool and filter. Reserve the precipitate for test B; the filtrate gives reaction A of ferrous salts (2.3.1).

B. Wash the precipitate reserved in test A with a mixture of 1 volume of *dilute hydrochloric acid* and 9 volumes of *water* and dry at 105°. Suspend 0.1 g of the residue in 2 ml of *sodium carbonate solution* and add *dilute potassium permanganate solution* dropwise; the permanganate is decolorised and a brownish solution is obtained.

C. Mix 0.5 g with 1 g of *resorcinol*. To 0.5 g of the mixture in a crucible, add a few drops of *sulphuric acid* and heat gently; a deep red semi-solid mass is formed. Add the mass to a large volume of *water*; an orange-yellow solution without any fluorescence is obtained.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *0.1 M hydrochloric acid*,
Speed and time. 75 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter. Titrate 100.0 ml of the filtrate with *0.01M ceric ammonium sulphate* using *ferroin solution* as indicator.

1 ml of *0.01 M ceric ammonium sulphate* is equivalent to 0.0005585 g of ferrous iron.

Q. Not less than 75 per cent of the stated amount of ferrous iron.

Ferric iron. Weigh a quantity of the powder prepared for the Assay, containing about 1.5 g of Ferrous Fumarate, in a stoppered flask, dissolve as completely as possible with the aid of heat in a mixture of 100 ml of freshly boiled and cooled *water* and 10 ml of *hydrochloric acid*, boil for 15 seconds, cool rapidly, add 3 g of *potassium iodide*, close the flask and allow to stand in the dark for 15 minutes. Titrate the liberated iodine with *0.1 M sodium thiosulphate* using *starch solution*, added towards the end of the titration, as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of iodine liberated by the ferric iron. Not more than 13.5 ml of *0.1 M sodium thiosulphate* is required.

Disintegration (2.5.1). Not more than 60 minutes.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 0.3 g of Ferrous Fumarate in 15 ml of *dilute sulphuric acid* with the aid of gentle heat. Cool, add 50 ml of *water* and immediately titrate with *0.1 M ceric ammonium sulphate* using *ferroin sulphate solution* as indicator.

1 ml of *0.1 M ceric ammonium sulphate* is equivalent to 0.01699 g of $C_4H_2FeO_4$.

Labelling. The label states the quantity of the active ingredient both as the amount of Ferrous Fumarate and in terms of the equivalent amount of ferrous iron in each tablet.

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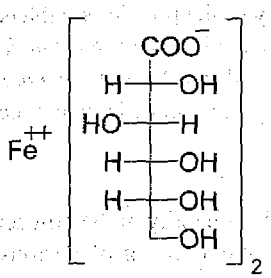
Ferrous Gluconate



$C_{12}H_{22}FeO_{14},xH_2O$ Mol. Wt. 446.1 (anhydrous)

Ferrous Gluconate is ferrous di(D-gluconate).

Ferrous Gluconate contains not less than 95.0 per cent and not more than 102.0 per cent of $C_{12}H_{22}FeO_{14}$, calculated on the dried basis.

Category. Haematinic.

Description. A yellowish grey or pale greenish-yellow, fine powder or granules.

Identification

A. Dissolve 5 g in *carbon dioxide-free water* at 60°, cool and dilute to 50 ml with *water*. 1 ml of the resulting solution gives reaction A of ferrous salts (2.3.1).

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *Silica gel G*.

Mobile phase. A mixture of 10 volumes of *concentrated ammonia*, 10 volumes of *ethyl acetate*, 30 volumes of *water* and 50 volumes of *ethanol (95 per cent)*.

Test solution. Dissolve 20 mg of the substance under examination in 2.0 ml of *water*, heating if necessary in a water-bath at 60°.

Reference solution. Dissolve 20 mg of *ferrous gluconate IPRS* in 2.0 ml of *water*, heating if necessary in a water-bath at 60°.

Apply to the plate 5µl of each solution. After development, dry the plate at 105° for 20 minutes and spray with 5 per cent w/v solution of *potassium dichromate* in a 40 per cent w/v solution of *sulphuric acid*. The principle spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Appearance of solution. Dissolve 5.0 g in *carbon dioxide-free water* at 60°, cool and dilute to 50 ml with the same solvent (solution A). Dilute 2 ml of solution A to 10 ml with *water*. When examined against the light, the resulting solution is clear (2.4.1).

pH (2.4.24). 4.0 to 5.5, determined in solution A, 3 to 4 hours after preparation.

Arsenic (2.3.10). To 5.0 g add 15 ml of *water* and 15 ml of *stannated hydrochloric acid*, distill 22 ml and add to the distillate 40 ml of *water* and 0.2 ml of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). Warm 2.0 g gently with 10 ml of *nitric acid* until reaction begins and allow to stand until the evolution of nitrous fumes subsides. Boil gently to complete oxidation, adding a further 5 ml of *nitric acid*, if necessary, and continue boiling until the volume is reduced to about 5 ml. Add 20 ml of *hydrochloric acid*, boil gently for 1 minute, cool and extract with three quantities, each of 20 ml, of *ether*. If the acid solution is still more than faintly yellow, extract with a fourth quantity of 20 ml of *ether* and discard the ether extracts. Transfer the acid solution to a narrow-necked flask, rinse the separator with 5 ml of *water*, and add the rinsings to the flask. Heat to remove the dissolved ether and part of the *hydrochloric acid*. Cool and dilute to 50 ml with *water*. 25 ml of the resulting solution complies with the limit test for heavy metals, Method A (20 ppm).

Chlorides (2.3.12). 0.4 g complies with the limit test for chlorides (625 ppm).

Sulphates (2.3.17). 0.3 g complies with the limit test for sulphates (500 ppm).

Barium. Dissolve 0.1 g in 50 ml of *distilled water*, and 5 ml of *dilute sulphuric acid*, and allow to stand for 5 minutes. The solution is not more opalescent than a mixture of 10 ml of solution A and 45 ml of *distilled water*, when examined against the light.

Ferric iron. Not more than 1.0 per cent, determined by the following method. Weigh 5.0 g, transfer to a glass-stoppered flask and dissolve in a mixture of 100 ml of freshly boiled and cooled *water* and 10 ml of *hydrochloric acid*. Add 3 g of *potassium iodide*, shake well and allow to stand in the dark for 5 minutes. Titrate the liberated iodine with *0.1 M sodium thiosulphate* using *starch solution*, added towards the end of the titration, as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of iodine liberated by the ferric iron.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.005585 g of ferric iron.

Oxalic acid. Dissolve 1 g in 5 ml of *water*, add 2 ml of *hydrochloric acid* and transfer to a separator. Extract with two quantities, each of 20 ml, of *ether*. Evaporate the combined ether extracts to dryness on a water-bath and dissolve the residue in 5 ml of *water*. Add 0.05 ml of *acetic acid* and 3 ml of *calcium chloride solution*; no turbidity is produced.

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Reducing sugars. Dissolve 0.5 g in 10 ml of *water* and make alkaline with *dilute ammonia solution*. Pass *hydrogen sulphide* into the solution and allow to stand for 30 minutes. Filter and wash the precipitate with two quantities, each of 5 ml, of *water*. Combine the filtrate and the washings and acidify with *dilute hydrochloric acid*. Add 2 ml of *dilute hydrochloric acid* in excess. Boil the solution until the vapours no longer darken *lead acetate paper* and, if necessary, boil further to concentrate the solution to about 10 ml. Cool and add 10 ml of *sodium carbonate solution*, set aside for 5 minutes, filter and dilute the filtrate to 100 ml with *water*. To 5 ml of the filtrate add 2 ml of *potassium cupri-tartrate solution* and boil for 1 minute; no red precipitate is formed.

Loss on drying (2.4.19). 5.0 per cent to 10.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 10^3 CFU per g determined by plate count.

Assay. Dissolve 0.5 g of *sodium bicarbonate* in a mixture of 70 ml of *water* and 30 ml of *1 M sulphuric acid*. When effervescence ceases, add about 1.0 g, accurately weighed, of the substance under examination, shake gently to dissolve and titrate with *0.1 M ceric ammonium nitrate*, using 0.1 ml of *ferroin solution* as indicator, until the red colour disappears.

1 ml of *0.1 M ceric ammonium nitrate* is equivalent to 0.04461 g of $C_{12}H_{22}FeO_{14}$.

Storage. Store protected from light.

Ferrous Gluconate Tablets

Ferrous Gluconate Tablets contain not less than 90.0 per cent and not more than 105.0 per cent of the stated amount of ferrous gluconate, $C_{12}H_{22}FeO_{14},2H_2O$.

Usual strength. 300 mg. (300 mg of ferrous gluconate is approximately equivalent to 35 mg of ferrous iron).

Identification

Dissolve a quantity of the powdered tablets containing about 1 g of Ferrous Gluconate in 10 ml of *water* and filter; the filtrate complies with the following tests.

A. 1 ml of the filtrate gives reaction (A) of ferrous salts (2.3.1).

B. To 7.5 ml of the filtrate add 1 ml of *glacial acetic acid* and 1 ml of freshly distilled *phenylhydrazine*. Heat the mixture on a water-bath for 30 minutes. Cool and scratch the inner surface of the test tube with a glass rod until crystals of gluconic acid phenylhydrazide begin to form. Set aside for 10 minutes, filter, dissolve the precipitate in hot *water*, mix a small amount of *decolorising charcoal* and filter into a test tube. Allow the filtrate to cool, and scratch the inner surface of the test tube; white crystals are obtained which melt at about 202°, with decomposition (2.4.21).

C. Shake a quantity of the powdered tablets containing 0.5 g of Ferrous Gluconate with 10 ml of *dilute hydrochloric acid*, filter and add to the filtrate 1 ml of *barium chloride solution*; an opalescence may be produced but no precipitate is formed.

Tests

Ferric iron. Weigh a quantity of the powder prepared for the Assay, containing about 5.0 g of Ferrous Gluconate, in a stoppered flask, dissolve as completely as possible without the aid of heat in a mixture of 100 ml of freshly boiled and cooled *water* and 10 ml of *hydrochloric acid*, add 3 g of *potassium iodide*, close the flask and allow to stand in the dark for 5 minutes. Titrate the liberated iodine with *0.1 M sodium thiosulphate* using *starch solution*, added towards the end of the titration, as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of iodine liberated by the ferric iron. Not more than 11.2 ml of *0.1 M sodium thiosulphate* is required.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing 1.0 g of Ferrous Gluconate in a mixture of 140 ml of *water* and 60 ml of *1 M sulphuric acid* and titrate with *0.1 M ceric ammonium sulphate*, using *ferroin solution* as indicator, until the red colour disappears.

1 ml of *0.1 M ceric ammonium sulphate* is equivalent to 0.04822 g of $C_{12}H_{22}FeO_{14},2H_2O$.

Storage. Store protected from light.

Labelling. The label states the quantity of the active ingredient both as the amount of Ferrous Gluconate and in terms of the equivalent amount of ferrous iron in each tablet.

Ferrous Sulphate

$FeSO_4,7H_2O$ Mol. Wt. 278.0

Ferrous Sulphate contains not less than 98.0 per cent and not more than 105.0 per cent of $FeSO_4,7H_2O$.

Category. Haematinic.

Description. Bluish green crystals or a light green, crystalline powder. Efflorescent in air. On exposure to moist air, the crystals rapidly oxidise and become brown.

Identification

It gives reaction (A) of ferrous salts and the reactions of sulphates (2.3.1).

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Tests

Appearance of solution. Dissolve 2.5 g in *carbon dioxide-free water*, add 0.5 ml of *1 M sulphuric acid* and dilute to 50.0 ml with *water* (solution A). The solution is not more opalescent than opalescence standard OS2 (2.4.1).

pH (2.4.24). 3.0 to 4.0, determined in a 5.0 per cent solution.

Arsenic (2.3.10). Dissolve 5.0 g in 10 ml of *water*, add 15 ml of *stannated hydrochloric acid* and distill 20 ml. To the distillate add a few drops of *bromine solution*, remove the excess of bromine with a few drops of *stannous chloride solution AsT* and add 40 ml of *water*. The resulting solution complies with the limit test for arsenic (2 ppm).

Copper. Dissolve 8.0 g in 40 ml of *hydrochloric acid*, add 10 ml of *nitric acid* and 15 ml of *water*, boil gently for 5 minutes and cool. Shake with four quantities, each of 30 ml, of *ether* and discard the ether extracts. Heat the acid solution on a water-bath to remove the dissolved *ether*, cool and add sufficient *water* to produce 100.0 ml (solution B). To 10.0 ml of solution B add 1 g of *citric acid*, make alkaline with *dilute ammonia solution*, add 25 ml of *water* and 5 ml of *sodium diethyldithiocarbamate solution*. Extract successively with 5, 3 and 2-ml quantities of *carbon tetrachloride*, mix the carbon tetrachloride extracts and add sufficient *carbon tetrachloride* to produce 100.0 ml. The resulting solution is not more intensely coloured than a solution prepared by treating 4.0 ml of *copper standard solution (10 ppm Cu)* and 7.5 ml of *water* in the same manner (50 ppm).

Lead. Make 25.0 ml of solution B alkaline with *dilute ammonia solution*, add 1 ml of *potassium cyanide solution* and sufficient *water* to produce 50.0 ml. Add 0.1 ml of *sodium sulphide solution*; the solution is not more intensely coloured than a mixture of 10 ml of *hydrochloric acid*, 0.5 ml of *nitric acid*, 5.0 ml of *lead standard solution (20 ppm Pb)*, 0.1 ml of *sodium sulphide solution* and sufficient *water* to produce 50.0 ml (50 ppm).

Zinc. To 2.5 ml of solution B add 1 g of *citric acid* and 1 g of *resorcinol*, neutralise the solution with *dilute ammonia solution* using *thymol blue solution* as indicator and shake for 1 minute with two quantities, each of 20 ml, of *dithizone solution*. To the combined extracts add 10 ml of *0.1 M hydrochloric acid* and shake for 1 minute. Separate the acid layer, add 3 ml of *1 M hydrochloric acid* and 20 ml of *ammonium chloride solution* and adjust the volume to 50.0 ml with *water*. Add 1.0 ml of *potassium ferrocyanide solution* and allow to stand for 15 minutes. Any turbidity produced is not greater than that developed in 15 minutes by the addition of 1.0 ml of *potassium ferrocyanide solution* to a freshly prepared mixture of 10.0 ml of *zinc standard solution (10 ppm Zn)*, 4 ml of *1 M hydrochloric acid*, 20 ml of *ammonium chloride solution* and sufficient *water* to produce 50.0 ml (500 ppm).

Manganese. Dissolve 1.0 g in 40 ml of *water*, add 10 ml of *nitric acid* and boil until red fumes are evolved. Add 0.5 g of *ammonium persulphate* and boil for 10 minutes. Discharge any pink colour by the dropwise addition of a 5 per cent w/v solution of *sodium sulphite* and boil until any odour of sulphur dioxide is eliminated. Add 10 ml of *water*, 5 ml of *phosphoric acid* and 0.5 g of *sodium periodate*, boil for 1 minute and allow to cool. The resulting solution is not more intensely coloured than that of a solution prepared at the same time and in the same manner using 1.0 ml of *0.02 M potassium permanganate* in place of the substance under examination (0.1 per cent).

Ferric ions. Not more than 0.5 per cent

Dissolve 5 g ferrous sulphate in a mixture of 10 ml of *hydrochloric acid* and 100 ml of *water* in a stoppered flask, add 3 g of *potassium iodide*, close the flask and allow to stand in the dark for 5 minutes. Titrate the liberated iodine with *0.1 M sodium thiosulphate*, using 0.5 ml of *starch solution* as indicator. Carry out the blank titration. Not more than 4.5 ml of *0.1 M sodium thiosulphate* is required.

Chlorides (2.3.12). 20 ml of solution A complies with the limit test for chlorides (250 ppm).

Assay. Dissolve 2.5 g of *sodium bicarbonate* in a mixture of 150 ml of *water* and 10 ml of *sulphuric acid*. When effervescence ceases, add about 0.5 g of the substance under examination, accurately weighed, shake gently to dissolve and titrate with *0.1 M ceric ammonium nitrate*, using 0.1 ml of *ferroin solution* as indicator, until the red colour disappears.

1 ml of *0.1 M ceric ammonium nitrate* is equivalent to 0.02780 g of $FeSO_4,7H_2O$.

Dried Ferrous Sulphate

Dried Ferrous Sulphate is Ferrous Sulphate from which a part of the water of crystallization has been removed by drying.

Dried Ferrous Sulphate contains not less than 86.0 per cent and not more than 90.0 per cent of $FeSO_4$.

Category. Haematinic.

Description. A greyish white to buff coloured powder.

Identification

It gives reaction (A) of ferrous salts and the reactions of sulphates (2.3.1).

Tests

Copper. Dissolve 8.0 g in 40 ml of *hydrochloric acid*, add 10 ml of *nitric acid* and 15 ml of *water*, boil gently for 5 minutes

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and cool. Shake with four quantities, each of 30 ml, of *ether* and discard the ether extracts. Heat the acid solution on a water-bath to remove the dissolved *ether*, cool and add sufficient *water* to produce 100.0 ml (solution A). To 10.0 ml of solution A add 1 g of *citric acid*, make alkaline with *dilute ammonia solution*, add 25 ml of *water* and 5 ml of *sodium diethyldithiocarbamate solution*. Extract successively with 5, 3 and 2 ml quantities of *carbon tetrachloride*, mix the carbon tetrachloride extracts and add sufficient *carbon tetrachloride* to produce 100.0 ml. The resulting solution is not more intensely coloured than a solution prepared by treating 4.0 ml of *copper standard solution (10 ppm Cu)* and 7.5 ml of *water* in the same manner (50 ppm).

Lead. Make 25.0 ml of solution A alkaline with *dilute ammonia solution*, add 1 ml of *potassium cyanide solution* and sufficient *water* to produce 50.0 ml. Add 0.1 ml of *sodium sulphide solution*; the solution is not more intensely coloured than a mixture of 10 ml of *hydrochloric acid*, 0.5 ml of *nitric acid*, 5.0 ml of *lead standard solution (20 ppm Pb)*, 0.1 ml of *sodium sulphide solution* and sufficient *water* to produce 50.0 ml (50 ppm).

Zinc. To 2.5 ml of solution A add 1 g of *citric acid* and 1 g of *resorcinol*, neutralise the solution with *dilute ammonia solution* using *thymol blue solution* as indicator and shake for 1 minute with two quantities, each of 20 ml, of *dithizone solution*. To the combined extracts add 10 ml of *0.1 M hydrochloric acid* and shake for 1 minute. Separate the acid layer, add 3 ml of *1 M hydrochloric acid* and 20 ml of *ammonium chloride solution* and adjust the volume to 50.0 ml with *water*. Add 1.0 ml of *potassium ferrocyanide solution* and allow to stand for 15 minutes. Any turbidity produced is not greater than that developed in 15 minutes by the addition of 1.0 ml of *potassium ferrocyanide solution* to a freshly prepared mixture of 10.0 ml of *zinc standard solution (10 ppm Zn)*, 4 ml of *1 M hydrochloric acid*, 20 ml of *ammonium chloride solution* and sufficient *water* to produce 50.0 ml (500 ppm).

Manganese. Dissolve 1.0 g in 40 ml of *water*, add 10 ml of *nitric acid* and boil until red fumes are evolved. Add 0.5 g of *ammonium persulphate* and boil for 10 minutes. Discharge any pink colour by the dropwise addition of a 5 per cent w/v solution of *sodium sulphite* and boil until any odour of sulphur dioxide is eliminated. Add 10 ml of *water*, 5 ml of *phosphoric acid* and 0.5 g of *sodium periodate*, boil for 1 minute and allow to cool. The resulting solution is not more intensely coloured than that of a solution prepared at the same time and in the same manner using 1.0 ml of *0.02 M potassium permanganate* in place of the substance under examination (0.1 per cent).

Arsenic (2.3.10). Dissolve 3.3 g in 10 ml of *water*, add 15 ml of *stannated hydrochloric acid* and distill 20 ml. To the distillate add a few drops of *bromine solution*, remove the excess of bromine with a few drops of *stannous chloride solution AsT* and add 40 ml of *water*. The resulting solution complies with the limit test for arsenic (3 ppm).

Basic sulphate. 2.0 g dissolves slowly in a mixture of 7.5 ml of freshly boiled and cooled *water* and 0.5 ml of *0.5 M sulphuric acid*, producing a solution that is not more than faintly turbid.

Ferric ions. Not more than 0.5 per cent

Dissolve 5 g ferrous sulphate in a mixture of 10 ml of *hydrochloric acid* and 100 ml of *water* in a stoppered flask, add 3 g of *potassium iodide*, close the flask and allow to stand in the dark for 5 minutes. Titrate the liberated iodine with *0.1 M sodium thiosulphate*, using 0.5 ml of *starch solution* as indicator. Carry out the blank titration. Not more than 4.5 ml of *0.1 M sodium thiosulphate* is required.

Assay. Weigh 0.5 g, dissolve in a mixture of 30 ml of *water* and 20 ml of *1 M sulphuric acid* and titrate with *0.1 M ceric ammonium sulphate* using *ferroin solution* as indicator.

1 ml of *0.1 M ceric ammonium sulphate* is equivalent to 0.01519 g of $FeSO_4$.

Ferrous Sulphate Tablets

Ferrous Sulphate Tablets contain Dried Ferrous Sulphate.

Ferrous Sulphate Tablets contain not less than 80.0 per cent and not more than 90.0 per cent of the stated amount of dried ferrous sulphate, $FeSO_4$. The tablets are coated.

Usual strength. 200 mg. (200 mg of Dried Ferrous Sulphate is approximately equivalent to 60 mg of ferrous iron).

Identification

A. The powdered tablets give reaction (A) of ferrous salts (2.3.1).

B. Extract the powdered tablets with *2 M hydrochloric acid* and filter. The filtrate gives reaction (A) of sulphates (2.3.1).

Tests

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 0.5 g of Dried Ferrous Sulphate in a mixture of 30 ml of *water* and 20 ml of *1 M sulphuric acid* and titrate with *0.1 M ceric ammonium sulphate* using *ferroin solution* as indicator.

1 ml of *0.1 M ceric ammonium sulphate* is equivalent to 0.01519 g of $FeSO_4$.

Labelling. The label states the quantity of the active ingredient both as the amount of Dried Ferrous Sulphate and in terms of the equivalent amount of ferrous iron in each tablet.

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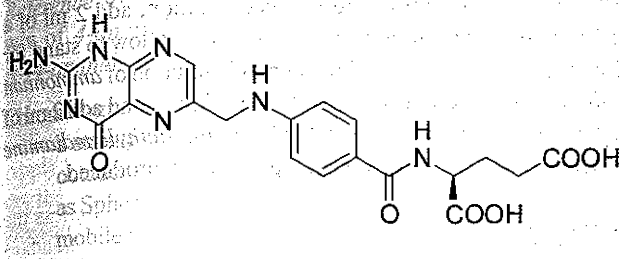
Folic Acid

Pteroylglutamic Acid



$C_{19}H_{19}N_7O_6$ Mol. Wt. 441.4

Folic Acid is (2*S*)-[4-[(2-amino-4-hydroroxypteridin-6-yl) methylamino]benzamido]glutamic acid.

Folic acid contains not less than 95.0 per cent and not more than 102.0 per cent of $C_{19}H_{19}N_7O_6$, calculated on the anhydrous basis.

Category. Haematinic.

Description. A yellow to yellowish-orange, crystalline powder.

Identification

A. When examined in the range 230 nm to 380 nm (2.4.7), a 0.001 per cent w/v solution in *0.1 M sodium hydroxide* shows three absorption maxima, at about 256 nm, 283 nm and 365 nm; absorbance at about 256 nm, about 0.59, at about 283 nm, about 0.575 and at about 365 nm, about 0.206.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 60 volumes of *ethanol (95 per cent)*, 20 volumes of *strong ammonia solution* and 20 volumes of *1-propanol*.

Solvent mixture. 9 volumes of *methanol* and 2 volumes of *strong ammonia solution*.

Test solution. Dissolve 50 mg of the substance under examination in 100 ml of the solvent mixture.

Reference solution. A 0.05 per cent w/v solution of *folic acid IPRS* in the solvent mixture.

Apply to the plate 2 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

Specific optical rotation (2.4.22). + 18° to + 22°, determined in a 1.0 per cent w/v solution in *0.1 M sodium hydroxide*.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in 5 ml of a 2.86 per cent w/v solution of *sodium carbonate* and dilute to 100.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 100 mg of *folic acid IPRS* in 5 ml of a 2.86 per cent w/v solution of *sodium carbonate* and dilute 100 ml with the mobile phase. Dilute 2.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). To 20 mg of *pteroic acid*, add 5 ml of a 2.86 per cent w/v solution of *sodium carbonate*, dilute to 100 ml with the same solution. Mix 1.0 ml of the solution with 1.0 ml of reference solution (a) and dilute to 100 ml with the mobile phase.

Reference solution (c). Dilute 2.0 ml of the test solution to 20 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (d). Dissolve 10 mg of *N-(4-amino-benzoyl)-l-gulatamic acid (folic acid impurity A)* in 1 ml of a 2.86 per cent w/v solution of *sodium carbonate* and dilute to 100 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (e). To 12 mg of *pteroic acid (folic acid impurity D)*, add 1 ml of a 2.86 per cent w/v solution of *sodium carbonate*, dilute to 100 ml with the same solution. Dilute 1.0 ml of the solution to 100 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 12 volumes of *methanol* and 88 volumes of a solution containing 1.1 per cent w/v solution of *potassium dihydrogen phosphate* and 0.6 per cent w/v solution of *dipotassium hydrogen phosphate*,
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 5 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to folic acid and folic acid impurity D is not less than 4.0. The relative retention time with reference to folic acid for N-(4-aminobenzoyl)-L-glutamic acid (folic acid impurity A) is about 0.5; for 2,5,6-triaminopyrimidin-4(1H)-one (folic acid impurity B) is about 0.6; for isofolic acid (folic acid impurity C) is about 0.9; for pteroic acid (folic acid impurity D) is about 1.33; for 6-pterinylfolic acid (folic acid impurity E) is about 1.27 and for 2-amino-7-(chloromethyl) pteridin-4(1*H*)-one (folic acid impurity F) is about 2.2.

Inject reference solutions (c), (d), (e) and the test solution. In the chromatogram obtained with the test solution the area of

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peak corresponding to folic acid impurity A is not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent), the area of the peak corresponding to folic acid impurity D is not more than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.6 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent), the sum of areas of all other secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent). Ignore any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.3 per cent.

Water (2.3.43). 5.0 to 8.5 per cent, determined on 0.15 g.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances.

Inject reference solution (a) and the test solution.

Calculate the content of $C_{19}H_{19}N_7O_6$.

Storage. Store protected from light.

Folic Acid Tablets

Folic Acid Tablets contain not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of folic acid, $C_{19}H_{19}N_7O_6$.

Usual strengths. 1 mg; 5 mg; 10 mg.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 60 volumes of *ethanol (95 per cent)*, 20 volumes of *strong ammonia solution* and 20 volumes of *1-propanol*.

Solvent mixture. 9 volumes of *methanol* and 2 volumes of *strong ammonia solution*.

Test solution. Extract a quantity of the powdered tablets containing 0.5 mg of Folic Acid with 1 ml of the solvent mixture.

Reference solution. A 0.05 per cent w/v solution of *folic acid IPRS* in the solvent mixture.

Apply to the plate 2 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. Dissolve a quantity of the powdered tablets containing 5 mg of Folic Acid as completely as possible in 5 ml of *0.1 M sodium hydroxide* and filter. To the filtrate add 45 ml of *0.5 M hydrochloric acid* and 5 g of *zinc powder* and shake for 30 minutes. To 5 ml of the reduced solution, add 2 ml of a 0.1 per cent w/v solution of *sodium nitrite*, allow to stand for 2 minutes, add 2 ml of a 0.5 per cent w/v solution of *ammonium sulphamate*, mix, allow to stand for 3 minutes and add 2 ml of a 0.1 per cent w/v solution of *N-(1-naphthyl)ethylenediamine dihydrochloride*; a deep magenta colour is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 500 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a suitable quantity of *folic acid IPRS* in the dissolution medium and dilute with the same medium to obtain a solution having known concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of a buffer solution prepared by dissolving 35.1 g of *sodium perchlorate* and 1.4 g of *monobasic potassium phosphate* in 900 ml of *water*, add 7 ml of *1M potassium hydroxide*, dilute to 1000 ml with *water*, adjusted to pH 7.2 with *1M potassium hydroxide* or *orthophosphoric acid* and 40 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 25 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{19}N_7O_6$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{19}H_{19}N_7O_6$.

Hydrolysis products. Determine by liquid chromatography (2.4.14).

NOTE — Protect the solutions from light.

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Test solution. Shake a quantity of the powdered tablets containing 5.0 mg of Folic Acid with 50 ml of the mobile phase, centrifuge and use the supernatant liquid.

Reference solution. A solution containing 0.00005 per cent w/v of *4-aminobenzoic acid* and 0.0002 per cent w/v of *N-(4-aminobenzoyl)-L-glutamic acid* in the mobile phase.

Chromatographic system
- a stainless steel column 20 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Spherisorb ODS 1),
- mobile phase: *0.05 M potassium dihydrogen phosphate*, adjusted to pH 5.5 with *5 M sodium hydroxide*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 269 nm,
- injection volume: 20 µl.

In the chromatogram obtained with the reference solution two peaks due to N-(4-aminobenzoyl)-L-glutamic acid and 4-aminobenzoic acid are obtained in the order of their emergence. The areas of the peaks due to *4-aminobenzoic-acid* and *N-(4-aminobenzoyl)-L-glutamic acid* in the chromatogram obtained with the reference solution are greater than the areas of any corresponding peaks in the chromatogram obtained with the test solution. The test is not valid unless the resolution between the two peaks in the chromatogram obtained with the reference solution is greater than 3.

Uniformity of content. Complies with the test stated under Tablets. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test solution. Shake 1 tablet with 5.0 ml of *0.1 M sodium hydroxide*, add sufficient mobile phase to produce a solution containing 0.001 per cent w/v of Folic Acid, centrifuge and use the supernatant liquid.

Reference solution. Add 1.0 ml of *0.5 M hydrochloric acid* to 5.0 ml of a 0.002 per cent w/v solution of *folic acid IPRS* in *0.1 M sodium hydroxide* and dilute to 10.0 ml with the mobile phase.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{19}N_7O_6$ in the tablet.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Disperse a quantity of the powder containing about 20 mg of Folic Acid with 50 ml of *0.1 M sodium hydroxide*, dilute to 100.0 ml with the same solvent, centrifuge and dilute 5.0 ml of the supernatant liquid to 100.0 ml with the mobile phase.

Reference solution. Dilute 5.0 ml of a 0.02 per cent w/v solution of *folic acid IPRS* in *0.1 M sodium hydroxide* to 100.0 ml with the mobile phase.

Chromatographic system
- a stainless steel column 20 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 93 volumes of *0.05 M potassium dihydrogen phosphate* and 7 volumes of *acetonitrile* adjusted to pH 6.0 with *5 M sodium hydroxide*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 283 nm,
- injection volume: 20 µl.

Inject the reference solution and the test solution.

Calculate the content of $C_{19}H_{19}N_7O_6$ in the tablets.

Storage. Store protected from light.

Folic Acid and Methylcobalamin Tablets

Folic acid and Methylcobalamin Tablets contain not less than 90.0 per cent of the stated amount of folic acid $C_{19}H_{19}N_7O_6$ and methylcobalamin, $C_{63}H_{91}CoN_{13}O_{14}P$.

Usual strength. Folic acid, 5 mg and Methylcobalamin, 750 mcg.

NOTE — Carry out the tests in the dark using low actinic glassware.

Identification

In the Assay, the principal peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 500 ml of *water*,
Speed and time. 100 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter, rejecting the first few ml of filtrate.

Determine by liquid chromatography (2.4.14).

Solvent mixture. *Water*, adjusted to about pH 11.5 with *dilute sodium hydroxide* solution.

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution (a). A 0.005 per cent w/v solution of *folic acid IPRS* in solvent mixture.

Reference solution (b). A 0.0015 per cent w/v solution of *mecobalamin IPRS* in dissolution medium.

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Reference solution (c). Dilute a suitable volume of reference solution (a) and reference solution (b) with *water* to obtain a concentration similar to the test solution.

Chromatographic system

- a stainless steel column 10 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 35°,
- mobile phase: A. a buffer solution prepared by dissolving 0.5 g of *sulphonate sodium salt* in 1000 ml of *water* and add 1 ml of *formic acid*,

  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
7	90	10
18	35	65
20	90	10
25	90	10

Inject reference solution (c) and the test solution.

Calculate the content of $C_{19}H_{19}N_7O_6$ and $C_{63}H_{91}CoN_{13}O_{14}P$ in the medium.

Q. Not less than 70 per cent of the stated amount of $C_{19}H_{19}N_7O_6$ and $C_{63}H_{91}CoN_{13}O_{14}P$.

Uniformity of content. Complies with the test stated under Tablets.

Determine by liquid chromatography (2.4.14), as described under Assay using the following solution.

Test solution. Transfer one tablet in 50-ml volumetric flask. Add 10 ml of the solvent mixture, and disperse with the aid of ultrasound and dilute to volume with *water*.

Calculate the content of $C_{19}H_{19}N_7O_6$ and $C_{63}H_{91}CoN_{13}O_{14}P$ in the tablets.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Transfer an accurately weighed quantity of the powder equivalent to the weight of one tablet into a 25-ml amber coloured volumetric flask. Add 5 ml of the solvent mixture, and disperse with the aid of ultrasound for 5 minutes in ice cold water with intermittent shaking and dilute to volume with *water*, filter. Dilute 5.0 ml of the solution to 10.0 ml with *water*.

Reference solution (a). Weigh 50 mg of *folic acid IPRS* and transfer to 100-ml amber colour volumetric flask, dissolve in the solvent mixture with the aid of ultrasound for 5 minutes in ice cold *water* and dilute to volume with *water*.

Reference solution (b). Weigh 30 mg of *methylcobalamin IPRS* and transfer to 100-ml amber colour volumetric flask, dissolve in the solvent mixture with the aid of ultrasound for 5 minutes in ice cold water and dilute to volume with *water*.

Reference solution (c). Dilute reference solution (a) and reference solution (b) to obtain a concentration similar to the test solution.

Use chromatographic system and solvent mixture as described under Dissolution, using sampler temperature: 15°.

Inject reference solution (c). The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

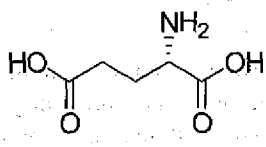
Inject reference solution (c) and the test solution.

Calculate the content of $C_{19}H_{19}N_7O_6$ and $C_{63}H_{91}CoN_{13}O_{14}P$ in the tablets.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Glutamic Acid

L-Glutamic Acid



$C_5H_9NO_4$ Mol. Wt. 147.1

Glutamic Acid is *S*-2-aminopentanedioic acid.

Glutamic Acid contains not less than 98.5 per cent and not more than 101.5 per cent of $C_5H_9NO_4$, calculated on the dried basis.

Category. Amino Acid.

Description. A white crystalline powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *glutamic acid IPRS* or with the reference spectrum of glutamic acid.

Tests

Specific optical rotation (2.4.22). +31.5° to +32.5°, determined on 10.0 per cent w/v solution in *2M hydrochloric acid* at 20°.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*

Mobile phase. A mixture of 6 volumes of *butyl alcohol*, 2 volumes of *glacial acetic acid* and 2 volumes of *water*.

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Test solution. Dissolve 0.1 g of the substances under examination in 10.0 ml of a mixture of equal volumes of 10 per cent w/v solution of *ammonia* and *water*.

Reference solution (a). A 0.005 per cent w/v solution of *glutamic acid IPRS* in *water*.

Reference solution (b). A solution containing 0.04 per cent w/v, each of, *aspartic acid IPRS* and *glutamic acid IPRS* in *water*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air, spray with a freshly prepared 0.2 per cent w/v solution of *ninhydrin* in a mixture of 95 volumes of *butyl alcohol* and 5 volumes of *2 M acetic acid*, dry the plate at 105° for 15 minutes and examine the plate under day light. Any secondary spot in the chromatogram obtained with the test solution is not larger or more intense than the spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

Iron (2.3.14). 4 g complies with the limit test for iron (10 ppm).

Chlorides (2.3.12). 1.25 g complies with the limit test for chlorides (200 ppm).

Sulphates (2.3.17). 0.75 g complies with the limit test for sulphates (200 ppm).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.1 per cent, determined by drying in an oven at 105° for 3 hours.

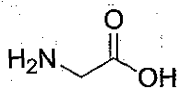
Assay. Dissolve 0.14 g in 6 ml of *formic acid* and 50 ml of *glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determine the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01471 g of glutamic acid.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Glycine

Aminoacetic acid



$C_2H_5NO_2$ Mol. Wt. 75.1

Glycine is 2-aminoethanoic acid.

Glycine contains not less than 98.5 per cent and not more than 101.5 per cent of $C_2H_5NO_2$, calculated on the dried basis.

Category. Bladder irrigation agent; pharmaceutical aid.

Description. A white, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *glycine IPRS*. Examine the substances as discs prepared using about 1 mg for 0.4 g of *potassium bromide IR* or with the reference spectrum of glycine.

B. Dissolve 50 mg in 5 ml of *water*, add 1 ml of *sodium hypochlorite solution (3 per cent Cl)*, boil for 2 minutes, add 1 ml of *hydrochloric acid* and boil for 4 to 5 minutes. To the resulting solution add 2 ml of *hydrochloric acid* and 1 ml of a 2 per cent w/v solution of *resorcinol*, boil for 1 minute, cool, add 10 ml of *water* and mix. To 5 ml of the solution add 6 ml of *2 M sodium hydroxide*. The resulting solution is violet with a greenish yellow fluorescence. After a few minutes the solution becomes orange and then yellow and the intense fluorescence remains.

Tests

Appearance of solution. A 10.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution YS7 (2.4.1).

pH (2.4.24). 5.9 to 6.3, determined in a 5.0 per cent w/v solution.

Chlorides (2.3.12). 2.5 g dissolved in 20 ml of *water* complies with the limit test for chlorides (100 ppm).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method A (10 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

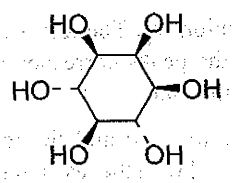
Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Assay. Weigh 0.15 g and dissolve in 100 ml of *anhydrous glacial acetic acid*. Immediately after dissolution titrate with *0.1 M perchloric acid*, using 0.05 ml of *crystal violet solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.00751 g of $C_2H_5NO_2$.

Inositol

myo-Inositol



$C_6H_{12}O_6$ Mol. Wt. 180.2

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Inositol is *cis*-1,2,3,5-trans-4,6-cyclohexanehexol.

Inositol contains not less than 97.0 per cent and not more than 102.0 per cent of $C_6H_{12}O_6$, calculated on the anhydrous basis.

Category. Carbohydrate.

Description. A white crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *inositol IPRS* or with the reference spectrum of inositol.

B. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Tests

Appearance of solution. A 10 per cent w/v solution is not more opalescent than opalescence standard OS2 (2.4.1), and not more intensely coloured than reference solution RS7 (2.4.1).

Conductivity (2.4.9). Not more than 20 μS cm^{-1}, determined on a 20 per cent w/v solution at 20°.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 2.5 g of the substance under examination in *water* and dilute to 50.0 ml with *water*.

Reference solution (a). A 5.0 per cent w/v solution of *inositol IPRS* in *water*.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 100.0 ml with *water*.

Reference solution (c). A solution containing 0.005 per cent w/v, each of, *inositol IPRS* and *mannitol IPRS* in *water*.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm, packed with a strong cation-exchange resin consisting of sulphonated cross-linked styrene-divinylbenzene copolymer in the calcium form (9 μm) (Such as Carbo CHO-820),
- column temperature: 85°,
- mobile phase: *water,*
- flow rate: 0.5 ml per minute,
- refractive index detector at 30° - 35°,
- injection volume: 20 μl.

Inject reference solution (c). The test is not valid unless the resolution between the peaks corresponding to inositol and mannitol is not less than 4.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent) and the sum of the areas of all the secondary peaks is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Ignore any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Lead (2.3.15). Not more than 0.5 ppm.

Determine by atomic absorption spectrophotometry (2.4.2), measuring at 283.3 nm using a lead hollow cathode discharge lamp and an air acetylene flame.

NOTE — Prepare the solutions immediately before use.

Test solution. Dissolve 20.0 g of inositol to 100 ml of *dilute acetic acid*. Add 2.0 ml of saturated *ammonium pyrrolidinedithiocarbamate* solution (containing about 1 per cent w/v of *ammonium pyrrolidinedithiocarbamate*), and 10.0 ml of *methyl isobutyl ketone*, and shake for 30 seconds. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.

Reference stock solution. Dissolve 0.16 g of lead nitrate in 100 ml of *water*, add 1 ml of *nitric acid* and dilute to 1000.0 ml with *water*. Dilute10.0 ml of the solution to 100.0 ml with *water* (Each ml contains 10 μg of lead).

Reference solutions. Prepare as directed for the test solution, except prepare three reference solutions 0.5, 1.0 and 1.5 ml, respectively of the reference stock solution in addition to the 20.0 g of inositol under examination.

Set zero using blank solution prepared in test solution without inositol. Introduce the test solution and each of the three reference solutions on the instrument and record the absorbance. Plot the absorbance reading against the known concentration of lead and draw a straight line.

Water (2.3.43). Not more than 0.5 per cent, determined on 1.0 g.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances using with the following modification.

- injection volume: 10 μl.

Inject reference solution (a) and (c). The test is not valid unless the resolution between the peaks corresponding to inositol and mannitol is not less than 4.0 in the chromatogram obtained with reference solution (c) and the relative standard deviation for replicate injection is not more than 2.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_6H_{12}O_6$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Iron and Ammonium Citrate

Ferric Ammonium Citrate

Iron and Ammonium Citrate is a complex of ammonium ferric citrate.

Iron and Ammonium Citrate contains not less than 20.5 per cent and not more than 22.5 per cent of iron, Fe.

Category. Haematinic.

Description. Thin, transparent, dark red scales or granules or a brownish red powder; deliquescent in moist air and is affected by light.

Identification

A. Ignite 0.1 g gently and dissolve the residue in 5 ml of *hydrochloric acid*; the solution gives the reactions of ferric salts (2.3.1).

B. Warm 50 mg with *5 M sodium hydroxide*; ammonia is evolved and the solution gives the reactions of citrates (2.3.1).

Tests

Arsenic (2.3.10). Mix 2.5 g with 1.5 g of *anhydrous sodium carbonate*, add 10 ml of *bromine solution* and mix thoroughly. Evaporate to dryness on a water-bath, gently ignite and dissolve the cooled residue in 20 ml of *brominated hydrochloric acid* and 10 ml of *water*. Transfer to a small flask, add sufficient *stannous chloride AsT* to remove the yellow colour, connect to a condenser and distill 22 ml. To the distillate add 40 ml of *water* and 0.15 ml of *stannous chloride AsT*. The resulting solution complies with the limit test for arsenic (4 ppm).

Lead. Dissolve 2.0 g in 20 ml of *hydrochloric acid* and 8 ml of *water*, add 0.5 ml of *nitric acid*, heat just to boiling, cool, transfer to a separating funnel and extract with three quantities, each of 20 ml, of *ether*. If the acid solution is still more than faintly yellow, extract with an additional 20 ml of *ether*. Reject the ether extracts. Transfer the acid solution to a narrow-necked flask, rinse the separating funnel with 5 ml of *water* and add the rinsings to the flask. Heat to remove the dissolved ether, cool, make alkaline with *ammonia solution*, add 1 ml of *potassium cyanide solution*, dilute to 50 ml with *water* and add 0.1 ml of *sodium sulphide solution*. Any colour produced is not more intense than that produced by mixing 10 ml of *hydrochloric acid*, 0.5 ml of *nitric acid* and 6 ml of *lead standard solution (10 ppm Pb)*, making alkaline with *ammonia solution* and adding 1 ml of *potassium cyanide solution* and 0.1 ml of *sodium sulphide solution* (30 ppm).

Zinc. Dissolve 2.0 g in a mixture of 20 ml of *hydrochloric acid* and 8 ml of *water*, add 0.5 ml of *nitric acid*, heat just to boiling, cool and extract with three quantities, each of 20 ml, of *ether*. If the acid solution is still more than faintly yellow, repeat the extraction with an additional 20 ml of *ether*. Reject the ether extracts. Warm the acid solution on a water-bath to remove dissolved ether, cool and add sufficient *water* to produce 100 ml. To 10 ml add 1 g of *citric acid* and 0.1 g of *resorcinol*, neutralise with *dilute ammonia solution*, using *thymol blue solution* as indicator, and shake for 1 minute with two successive quantities, each of 20 ml, of *dithizone solution*. To the combined extracts add 10 ml of *0.1 M hydrochloric acid*, shake for 1 minute, separate the acid layer and wash it with 2 ml of *chloroform*. To the acid layer add 3 ml of *1 M hydrochloric acid* and 20 ml of *ammonium chloride solution* and adjust the volume to 50 ml with *water*. Add 1 ml of *potassium ferrocyanide solution* and allow to stand for 15 minutes. Any turbidity produced is not more than that produced by the addition of 1 ml of *potassium ferrocyanide solution* to a freshly prepared mixture of 1.0 ml of *zinc standard solution (10 ppm Zn)*, 4 ml of *1 M hydrochloric acid*, 20 ml of *ammonium chloride solution* and sufficient *water* to produce 50 ml (50 ppm).

Free ferric compounds. A 1.0 per cent w/v solution gives no blue precipitate with *potassium ferrocyanide solution* unless acidified with *hydrochloric acid*.

Chlorides (2.3.12). Dissolve 0.1 g in 5 ml of *water* and boil with 2 ml of *nitric acid*; the solution complies with the limit test for chlorides (0.25 per cent).

Sulphates (2.3.17). 10 ml of a 0.5 per cent w/v solution complies with the limit test for sulphates (0.3 per cent).

Assay. Weigh 0.5 g, dissolve in a mixture of 15 ml of *water* and 1 ml of *sulphuric acid* and warm until the dark brown colour becomes yellow. After cooling the solution to 15° add dropwise *0.02 M potassium permanganate* till a pink colour persisting for 5 seconds is obtained. Add 15 ml of *hydrochloric acid* and 2 g of *potassium iodide*, allow to stand for 3 minutes, add about 60 ml of *water* and titrate with *0.1 M sodium thiosulphate* using *starch solution* as indicator.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.005585 g of Fe.

Storage. Store protected from light and moisture.

Iron and Folic Acid Syrup

Ferrous Sulphate and Folic Acid Syrup

Iron and Folic Acid Syrup is a mixture of ferrous sulphate and folic acid with a suitable flavoring agent and other excipients as required. It is filled in a sealed container.

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Iron and Folic Acid Syrup contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ferrous sulphate, $FeSO_4,7H_2O$ equivalent to elemental ferrous iron and not less than 90.0 per cent of the stated amount of folic acid, $C_{19}H_{19}N_7O_6$.

Usual strength. Ferrous sulphate equivalent to 100 mg elemental ferrous iron and 0.5 mg of folic acid per 5 ml.

Identification

Mix a volume of syrup containing 0.3 g of Ferrous Sulphate in 10 ml of a mixture of equal volumes of *hydrochloric acid* and *water*, warm, cool and filter (Solution A).

A. To 1.0 ml of Solution A, add 1 ml of *potassium ferricyanide solution*; a dark blue precipitate is formed that is insoluble in *dilute hydrochloric acid* and is decomposed by *sodium hydroxide solution*.

B. To 5 ml of the Solution A, add 1 ml of *dilute hydrochloric acid* and 1 ml of *barium chloride solution*; a white precipitate is formed.

C. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 4.5 to 5.5.

Weight per ml (2.4.29). Not less than 1.0677 g.

Free ferric compounds. Mix a quantity of syrup containing 0.4 g of ferrous sulphate in a mixture of 100 ml of *water* and 3 ml of *hydrochloric acid* by rapidly heating to the boiling point. Boil for 15 seconds and cool rapidly, add 1 g of *potassium iodide*, allow to stand in dark for 15 minutes and titrate the liberated iodine with *0.1 M sodium thiosulphate* using starch as indicator. Carry out a blank titration.

The difference is not more than 3.6 ml (5 per cent of ferric iron in ferrous sulphate).

Other tests. Comply with the tests stated under Oral Liquids.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 1000 CFU per ml and 1 ml is free from *E. coli*.

Assay. *For ferrous sulphate* — Mix a quantity of syrup containing 0.2 g of elemental iron in a mixture of 100 ml of *water* and 20 ml of *1 M sulphuric acid* and titrate with *0.05 M ceric ammonium sulphate* using *ferroin solution* as indicator.

1 ml of *0.05 M ceric ammonium sulphate* is equivalent to 0.002792 g of Fe(II).

Determine the weight per ml (2.4.29) of the syrup and calculate the content of Fe(II), weight in volume.

For folic acid — Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of a 0.57 per cent w/v solution of *dipotassium hydrogen orthophosphate* and 13.5 volumes of *methanol*.

Test solution. Dilute a volume of syrup containing 0.5 mg of Folic acid in 40 ml of the solvent mixture with the aid of ultrasound for 15 minutes and dilute to 50.0 ml with the solvent mixture and filter.

Reference solution. A 0.001 per cent w/v solution of *folic acid IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 13.5 volumes of *methanol* and 80 volumes of a solution containing 0.938 per cent w/v of *sodium perchlorate* and 0.075 per cent w/v of *potassum dihydrogen orthophosphate*, adjusted to pH 7.2 with *0.1 M potassium hydroxide* and dilute to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 277 nm,
- injection volume: 20 µl.

Inject the reference solutions. The test is not valid unless the column efficiency if not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject the reference solution and the test solution.

Determine the weight per ml of the syrup (2.4.29) and calculate the content of $C_{19}H_{19}N_7O_6$.

Labelling. The label states the strength in terms of ferrous sulphate, equivalent ferrous elemental iron and folic acid.

Iron and Folic Acid Tablets

Ferrous Sulphate and Folic Acid Tablets

Iron and Folic Acid Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of dried ferrous sulphate, $FeSO_4$ equivalent to elemental iron and not less than 90.0 per cent of the stated amount of folic acid, $C_{19}H_{19}N_7O_6$.

Identification

For folic acid — In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

For dried ferrous sulphate — Dissolve a quantity of the powdered tablets containing 10 mg of Iron in 2 ml of *water*, add 1 ml of *potassium ferricyanide solution*; a blue precipitate

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is formed that does not dissolve on addition of 5 ml of *dilute hydrochloric acid*.

Tests

Uniformity of content. Complies with the test stated under tablets.

For folic acid—Determine by liquid chromatography (2.4.14).

Solvent mixture. 80 volumes of 0.57 per cent w/v solution of *dipotassium hydrogen orthophosphate* and 13.5 volumes of *methanol*.

Test solution. Disperse 1 tablet in the solvent mixture and dilute to obtain a solution containing 0.0004 per cent w/v of folic acid in the solvent mixture and filter.

Reference solution. A 0.0004 per cent w/v solution of *folic acid IPRS* in the solvent mixture.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 135 volumes of *methanol* and 800 volumes of a solution containing 0.938 per cent w/v of *sodium perchlorate* and 0.075 per cent w/v of *potassium dihydrogen orthophosphate* adjusted to pH 7.2 with *0.1 M potassium hydroxide* and diluted to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at: 277 nm,
- injection volume: 100 µl.

Inject the reference solution and the test solution.

Calculate the content of folic acid in the tablet.

Free ferric compounds. Dissolve a quantity of powdered tablets containing 1.5 g of ferrous sulphate in a mixture of 100 ml of *water* and 10 ml of *hydrochloric acid* by rapidly heating to the boiling point. Boil for 15 seconds and cool rapidly, add 3 g of *potassium iodide*, allow to stand in dark for 15 minutes and titrate the liberated iodine with *0.1 M sodium thiosulphate* using *starch* as indicator. Carry out a blank titration.

The difference is not more than 13.4 ml (5 per cent of ferric iron in dried ferrous sulphate).

Other tests. Comply with the tests stated under Tablets.

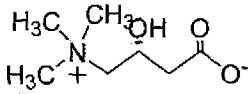
Assay. *For dried ferrous sulphate equivalent to ferrous iron* —Weigh and powder 20 tablets. Disperse a quantity of powder containing 0.4 g of dried Ferrous Sulphate in a mixture of 30 ml of *water* and 20 ml of *1 M sulphuric acid* and titrate with *0.1 M ceric ammonium sulphate* using *ferroin solution* as indicator.

1 ml of *0.1 M ceric ammonium sulphate* is equivalent to 0.005584 g of Fe (II).

For folic acid — For Assay, use the average content of the results obtained in the test for uniformity of content in folic acid.

Labelling. The label states the strength in terms of ferrous sulphate, equivalent ferrous elemental iron and folic acid.

Levocarnitine



$C_7H_{15}NO_3$ Mol. Wt. 161.20

Levocarnitine is (*R*)-3-Carboxy-2-hydroxy-*N*,*N*,*N*-trimethyl-1-propanaminium, inner salt; (*R*)-(3-Carboxy-2-hydroxypropyl) trimethylammonium, inner salt.

Levocarnitine contains not less than 97.0 per cent and not more than 103.0 per cent of $C_7H_{15}NO_3$, calculated on the anhydrous basis.

Category. Carnitine deficiency.

Description. A white crystals or crystalline powder; hygroscopic.

Identification

Dry the sample and reference standard under vacuum at 50° for 5 hours and determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *levocarnitine IPRS* or with the reference spectrum of levocarnitine.

Tests

Specific optical rotation (2.4.22). −32.0° to −29.0°, determined in a 10.0 per cent w/v solution in *carbon dioxide-free water*.

pH (2.4.24). 5.5 to 9.5, determined in a 5.0 per cent w/v solution in *carbon dioxide-free water*.

Chloride. Not more than 4000 ppm.

Test solution. Dissolve 0.09 g of levocarnitine in 30 to 40 ml of *water*, neutralise the solution with *nitric acid*, to litmus. Add 1 ml of *nitric acid* and 1 ml of *silver nitrate solution*, make up the volume to 50.0 ml with *water*.

Reference solution. Mix 0.5 ml of *0.02 M hydrochloric acid* in 30 to 40 ml of *water*. Add 1 ml of *nitric acid* and 1 ml of *silver nitrate solution*, make up the volume to 50.0 ml with *water*.

Allow to stand for 5 minutes protected from direct sunlight. Compare the turbidity of test solution, if any, with that produced with reference solution.

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Specific optical rotation (2.4.22). – 1.0° to + 1.0°, determined in 5.0 per cent w/v solution in *ethanol*.

Related substances. A. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.1 g of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). A 0.1 cent w/v solution of *alpha lipoic acid IPRS* in the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase. Further dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 46 volumes of 0.068 per cent w/v solution of *potassium dihydrogen phosphate*, 58 volumes of *methanol* and 9 volumes of *acetonitrile*, adjusted to pH 3.0 with *orthophosphoric acid*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the signal-to-noise ratio is not less than 10 and the relative standard deviation for replicate injections is not more than 10.0 per cent.

Inject the test solution. The area of any secondary peak is not more than 0.1 per cent and the sum of areas of all the secondary peaks is not more than 2.0 per cent, calculated by area normalization.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

NOTE — Carry out the test using low actinic glassware.

Mobile phase. A mixture of 40 volumes of *1-propanol*, 40 volumes of *ethyl acetate*, 10 volumes of *water* and 5 volumes of *ammonia*.

Test solution. Dissolve 0.4 g of the substance under examination in 10.0 ml of *dimethylformamide*.

Reference solution. A 4.0 per cent w/v solution of *alpha lipoic acid IPRS* in *dimethylformamide*.

Apply to the plate 5µl of each solution. Allow the mobile phase to rise 10 cm. Dry the plate in air, until the ammonia disappears completely, heat at 50° for 20 minutes allow to cool and place in iodine vapour saturated chamber until spots are visible. The R_f values of alpha lipoic acid spot is, 0.25 - 0.30 and for polymeric lipoic acid is 0. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot at R_f 0 in the chromatogram obtained with the reference solution.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.2 per cent, by drying under vacuum at 40° for 3 hours.

Assay. Determine by liquid chromatography (2.4.14), as described under Related substances A.

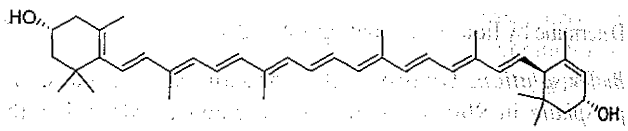
Inject reference solution (a). The test is not valid unless the column efficiency is not less than 10000 theoretical plates, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of $C_8H_{14}O_2S_2$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Lutein



$C_{40}H_{56}O_2$ Mol. Wt. 568.9

Lutein is β-ε-Carotene-3,3' -diol (3*R*,3'*R*,6'*R*). It is the purified fraction obtained from saponification of the oleoresin of *Tagetes eracta L.*

Lutein contains not less than 80.0 per cent of total carotenoids calculated as $C_{40}H_{56}O_2$. It contains not less than 74.0 per cent of lutein and not more than 8.5 per cent of zeaxanthin, both calculated as $C_{40}H_{56}O_2$ on the anhydrous basis.

Category. Antioxident

Description. A red crystalline powder.

Identification

A. When examined in the range 300 nm to 700 nm (2.4.7), the test solution prepared in the test of content of total carotenoids shows the ratio of the absorbance at 446 nm to that at 474 nm is between 1.09 to 1.14.

B. In the test of Content of lutein, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

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Tests

Content of Total Carotenoids.

NOTE — Carry out the test using low actinic glassware.

Solvent mixture. 10 volumes of *hexane*, 7 volumes of *acetone*, 7 volumes of *toluene* and 6 volumes of *ethanol*.

A 0.03 per cent w/v solution of the substance under examination in the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with *ethanol*. Measure the absorbance of the resulting solution at the maximum at about 446 nm (2.4.7). Calculate the content of $C_{40}H_{56}O_2$, taking 2550 as the specific absorbance at 446 nm.

Content of Lutein. Determine by liquid chromatography (2.4.14).

NOTE — Carry out the test using low actinic glassware.

Test solution. Evaporate 1.0 ml of a 0.03 per cent w/v solution of the substance under examination in the solvent mixture, under a stream of nitrogen to dryness. Dissolve the residue in 1.0 ml of the mobile phase, with the aid of ultrasound.

Reference solution. A 0.015 per cent w/v solution of *lutein IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with porous silica (5 µm),
- mobile phase: a mixture of 75 volumes of *hexane* and 25 volumes of *ethyl acetate*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 446 nm,
- injection volume: 10 µl.

The relative retention time with reference to lutein for zeaxanthin is about 1.05.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to lutein and zeaxanthin is not less than 1.0, the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the test solution. The sum of the areas of all the peaks due to lutein is not more than 85.0 per cent, calculated by area normalization.

Calculate the content of lutein $C_{40}H_{56}O_2$, using the following expression:

$$\text{Result} = \frac{A_U}{A_T} \times T$$

Where,

A_U = peak area of lutein;
A_T = sum of the areas of all the peaks;
T = percentage of total carotenoids as determined in the test for Content of total carotenoids.

Zeaxanthin and other related compounds. Determine by liquid chromatography (2.4.14), as described under Content of lutein.

Inject the test solution. The area of zeaxanthin peak is not more than 9.0 per cent, calculated by area normalization.

Calculate the content of zeaxanthin, using the following expression:

$$\text{Result} = \frac{B_U}{B_T} \times T$$

Where,

B_U = peak area of zeaxanthin;
B_T = sum of the areas of all the peaks;
T = percentage of total carotenoids as determined in the test for Content of total carotenoids.

The area of any secondary peak other than lutein and zeaxanthin is not more than 1.0 per cent, calculated by area normalization.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

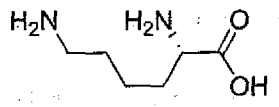
Sulphated ash (2.3.18). Not more than 2.0 per cent.

Water (2.3.43). Not more than 1.0 per cent, determined on 0.5 g.

Storage. Store protected from light, in hermetically oxygen resistant container, at temperature between 8°-15°.

Lysine Hydrochloride

L-Lysine Hydrochloride



$C_6H_{14}N_2O_2,HCl$ Mol. Wt. 182.7

Lysine Hydrochloride contains not less than 98.5 per cent and not more than 101.5 per cent of $C_6H_{14}N_2O_2,HCl$ calculated on the dried basis.

Category. Amino acid

Description. A white powder.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *L-lysine hydrochloride IPRS* or with the reference spectrum of lysine hydrochloride.

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Tests

Specific optical rotation (2.4.22). +20.4° to +21.4°, determined in a 8.0 per cent w/v solution in 6 *M hydrochloric acid*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 70 volumes of *isopropyl alcohol* and 30 volumes of *ammonia*.

Test solution. A 1.0 per cent w/v solution of the substance under examination in *water*.

Reference solution (a). A 0.005 per cent w/v solution of *L- lysine hydrochloride IPRS* in *water*.

Reference solution (b). A solution containing 0.04 per cent w/v, each of, *L-lysine hydrochloride IPRS* and *arginine hydrochloride IPRS* in *water*.

Apply to the plate 5 µl of each solution. After development, dry the plate at 105° until the ammonia completely disappears, cool and spray with a 0.2 per cent w/v solution of *ninhydrin* in a mixture of 95 volumes of *butyl alcohol* and 5 volumes of *2M acetic acid*. Heat at 105° for 15 minutes and examine under day light. Any secondary spot in the chromatogram obtained with the test solution is not more intense or larger than the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

Chloride content. 19.0 per cent to 19.6 per cent.

Dissolve 0.35 g in 140 ml of *water* and 1 ml of *2,7 dichlorofluorescein solution* in a porcelain casserole. Titrate with *0.1 M silver nitrate* until the silver chloride flocculates and the mixture acquires a faint pink colour. Carry out a blank titration.

1 ml of *0.1 M silver nitrate* is equivalent to 0.003545 g of chloride.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Iron (2.3.14). 1.33 g complies with the limit test for iron (30 ppm).

Sulphates (2.3.17). 0.5 g complies with the limit test for sulphates (300 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.4 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 90 mg in a mixture of 3 ml of *formic acid* and 50 ml of *anhydrous glacial acetic acid*, add 10 ml of *mercuric acetate solution* and titrate with *0.1 M perchloric acid*. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.009133 g of $C_6H_{14}N_2O_2.HCl$.

Heavy Magnesium Carbonate

Heavy Magnesium Carbonate is a hydrated basic magnesium carbonate.

Heavy Magnesium Carbonate contains the equivalent of not less than 40.0 per cent and not more than 45.0 per cent of MgO.

Category. Antacid; osmotic laxative.

Description. A white powder. 15 g occupies a volume of about 30 ml.

Identification

A. It gives reaction (A) of carbonates (2.3.1).

B. Dissolve about 15 mg in 2 ml of *2 M nitric acid* and neutralise with *2 M sodium hydroxide*. The resulting solution gives reaction (A) of magnesium salts (2.3.1).

Tests

Appearance of solution. Dissolve 5.0 g in a mixture of 70 ml of *5 M acetic acid* and 30 ml of *water*. Filter, if necessary, through a previously ignited and weighed porcelain or silica crucible of a suitable porosity to give a clear filtrate (solution A). Reserve any residue (residue R) for the test for Substances insoluble in *acetic acid*. Solution A is not more intensely coloured than reference solution BS3 (2.4.1).

Arsenic (2.3.10). Dissolve 5.0 g in 15 ml of *brominated hydrochloric acid* and 45 ml of *water* and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). To 20 ml of solution A add 15 ml of *7 M hydrochloric acid* and shake with 25 ml of *4-methylpentan-2-one* for 2 minutes. Separate the layers, evaporate the aqueous layer to dryness, dissolve the residue in 1 ml of *5 M acetic acid* and dilute to 20 ml with *water*. 12 ml of the resulting solution complies with the limit test for heavy metals, Method D (20 ppm). Use *lead standard solution (1 ppm Pb)* for standard preparation.

Iron (2.3.14). Dissolve 0.1 g in 3 ml of *2 M hydrochloric acid* and dilute to 10 ml with *water*. The resulting solution complies with the limit test for iron (400 ppm).

Chlorides (2.3.12). 5.0 ml of solution A complies with the limit test for chlorides (0.1 per cent).

Sulphates (2.3.17). 0.5 ml of solution A diluted to 15 ml with *distilled water* complies with the limit test for sulphates (0.6 per cent).

Calcium. To 0.2 ml of *ethanolic calcium standard solution (100 ppm Ca)* add 1 ml of *ammonium oxalate solution*. After 1 minute add a mixture of 1 ml of *2 M acetic acid* and 15 ml of a solution prepared by diluting 2.6 ml of solution A to 150 ml with *distilled water* and shake. After 15 minutes any opalescence produced is not more intense than that of a standard prepared in the same manner using a mixture of 10 ml of *calcium standard solution (10 ppm Ca)* and 5 ml of *distilled water* in place of solution A (0.75 per cent).

Copper. Dissolve 1 g in 5 ml of *hydrochloric acid* and 25 ml of *water*, boil to remove carbon dioxide, make alkaline with *dilute ammonia solution*; no blue colour is produced.

Soluble substances. Mix 2.0 g with 100 ml of *water*, boil for 5 minutes, filter whilst hot through a sintered-glass filter (porosity No. 3), allow to cool and dilute to 100.0 ml with *water*. Evaporate 50.0 ml of the filtrate to dryness and dry the residue at 105°; the residue weighs not more than 10 mg (1.0 per cent).

Substances insoluble in acetic acid. Residue when washed, dried and ignited at 600°, weighs not more than 2.5 mg (0.05 per cent).

Assay. Weigh 0.15 g, dissolve in a mixture of 20 ml of *water* and 2 ml of *2 M hydrochloric acid* and add 50 ml of *water*, 10 ml of *strong ammonia-ammonium chloride solution* and titrate with *0.05 M disodium edetate*, using 0.1 g of *mordant black II mixture* as indicator, until a blue colour is obtained. Carry out a blank titration.

1 ml of *0.05 M disodium edetate* is equivalent to 0.002015 g of MgO.

Storage. Store protected from moisture.

Light Magnesium Carbonate

Light Magnesium Carbonate is a hydrated basic magnesium carbonate.

Light Magnesium Carbonate contains the equivalent of not less than 40.0 per cent and not more than 45.0 per cent of MgO.

Category. Antacid; osmotic laxative.

Description. A very light, white powder. 15 g occupies a volume of about 125 ml.

Identification

A. It gives reaction (A) of carbonates (2.3.1).

B. Dissolve about 15 mg in 2 ml of *2 M nitric acid* and neutralise with *2 M sodium hydroxide*. The resulting solution gives reaction A of magnesium salts (2.3.1).

Tests

Appearance of solution. Dissolve 5.0 g in a mixture of 70 ml of *5 M acetic acid* and 30 ml of *water*. Filter, if necessary, through a previously ignited and weighed porcelain or silica crucible of a suitable porosity to give a clear filtrate (solution A). Reserve any residue (residue R) for the test for Substances insoluble in *acetic acid*. Solution A is not more intensely coloured than reference solution BS3 (2.4.1).

Arsenic (2.3.10). Dissolve 5.0 g in 15 ml of *brominated hydrochloric acid* and 45 ml of *water* and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). To 20 ml of solution A add 15 ml of *7 M hydrochloric acid* and shake with 25 ml of *4-methylpentan-2-one* for 2 minutes. Separate the layers, evaporate the aqueous layer to dryness, dissolve the residue in 1 ml of *5 M acetic acid* and dilute to 20 ml with *water*. 12 ml of the resulting solution complies with the limit test for heavy metals, Method D (20 ppm). Use *lead standard solution (1 ppm Pb)* to prepare the standard.

Iron (2.3.14). Dissolve 0.1 g in 3 ml of *2 M hydrochloric acid* and dilute to 10 ml with *water*. The resulting solution complies with the limit test for iron (400 ppm).

Chlorides (2.3.12). 5.0 ml of solution A complies with the limit test for chlorides (0.1 per cent).

Calcium. To 0.2 ml of *ethanolic calcium standard solution (100 ppm Ca)* add 1 ml of *ammonium oxalate solution*. After 1 minute add a mixture of 1 ml of *2 M acetic acid* and 15 ml of a solution prepared by diluting 2.6 ml of solution A to 150 ml with *distilled water* and shake. After 15 minutes any opalescence produced is not more intense than that of a standard prepared in the same manner using a mixture of 10 ml of *calcium standard solution (10 ppm Ca)* and 5 ml of *distilled water* in place of solution A (0.75 per cent).

Copper. Dissolve 1 g in 5 ml of *hydrochloric acid* and 25 ml of *water*, boil to remove carbon dioxide, make alkaline with *dilute ammonia solution*; no blue colour is produced.

Soluble substances. Mix 2.0 g with 100 ml of *water*, boil for 5 minutes, filter whilst hot through a sintered-glass filter (porosity No. 3), allow to cool and dilute to 100.0 ml with *water*. Evaporate 50.0 ml of the filtrate to dryness and dry the residue at 105°; the residue weighs not more than 10 mg (1.0 per cent).

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Substances insoluble in acetic acid. Residue R when washed, dried and ignited at 600°, weighs not more than 2.5 mg (0.05 per cent).

Sulphates (2.3.17). 1 ml of solution A diluted to 15 ml with *distilled water* complies with the limit test for sulphates (0.3 per cent).

Assay. Weigh 0.15 g, dissolve in a mixture of 20 ml of *water* and 2 ml of *2 M hydrochloric acid* and add 50 ml of *water*, 10 ml of *strong ammonia-ammonium chloride solution* and titrate with *0.05 M disodium edetate*, using 0.1 g of *mordant black II mixture* as indicator, until a blue colour is obtained.

1 ml of *0.05 M disodium edetate* is equivalent to 0.002015 g of MgO.

Storage. Store protected from moisture

Magnesium Chloride

$MgCl_2,6H_2O$ Mol. Wt. 203.3

Magnesium Chloride contains not less than 98.0 per cent and not more than 101.0 per cent of $MgCl_2,6H_2O$.

Category. Constituent of peritoneal dialysis solutions.

Description. Colourless crystals; hygroscopic.

Identification

A. It gives reaction (A) of chlorides (2.3.1).

B. It gives reaction (A) of magnesium salts (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. Dissolve 10.0 g in *carbon dioxide-free water* prepared from *distilled water* and dilute to 100 ml with the same solvent (solution A). To 5 ml of solution A add 0.05 ml of *phenol red solution*. Not more than 0.3 ml of either *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the solution.

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). Dissolve 1.0 g in 20 ml of *water*, adding 1 g of *ammonium chloride* and sufficient *water* to produce 25 ml. The resulting solution complies with the limit test for heavy metals, Method A (20 ppm).

Iron (2.3.14). 4.0 g complies with the limit test for iron (10 ppm).

Calcium. To 25 ml of solution A add sufficient *water* to produce 50 ml and determine by Method B for flame photometry (2.4.4), or by Method B for atomic absorption spectrophotometry (2.4.2), measuring at 423 nm and using *calcium solution FP*, or *calcium solution AAS*, as appropriate, diluted if necessary with *0.1 M hydrochloric acid* for the standard solution (0.1 per cent).

Sulphates (2.3.17). 15 ml of solution A complies with the limit test for sulphates (100 ppm).

Assay. Dissolve 0.3 g in 30 ml of *water*, add 10 ml of *strong ammonia-ammonium chloride solution* and titrate with *0.05 M disodium edetate* using 0.1 g of *mordant black II mixture* as indicator.

1 ml of *0.05 M disodium edetate* is equivalent to 0.01017 g of $MgCl_2,6H_2O$.

Magnesium Chloride intended for use in the preparation of dialysis solutions complies with the following additional requirement.

Aluminium. Dissolve 4.0 g in 100 ml of *water* and add 10 ml of *acetate buffer pH 6.0*. Extract with successive quantities of 20, 20 and 10 ml of a 0.5 per cent w/v solution of *8-hydroxyquinoline* in *chloroform* and dilute the combined extracts to 50 ml with *chloroform*. Use as the standard solution a mixture of 2 ml of *aluminium standard solution (2 ppm Al)*, 10 ml of *acetate buffer pH 6.0* and 98 ml of *water* treated in the same manner and as the blank solution a mixture of 10 ml of *acetate buffer pH 6.0* and 100 ml of *water* treated in the same manner. Measure the fluorescence of the test solution and the standard solution (2.4.5), using an excitation wavelength of about 392 nm and emission wavelength of about 518 nm, and setting the instrument to zero with the blank solution in each case. The fluorescence of the test solution is not greater than that of the standard solution (1 ppm).

Storage. Store protected from moisture.

Heavy Magnesium Oxide

Heavy Magnesia

MgO Mol. Wt. 40.3

Heavy Magnesium Oxide contains not less than 98.0 per cent and not more than 100.5 per cent of MgO, calculated on the ignited basis.

Category. Antacid; osmotic laxative.

Description. A fine, white powder. 15 g occupies a volume of about 30 ml.

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Identification

Dissolve about 15 mg in 2 ml of *2 M nitric acid* and neutralise with *2 M sodium hydroxide*. The resulting solution gives reaction (A) of magnesium salts (2.3.1).

Tests

Appearance of solution. Dissolve 5.0 g in a mixture of 70 ml of *5 M acetic acid* and 30 ml of *water*. Filter, if necessary, through a previously ignited and weighed porcelain or silica crucible of a suitable porosity to give a clear filtrate (solution A). Reserve any residue (residue R). Solution A is not more intensely coloured than reference solution BS3 (2.4.1).

Arsenic (2.3.10). Dissolve 2.5 g in 15 ml of *brominated hydrochloric acid*, add 45 ml of *water* and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (4 ppm).

Heavy metals (2.3.13). 12 ml of solution A complies with the limit test for heavy metals, Method D (30ppm), using *lead standard solution (1ppm Pb)*.

Iron (2.3.14). Dissolve 0.2 g in 7 ml of *2 M hydrochloric acid* and dilute to 20 ml with *water*. 5 ml of the resulting solution complies with the limit test for iron (800 ppm).

Chlorides (2.3.12). 5.0 ml of solution A complies with the limit test for chlorides (0.1 per cent).

Sulphates (2.3.17). 0.3 ml of solution A complies with the limit test for sulphates (1.0 per cent).

Calcium. To 0.2 ml of *ethanolic calcium standard solution (100 ppm Ca)* add 1 ml of *ammonium oxalate solution*. After 1 minute add a mixture of 1 ml of *2 M acetic acid* and 15 ml of a solution prepared by diluting 1.3 ml of solution A to 150 ml with *distilled water* and shake. After 15 minutes any opalescence produced is not more intense than that of a standard prepared in the same manner using a mixture of 10 ml of *calcium standard solution (10 ppm Ca)* and 5 ml of distilled water in place of solution A (1.5 per cent).

Soluble substances. Mix 2.0 g with 100 ml of *water*, boil for 5 minutes, filter whilst hot through a sintered-glass filter (porosity No. 3), allow to cool and dilute to 100.0 ml with *water*. Evaporate 50.0 ml of the filtrate to dryness and dry the residue at 105°. The residue weighs not more than 20 mg (2.0 per cent).

Substances insoluble in acetic acid. Residue R when washed with *water*, dried and ignited at 600°, weighs not more than 5 mg (0.1 per cent).

Loss on ignition (2.4.20). Not more than 8.0 per cent, determined on 0.5 g when ignited at 900°.

Assay. Dissolve 0.35 g in 10 ml of *2 M hydrochloric acid* and dilute with *water* to 100.0 ml. To 10.0 ml add 5 ml of *strong ammonia-ammonium chloride solution* and titrate with *0.05 M disodium edetate* using about 50 mg of *mordant black II mixture* as indicator.

1 ml of *0.05 M disodium edetate* is equivalent to 0.002015 g of MgO.

Storage. Store protected from moisture.

Light Magnesium Oxide

Light Magnesia

MgO Mol. Wt. 40.3

Light Magnesium Oxide contains not less than 98.0 per cent and not more than 100.5 per cent of MgO, calculated on the ignited basis.

Category. Antacid; osmotic laxative.

Description. A very fine, light, white powder. 15 g occupies a volume of about 150 ml.

Identification

Dissolve about 15 mg in 2 ml of *2 M nitric acid* and neutralise with *2 M sodium hydroxide*. The resulting solution gives reaction (A) of magnesium salts (2.3.1).

Tests

Appearance of solution. Dissolve 5.0 g in a mixture of 70 ml of *5 M acetic acid* and 30 ml of *water*. Filter, if necessary, through a previously ignited and weighed porcelain or silica crucible of a suitable porosity to give a clear filtrate (solution A). Reserve any residue (residue R) for the test for substances insoluble in acetic acid. Solution A is not more intensely coloured than reference solution BS2 (2.4.1).

Arsenic (2.3.10). Dissolve 2.5 g in 15 ml of *brominated hydrochloric acid*, add 45 ml of *water* and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (4 ppm).

Heavy metals (2.3.13). To 20 ml of solution A add 15 ml of *7 M hydrochloric acid* and shake with 25 ml of *4-methylpentan-2-one* for 2 minutes. Separate the layers, evaporate the aqueous layer to dryness, dissolve the residue in 1 ml of *5 M acetic acid* and dilute to 20 ml with *water*. 12 ml of the resulting solution complies with the limit test for heavy metals, Method D (30 ppm). Use 7.5 ml of *lead standard solution (2 ppm Pb)* for preparing the standard.

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Iron (2.3.14). Dissolve 40 mg in 5 ml of *2 M hydrochloric acid* and dilute to 10 ml with *water*. The resulting solution complies with the limit test for iron (0.1 per cent).

Chlorides (2.3.12). 4.0 ml of solution A complies with the limit test for chlorides (0.125 per cent).

Sulphates (2.3.17). 0.3 ml of solution A complies with the limit test for sulphates (1.0 per cent).

Calcium. To 0.2 ml of *ethanolic calcium standard solution (100 ppm Ca)* add 1 ml of *ammonium oxalate solution*. After 1 minute add a mixture of 1 ml of *2 M acetic acid* and 15 ml of a solution prepared by diluting 1.3 ml of solution A to 150 ml with *distilled water* and shake. After 15 minutes any opalescence produced is not more intense than that of a standard prepared in the same manner using a mixture of 10 ml of *calcium standard solution (10 ppm Ca)* and 5 ml of *distilled water* in place of solution A (1.5 per cent).

Soluble substances. Mix 2.0 g with 100 ml of *water*, boil for 5 minutes, filter whilst hot through a sintered-glass filter (porosity No. 3), allow to cool and dilute to 100.0 ml with *water*. Evaporate 50.0 ml of the filtrate to dryness and dry the residue at 105°. The residue weighs not more than 20 mg (2.0 per cent).

Substances insoluble in acetic acid. Residue R when washed with *water*, dried and ignited at 600°, weighs not more than 5 mg (0.1 per cent).

Loss on ignition (2.4.20). Not more than 8.0 per cent, determined on 0.5 g when ignited at 900°.

Assay. Dissolve 0.35 g in 10 ml of *2 M hydrochloric acid* and dilute with *water* to 100.0 ml. To 10.0 ml add 5 ml of *strong ammonia-ammonium chloride solution* and titrate with *0.05 M disodium edetate* using about 50 mg of *mordant black II mixture* as indicator.

1 ml of *0.05 M disodium edetate* is equivalent to 0.002015 g of MgO.

Storage. Store protected from moisture.

Magnesium Sulphate

Epsom Salts

$MgSO_4,7H_2O$ Mol. Wt. 246.5

Magnesium Sulphate contains not less than 99.0 per cent and not more than 100.5 per cent of $MgSO_4$, calculated on the dried basis.

Category. Osmotic laxative; used in the treatment of electrolyte deficiency.

Description. Colourless crystals or a white, crystalline powder.

Identification

A. It gives the reactions of sulphates (2.3.1).

B. It gives reaction (A) of magnesium salts (2.3.1).

Tests

Appearance of solution. Dissolve 5.0 g in sufficient *carbon dioxide-free water* to produce 50 ml (solution A). Solution A is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. To 10 ml of solution A add 0.05 ml of *phenol red solution*. Not more than 0.2 ml of either *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the solution.

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). Dissolve 2.0 g in 10 ml of *water*, 2 ml of *dilute acetic acid* and sufficient *water* to produce 25 ml. The solution complies with the limit test for heavy metals, Method A (10 ppm).

Iron (2.3.14). 2.0 ml of solution A diluted to 20 ml with *water* complies with the limit test for iron (200 ppm).

Chlorides (2.3.12). 8.0 ml of solution A complies with the limit test for chlorides (300 ppm).

Loss on drying (2.4.19). 48.0 to 52.0 per cent, determined on 0.5 g by drying in an oven at 110° to 120° for 1 hour and then at 400°.

Assay. Dissolve 0.3 g in 50 ml of *water*, add 10 ml of *strong ammonia-ammonium chloride solution* and titrate with *0.05 M disodium edetate*, using 0.1 g of *mordant black II mixture* as indicator, until a blue colour is obtained.

1 ml of *0.05 M disodium edetate* is equivalent to 0.00602 g of $MgSO_4$.

Storage. Store protected from moisture.

Magnesium Sulphate Injection

Magnesium Sulphate Heptahydrate Injection

Magnesium Sulphate Injection is a sterile solution of Magnesium Sulphate Heptahydrate in Water for Injection.

Magnesium Sulphate Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of $MgSO_4,7H_2O$.

Usual strength. 500 mg per ml.

Description. A clear, colourless solution.

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Identification

A. It gives the reactions of sulphates (2.3.1).

B. It gives the reactions of magnesium (2.3.1).

Tests

pH (2.4.24). 5.5 to7.0, in the injection diluted, if necessary, to contain 5.0 per cent w/v solution of Magnesium Sulphate Heptahydrate.

Bacterial endotoxins (2.2.3). Not more than 0.09 Endotoxin Unit per mg of magnesium sulphate.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Dilute an accurate volume containing about 1.25 g of Magnesium Sulphate Heptahydrate to 100.0 ml with *water*. To 20.0 ml of the solution, add 10 ml of *ammonia buffer pH 10.9* and titrate with *0.05 M disodium edetate* using 0.1 g of *mordant black11 mixture* as indicator until the pink tint is discharged from the blue colour.

1 ml of *0.05 M disodium edetate* is equivalent to 0.01232 g of $MgSO_4, 7H_2O$.

Labelling. The label states the strength as the percentage w/v of Magnesium Sulphate Heptahydrate and as concentration of Magnesium ions (Mg^{+2}) in millimoles per ml.

Magnesium Trisilicate

Magnesium Trisilicate is a hydrated magnesium silicate of the approximate composition $2MgO,3SiO_2,xH_2O$.

Magnesium Trisilicate contains not less than the equivalent of 29.0 per cent of MgO and not less than the equivalent of 65.0 per cent of SiO_2, both calculated on the ignited basis.

Category. Antacid.

Description. A fine, white or nearly white powder, free from grittiness; slightly hygroscopic.

Identification

A. To 2.0 g add a mixture of 4 ml of *nitric acid* and 4 ml of *distilled water* and heat to boiling, shaking frequently. Add 12 ml of *distilled water*, allow to cool, filter or centrifuge to obtain a clear solution and dilute the filtrate to 20 ml with *distilled water* (solution A). 1 ml of solution A, after neutralisation with *2 M sodium hydroxide*, gives reaction (A) of magnesium salts (2.3.1).

B. 0.25 g gives the reaction of silicates (2.3.1).

Tests

Alkalinity. In a tared 200-ml conical flask on a water-bath heat 10 g with 100 ml of *water* for 30 minutes, allow to cool and restore the initial weight with *water*. Allow to stand and filter or centrifuge until a clear liquid is obtained. To 10 ml of the clear liquid add 0.1 ml of *phenolphthalein solution*. Not more than 1.0 ml of *0.1 M hydrochloric acid* is required to change the colour of the solution.

Arsenic (2.3.10). Disperse 2.5 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (4 ppm).

Heavy metals (2.3.13). Neutralise 7.5 ml of solution A with *dilute ammonia solution* using *metanil yellow solution* as external indicator, dilute to 15 ml with *water* and filter if necessary. 12 ml of the resulting solution complies with the limit test for heavy metals, Method D (40 ppm), using *lead standard solution (2 ppm Pb)*.

Chlorides (2.3.12). 5.0 ml of solution A diluted to 15 ml with *distilled water* complies with the limit test for chlorides (500 ppm).

Sulphates (2.3.17). 0.3 ml of solution A diluted to 15 ml with *distilled water* complies with the limit test for sulphates (0.5 per cent).

Acid absorption. Not less than 100 ml of *0.1 M hydrochloric acid* per g, determined by the following method. Suspend 0.25 g in 100.0 ml of *0.1 M hydrochloric acid*, allow to stand in a water-bath at 37° ± 0.5° for 2 hours, shaking frequently and allow to cool. Add 0.1 ml of *bromophenol blue solution* to 20.0 ml of the supernatant liquid and titrate with *0.1 M sodium hydroxide* until a blue colour is produced.

Water-soluble salts. In a tared platinum dish evaporate to dryness on a water-bath 20 ml of the clear liquid obtained in the test for Alkalinity and ignite the residue to constant weight at 900°. The residue weighs not more than 30 mg.

Loss on ignition (2.4.20). 17.0 to 34.0 per cent, determined on 0.5 g when ignited in a platinum crucible at 900°.

Assay. *For magnesium oxide* — Weigh 1.0 g, add 35 ml of *hydrochloric acid* and 50 ml of *water* and allow to stand for 15 minutes on a water-bath. Allow to cool, filter, wash the residue with *water* and dilute the combined filtrate and washings to 250.0 ml with *water*. Neutralise 50.0 ml with about 8 ml of *10 M sodium hydroxide*, add 10 ml of *ammonia buffer pH 10.0*, 50 mg of *mordant black II mixture*, heat to 40° and titrate with *0.05 M disodium edetate* until the colour changes to a deep blue.

1 ml of *0.05 M disodium edetate* is equivalent to 0.002015 g of MgO.

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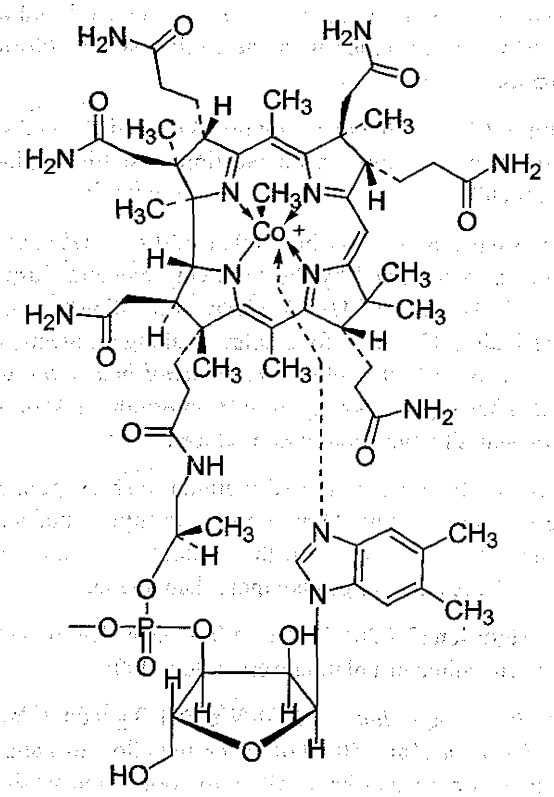
For silicon dioxide — Weigh 0.7 g, add 10 ml of *1 M sulphuric acid* and 10 ml of water and heat on a water-bath for 1.5 hours, shaking frequently and replacing the evaporated *water*. Allow to cool, decant onto an ashless filter paper (7 cm in diameter), wash the precipitate by decantation with three quantities, each of 5 ml, of hot *water*, transfer it to the filter paper and wash it with hot water until 1 ml of the filtrate remains clear on the addition of 2 ml of *barium chloride solution* and 0.05 ml of *2 M hydrochloric acid*. Ignite the filter paper and its contents in a tared platinum crucible at 900° to constant weight; the residue is SiO_2.

Storage. Store protected from moisture.

Methylcobalamin

Mecobalamin



$C_{63}H_{91}CoN_{13}O_{14}P$ Mol. Wt. 1344.4

Mecobalamin is Coα-[α-(5,6-dimethyl-1*H*-benzoimidazole-1-yl)]-Coβ-methylcobamide.

Mecobalamine contains not less than 98.0 per cent and not more than 102.0 per cent of the stated amount of $C_{63}H_{91}CoN_{13}O_{14}P$, calculated on the anhydrous basis.

Category. Vitamin.

Description. A dark red crystalline powder.

NOTE — perform the tests and assay in the dark in low-actininc glassware.

Identification

A. When examined in the range 230 nm to 360 nm (2.4.7), a 0.005 per cent w/v solution of methylcobalamin in *hydrochloric acid- potassium chloride buffer solution pH 2.0* shows similar maxima and intensity as that of reference solution. The same solution of methylcobalamin in phosphate buffer pH7.0, shows similar maxima and intensity as that of reference solution.

B. Mix 1 mg of methylcobalamin with 50 mg of *potassium bisulphate*, and fuse by igniting. Cool, break up the mass with glass rod, add 3 ml of *water*, and dissolve by boiling. Add 1 drop of *phenolphthalein solution*, then add dropwise *sodium hydroxide solution* until a light red colour develops. Add 0.5 g of *sodium acetate*,0.5 ml of *dilute acetic acid* and 0.5 ml of a solution of *disodium 1-nitroso-2naphthol-3,6-disulphonate*, a red orange colour is immediately produced. Then add 0.5 ml of *hydrochloric acid*, and boil for 1 minute, the red colour does not disappear.

Tests

Appearance of solution. A 0.2 per cent w/v solution in *water* is clear and having red colour. (2.4.1)

Related substances. Determine by liquid chromatography (2.4.14), as described under Assay with the following modifications.

Test Solution. Dissolve 50.0 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the same solvent.

Inject the test solution. Run the chromatogram 2.5 times the retention time of peak due to mecobalamin. In the chromatogram obtained with test solution the area of any secondary peak is not more than 0.5 per cent and the sum of areas of all the secondary peaks is not more than 2.0 per cent, calculated by area normalisation.

Water (2.3.43). Not more than 12.0 per cent, determined on 0.1 g.

Assay. Determine by liquid chromatography (2.4.14).

Test Solution. Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 50.0 ml with the same solvent.

Reference solution (a). A solution containing 0.005 per cent w/v each of *cyanocobalamin* and *hydroxocobalamin acetate* in the mobile phase.

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Reference solution (b). A 0.1 per cent w/v solution of *mecobalamin IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- Column temperature 40°,
- mobile phase: a mixture of 200 volumes of *acetonitrile* and 800 volumes of *0.02 M phosphate buffer solution* pH 3.5, add 3.76 g of *sodium 1-hexane sulphonate.*
- flow rate: 1 ml per minute,
- spectrophotometer set at 266 nm,
- injection volume: 10 µl.

Retention time of the principal peak is about 12 minutes.

Inject reference solution (a) and (b). The test is not valid unless, the resolution between the peaks due to cyanocobalamin and hydroxocobalamin is not less than 3.0 obtained with reference solution (a) and the column efficiency of the principal peak is not less than 6000 theoretical plates obtained with reference solution (b), and the relative standard deviation for replicate injections is not more than 1.0 per cent obtained with reference solution (b).

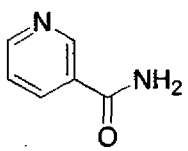
Inject reference solution (b) and the test solution.

Calculate the content of $C_{63}H_{91}CoN_{13}O_{14}P$.

Storage. Store protected from light and moisture.

Nicotinamide

Niacinamide



$C_6H_6N_2O$ Mol. Wt. 122.1

Nicotinamide is pyridine-3-carboxamide.

Nicotinamide contains not less than 99.0 per cent and not more than 101.0 per cent of $C_6H_6N_2O$, calculated on the dried basis.

Category. B-group vitamin.

Description. Colourless crystals or a white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nicotinamide IPRS* or with the reference spectrum of nicotinamide.

B. Heat about 5 mg in a dry tube; pyridine is evolved.

C. Boil 0.1 g with 1 ml of *dilute sodium hydroxide solution*; ammonia is evolved.

Tests

Appearance of solution. A 5.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution BYS7 (2.4.1).

pH (2.4.24). 6.0 to 7.5, determined in a 5.0 per cent w/v solution.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 48 volumes of *chloroform*, 45 volumes of *ethanol* and 4 volumes of *water.*

Test solution. Dissolve 0.8 g of the substance under examination in 10 ml of *ethanol (50 per cent).*

Reference solution. A 0.02 per cent w/v solution of the substance under examination in *ethanol (50 per cent).*

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 10 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Heavy metals (2.3.13). Dissolve 0.67 g in 10 ml of *water*, 7.5 ml of *1 M hydrochloric acid* and sufficient *water* to produce 25 ml. The solution complies with the limit test for heavy metals, Method A (30 ppm).

Chlorides (2.3.12). 1.0 g complies with the limit test for chlorides (250 ppm).

Sulphates (2.3.17). 1.2 g complies with the limit test for sulphates (125 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying over *phosphorus pentoxide* at a pressure of 1.5 to 2.7 kPa for 18 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 40 mg of the substance under examination in 3.0 ml of *water* and dilute to 100.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 40 mg of *nicotinamide IPRS* in 3.0 ml of *water* and dilute to 100.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 40 mg of *nicotinic acid IPRS* in 3.0 ml of *water* and dilute to 100.0 ml with the mobile phase.

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Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase. To 5.0 ml of the solution add 5.0 ml of reference solution (a).

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (1.5 to 10 µm),
- mobile phase: a mixture of 70 volumes of *0.005 M sodium-1-heptanesulphonate* and 30 volumes of *methanol*;
- flow rate: 2 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solutions (a) and (b). The test is not valid unless the resolution between the peaks corresponding to nicotinamide and nicotinic acid is not less than 3.0 in the chromatogram obtained with reference solution (b) and the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (a).

Inject reference solution (a) and the test solution.

Calculate the content of $C_6H_6N_2O$.

Storage. Store protected from moisture.

Nicotinamide Tablets

Niacinamide Tablets

Nicotinamide Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of nicotinamide, $C_6H_6N_2O$.

Usual strength. 50 mg.

Identification

Shake a quantity of the powdered tablets containing 0.2 g of Nicotinamide with 50 ml of *ethanol* for 15 minutes, filter and evaporate the filtrate to dryness on a water-bath. The residue complies with the following tests.

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nicotinamide IPRS* or with the reference spectrum of nicotinamide.

B. Boil 0.1 g with 1 ml of *dilute sodium hydroxide solution*; ammonia is evolved.

C. When examined in the range 230 nm to 360 nm (2.4.7), the solution obtained in the Assay shows an absorption maximum only at about 262 nm and two shoulders at about 258 nm and 269 nm.

Tests

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 48 volumes of *chloroform*, 45 volumes of *ethanol* and 4 volumes of *water*.

Test solution. Shake a quantity of the powdered tablets containing 0.1 g of Nicotinamide with 15 ml of *ethanol* for 15 minutes, filter, evaporate to dryness on a water-bath and dissolve the residue as completely as possible in 1 ml of *ethanol*.

Reference solution. Dilute 1 volume of the test solution to 400 volumes with *ethanol*.

Apply to the plate 5 µl of each solution. Allow the mobile phase to rise 10 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

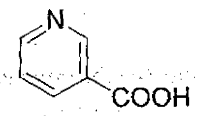
Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 50 mg of Nicotinamide, shake with 50 ml of *ethanol (95 per cent)* for 15 minutes and dilute to 100.0 ml with *ethanol (95 per cent)*. Mix, filter, dilute 5.0 ml of the filtrate to 100.0 ml with *ethanol (95 per cent)* and measure the absorbance of the resulting solution at the maximum at about 262 nm (2.4.7). Calculate the content of $C_6H_6N_2O$ taking 241 as the specific absorbance at 262 nm.

Storage. Store protected from light and moisture.

Nicotinic Acid

Niacin



$C_6H_5NO_2$ Mol. Wt. 123.1

Nicotinic Acid is pyridine-3-carboxylic acid.

Nicotinic Acid contains not less than 99.5 per cent and not more than 100.5 per cent of $C_6H_5NO_2$, calculated on the dried basis.

Category. B-group vitamin.

Description. A white or creamy-white, crystalline powder.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

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A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *nicotinic acid BPRS* or with the reference spectrum of nicotinic acid.

B. Heat a small quantity with twice its weight of *soda lime*; pyridine is evolved.

C. Dissolve about 50 mg in 20 ml of *water*, neutralise to *litmus paper* with *0.1 M sodium hydroxide*, add 3 ml of *copper sulphate solution*; a blue precipitate is gradually produced.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.12 g of the substance under examination in 200 µl of *dilute ammonia* and dilute to 10.0 ml with mobile phase A.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with mobile phase A.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- column temperature: 15°,
- mobile phase: A. dilute 2 ml of *acetic acid* in 950 ml of *water*, adjusted to pH 5.6 with *ammonia* and dilute to 1000 ml with *water*,
  B. a mixture of equal volumes of *acetonitrile* and *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 250 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
10	100	0
30	20	80
35	20	80
36	100	0

Name	Relative retention time
Nicotinic acid (Retention time: about 6 minutes)	1.0
Nicotinic acid impurity A[1]	2.7
Nicotinic acid impurity B[2]	2.8

[1]6-methylnicotinic acid,
[2]6,6'-dinicotinic acid.

Inject reference solution (a). The test is not valid unless the column efficiency is not less than 2000 theoretical plates and the tailing factor is not more than 2.0.

Inject reference solution (b) and the test solution. In the chromatogram obtained with the test solution, the area of any secondary peak is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent), the sum of areas of all the secondary peaks is not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent). Ignore any peak with an area less than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent).

Heavy metals (2.3.13). Mix 1.0 g with 1.5 ml of *dilute hydrochloric acid* and sufficient *water* to produce 25 ml, heat gently and cool to room temperature. The resulting solution complies with the limit test for heavy metals, Method A (20 ppm).

Chlorides (2.3.12). 1.0 g complies with the limit test for chlorides (250 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 1 hour.

Assay. Weigh 0.25 g, dissolve in 50 ml of *carbon dioxide-free water* and titrate with *0.1 M sodium hydroxide* using *phenol red solution* as indicator. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of sodium hydroxide required.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01231 g of $C_6H_5NO_2$.

Storage. Store protected from light and moisture.

Nicotinic Acid Tablets

Niacin Tablets

Nicotinic Acid Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the stated amount of nicotinic acid, $C_6H_5NO_2$.

Usual strengths. 25 mg; 50 mg; 100 mg.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 48 volumes of *chloroform*, 45 volumes of *ethanol (95 per cent)* and 8 volumes of *water*.

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Test solution. Shake a quantity of the powdered tablets containing 50 mg of Nicotinic Acid with 50 ml of hot *ethanol (95 per cent)*, filter and allow the filtrate to cool.

Reference solution. A 0.1 per cent w/v solution of *nicotinic acid IPRS* in *ethanol (95 per cent)*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. Shake a quantity of the powdered tablets containing 0.1 g of Nicotinic Acid with *ethanol (95 per cent)*, filter and evaporate the filtrate to dryness. Add to the residue 10 mg of *citric acid* and 0.15 ml of *acetic anhydride* and heat on a water-bath; a reddish-violet colour is produced.

Tests

Other tests. Comply with the tests stated under Tablets.

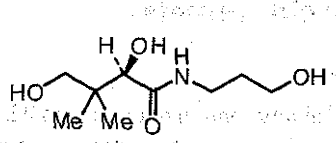
Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 0.25 g of Nicotinic Acid, add 40 ml of hot *ethanol (95 per cent)*, previously neutralised to *phenolphthalein solution*, and shake. Allow to stand for 15 minutes, swirling occasionally, and then shake for 10 minutes. Filter through a plug of cotton and wash the filter with *ethanol (95 per cent)*. Add 50 ml of *carbon dioxide-free water* and titrate with *0.1 M sodium hydroxide* using *phenol red solution* as indicator.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01231 g of $C_6H_5NO_2$.

Storage. Store protected from light and moisture.

D-Panthenol

Pantothenol; Dextro-pantothenyl Alcohol



$C_9H_{19}NO_4$ Mol. Wt. 205.3

D-Panthenol is (*R*)-2,4-dihydroxy-*N*-(3-hydroxypropyl)-3, 3-dimethylbutanamide.

D-Panthenol contains not less than 6.60 per cent and not more than 6.95 per cent of nitrogen, N.

Category. Vitamin B (enzyme co-factor).

Description. A clear, colourless or slightly yellow, viscous liquid.

Identification

Boil 50 mg with 5 ml of *0.1 M sodium hydroxide* for 1 minute, cool and add 5 ml of *1 M hydrochloric acid* and 0.1 ml of *ferric chloride test solution*; a deep yellow colour is produced.

Tests

pH (2.4.24). Not more than 10.5, determined in a 5.0 per cent w/v solution in *carbon dioxide-free water*.

Specific optical rotation (2.4.22). +29.0° to +31.5°, determined in a 5.0 per cent w/v solution.

Refractive index (2.4.27). 1.495 to 1.502, determined at 20°.

Heavy metals (2.3.13). 1.0 g dissolved in 25 ml of *water* complies with the limit test for heavy metals, Method A (20 ppm).

Assay. Weigh 0.5 g and carry out Method A for the determination of nitrogen (2.3.30).

1 ml of *0.05 M sulphuric acid* is equivalent to 0.001401 g of N.

Storage. Store protected from moisture.

Pepsin

Pepsin is obtained from the gastric mucosa of pigs, cattle or sheep. It contains gastric proteinases that are active in an acid medium, pH 1 to 5. It may contain a suitable diluent such as Lactose.

Pepsin has an activity equivalent to its ability to digest not less than 3000 times its weight of coagulated egg albumin when determined by the method given under Assay.

Category. Proteolytic enzyme.

Description. A white or light buff-coloured, crystalline or amorphous powder or translucent scales; hygroscopic.

Identification

A. Place 1 ml of *congo red fibrin* on a filter paper and wash until a colourless filtrate is obtained with a solution prepared by diluting 30 ml of *1 M hydrochloric acid* to 1000 ml with *water* and adjusted to pH 1.5 to 1.7. Perforate the filter paper and wash the congo red fibrin through it with 20 ml of the same hydrochloric acid solution. Shake this suspension before use. Dissolve about 10 mg of the substance under examination in 2 ml of the hydrochloric acid solution and adjusted the pH 1.5 to 1.7. Place 4 ml of the congo red fibrin suspension in each of two tubes. To one of the tubes add 1 ml of the solution of the substance under examination and to the other tube add 1 ml of *water* (control solution). Mix the contents of each tube and place in a water-bath at 25° with gentle shaking for 15 minutes; the control solution is colourless and the solution of the substance under examination is violet blue.

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B. The proteolytic activity of a solution in *water* is destroyed at once by boiling. It is destroyed by warming for 10 minutes at 40° at a pH of 8.0.

Tests

Microbial contamination (2.2.9). 1 g is free from *Escherichia coli*; 10 g is free from *Salmonellae* and *Shigella*.

Sulphated ash (2.3.18). Not more than 5.0 per cent.

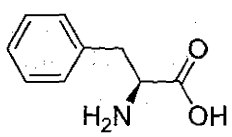
Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 4 hours.

Assay. Weigh 0.25 g, triturate with 1.0 g of *sodium chloride*, add slowly acidified *water* prepared by diluting 65 ml of *1 M hydrochloric acid* to 1000 ml with *water*, continue the trituration, dilute to 1000.0 ml with the acidified *water* and shake for 15 minutes. Prepare coagulated egg albumin by boiling fresh hen-eggs in *water* for 15 minutes, cooling rapidly to room temperature by immersion in cold *water*, separating the whites and rubbing through a no. 44 sieve. Reject the first portion that passes through the sieve and triturate 15.0 g of freshly prepared coagulated egg albumin with 50 ml of the acidified *water* ensuring that the particles of egg albumin are thoroughly disintegrated, add a further 50 ml of the acidified *water* and keep in a water-bath at 51° ± 1° for 15 minutes. Add 20.0 ml of the prepared solution of the substance under examination and digest at 51° ± 1° for 4 hours, shaking at intervals of 15 minutes. Centrifuge and decant off most of the clear supernatant liquid, wash the remainder into a 10-ml graduated cylinder and allow to stand for 30 minutes. The volume of the undissolved albumin is not more than 2 ml.

Storage. Store protected from moisture.

Phenylalanine

L-Phenylalanine



$C_9H_{11}NO_2$ Mol. Wt. 165.2

Phenylalanine contains not less than 98.5 per cent and not more than 101.5 per cent of $C_9H_{11}NO_2$, calculated on the dried basis.

Category. Amino acid

Description. A white crystals.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *L- phenylalanine IPRS* or with the reference spectrum of *L*-phenylalanine.

Tests

pH (2.4.24). 5.4 to 6.0, determined in 1.0 per cent w/v solution.

Specific optical rotation (2.4.22). – 34.7° to – 32.7°, determined in 2.0 per cent w/v solution in *water*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 30 volumes of *butyl alcohol*, 10 volumes of *glacial acetic acid* and 10 volumes of *water*.

Solvent mixture. 50 volumes of *glacial acetic acid* and 50 volumes of *water*.

Test solution. Dissolve 100 mg of the substance under examination in 10.0 ml of the solvent mixture.

Reference solution (a). A 0.005 per cent w/v solution of *L-phenylalanine IPRS* in the solvent mixture.

Reference solution (b). A solution containing 0.04 per cent w/v, each of, *L-phenylalanine IPRS* and *L-tyrosine IPRS* in the solvent mixture.

Apply to the plate 5 µl of each solution. Dry the plate in air and spray the plate with a 0.2 per cent w/v solution of *ninhydrin* in a mixture of 95 volumes of *butyl alcohol* and 5 volumes of *2 M acetic acid* and heat the plate at 100° to 105° for 15 minutes. Cool and examine in day light. Any secondary spot in the chromatogram obtained with the test solution is not larger or more intense than the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

Iron (2.3.14). 1.33 g complies with the limit test for iron (30 ppm).

Chlorides (2.3.12). 0.5 g complies with the limit test for chlorides (500 ppm).

Sulphates (2.3.17). 0.5 g complies with the limit test for sulphates (300 ppm).

Sulphated ash (2.3.18). Not more than 0.4 per cent.

Loss on drying (2.4.19). Not more than 0.3 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Weigh and dissolve 0.16 g of the substance under examination in a mixture of 3 ml of *formic acid* and 50 ml of *glacial acetic acid* and titrate with *0.1M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

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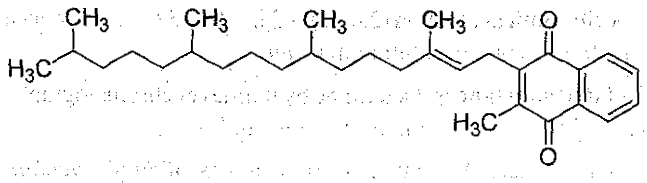
1 ml of *0.1 M perchloric acid* is equivalent to 0.01652g of $C_9H_{11}NO_2$.

Storage. Store protected from moisture.

Phytomenadione

Phytonadione



$C_{31}H_{46}O_2$ Mol. Wt. 450.7

Phytomenadione is 2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)naphthoquinone.

Phytomenadione contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{31}H_{46}O_2$.

Phytomenadione is a mixture of E- and Z-isomers containing not more than 21.0 per cent of the Z-isomers.

Category. Vitamin K analogue, anticoagulant.

Description. A clear, intense yellow, viscous, oily liquid.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *phytomenadione IPRS* or with the reference spectrum of phytomenadione.

B. In a 5 per cent w/v solution in *methanol*, add 1.0 ml of 20 per cent w/v solution of *potassium hydroxide* in *methanol*. A green colour is produced which becomes violet red on heating in a water-bath at 40° and then reddish brown on standing.

Tests

Appearance of solution. A 10.0 per cent w/v solution in *trimethylpentane* is clear.

Z-isomer content. Determine by liquid chromatography (2.4.14), as described under Assay using the following modication.

NOTE — Protect the solutions from light.

Calculate the content of Z-isomer in the portion of phytomenadione taken:

$$= \frac{tz}{tz + te} \times 100$$

where, tz = peak area of the (Z)- phytomenadione isomer from the test solution.

te = peak area of the (E)- phytomenadione isomer from the test solution.

Limit of Menadione. Mix 20 mg of phytomenadione with 0.5 ml of an equal mixture of *6M ammonium hydroxide* and *ethanol*. Add 1 drop of *ethyl cyanoacetate*, and shake gently. No purple or blue colour is produced.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Acid value (2.3.23). Not more than 2.0, determined on 2.0 g.

Assay. Determine by liquid chromatography (2.4.14).

Internal standard solution. A 0.25 per cent w/v solution of *cholesteryl benzoate* in mobile phase.

Test solution. Dilute a suitable quantity of the substance under examination with mobile phase to obtain a solution containing 0.01 per cent w/v of Phytomenadione. Transfer 10.0 ml of the solution and 7.0 ml of internal standard solution into 25-ml volumetric flask and dilute to volume with mobile phase.

Reference solution. A 0.01 per cent w/v solution of *phytomenadione IPRS* in mobile phase. Transfer 10.0 ml of the solution and 7.0 ml of internal standard solution into 25-ml volumetric flask and dilute to volume with mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with porous silica (5 µm) (Such as Kromasil silica column),
- mobile phase: a mixture of 2000.0 ml of *n-hexane* and 1.5 ml of *n-amyl alcohol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 50 µl.

The relative retention time for internal standard is 0.7, (Z)-phytomenadione is 0.9 and (E)- phytomenadione is 1.0.

Inject the reference solution. The test is not valid unless the resolution between the peaks due to (Z)-phytomenadione and (E)-phytomenadione is not less than 1.5 and the relative standard relation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Determine the ratio of the area of sum of the peaks due to Z-isomer and E-isomer and internal standard.

Calculate the content of $C_{31}H_{46}O_2$.

Storage. Store protected from light.

Phytomenadione Injection

Phytomenadione Injection is a sterile solution of Phytomenadione in Water for Injections with auxiliary substances.

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Phytomenadione Injection contains not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of phytomenadione, $C_{31}H_{46}O_2$.

Usual strengths. 1 mg per 0.5 ml; 1 mg per ml; 10 mg per ml.

Description. A clear to slightly opalescent pale yellow coloured liquid, when maintained at a temperature below 25°.

Identification

A. When examined in the range 230 nm to 350 nm (2.4.7), a 0.01 per cent w/v solution in *ethanol* shows absorption maxima at 328 nm and minima at 292 nm.

B. When examined in the range 230 nm to 350 nm (2.4.7), a 0.001 per cent w/v solution in *ethanol* shows absorption maxima at 245, 249, 263 and 271 nm and minima at 256 nm and 266 nm.

Tests

pH (2.4.24). 5.0 to 7.5.

Bacterial endotoxins (2.2.3). Not more than 14.0 Endotoxin Units per mg.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute the volume of injection containing 10 mg of Phytomenadione to 100.0 ml with the mobile phase.

Reference solution. 0.01 per cent w/v of *phytomenadione IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: 98 volumes of *methanol* and 2 volumes of *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 50 µl.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject reference solution and the test solution.

Calculate the content of $C_{31}H_{46}O_2$ in the injection.

Storage. Store protected from light, at a temperature not exceeding 25°. Do not freeze.

Labelling. The label states that the injection should not be used, if separation has occurred or if oil droplets have appeared.

Potassium Chloride

KCl Mol. Wt. 74.6

Potassium Chloride contains not less than 99.0 per cent and not more than 100.5 per cent of KCl, calculated on the dried basis.

Category. Electrolyte replenisher.

Description. Colourless crystals or a white, crystalline powder.

Identification

Dissolve 10 g in 100 ml of *carbon dioxide-free water* prepared from *distilled water* (solution A). The solution gives the reactions of potassium salts and of chlorides (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. 5.0 g dissolved in 50 ml of *carbon dioxide-free water* requires not more than 0.5 ml of *0.01 M sodium hydroxide* or *0.01 M hydrochloric acid* for neutralisation to *bromothymol blue solution*.

Arsenic (2.3.10). Dissolve 10.0 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (1 ppm).

Barium. To 5 ml of solution A add 5 ml of *water* and 1 ml of *1 M sulphuric acid*; the solution, after not less than 15 minutes, is not more opalescent than a mixture of 5 ml of solution A and 6 ml of *water*.

Heavy metals (2.3.13). Dissolve 2.0 g in 10 ml of *water* to which are added 2 ml of *dilute acetic acid* and 13 ml of *water*. The solution complies with the limit test for heavy metals, Method A (10 ppm).

Calcium and magnesium. Dissolve 1 g in 20 ml of *water*, add 1 ml of *dilute ammonia solution* and 1 ml of *sodium phosphate solution*; the solution remains clear.

Iron (2.3.14). 20 ml of solution A complies with the limit test for iron (20 ppm).

Bromides. Dilute 1.0 ml of solution A to 50.0 ml with *water*. To 5.0 ml of the solution add 2.0 ml of *phenol red reagent* and 1.0 ml of a 0.01 per cent solution of *chloramine T* and mix immediately. After exactly 2 minutes, add 0.15 ml of *0.1 M sodium thiosulphate*, mix and dilute to 10.0 ml with *water*. The absorbance of the solution measured at about 590 nm (2.4.7), using *water* as the blank, is not more than that of a standard prepared at the same time and in the same manner, using 5.0 ml of a 0.0003 per cent w/v solution of *potassium bromide* (0.1 per cent).

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Iodides. Moisten 5 g by adding dropwise, a solution freshly prepared by mixing 25 ml of *iodide-free starch solution*, 2 ml of *0.5 M sulphuric acid*, 0.15 ml of *sodium nitrite solution* and 25 ml of *water* and examine the mixture in daylight; the substance shows no blue colour after 5 minutes.

Sulphates (2.3.17). 0.5 g complies with the limit test for sulphates (300 ppm).

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.15 g in 50 ml of *water* and titrate with *0.1 M silver nitrate* using *potassium chromate solution* as indicator.

1 ml of *0.1 M silver nitrate* is equivalent to 0.007455 g of KCl.

Potassium Chloride intended for use in the manufacture of dialysis solutions complies with the following additional requirement.

Aluminium. Dissolve 4.0 g in 100 ml of *water* and add 10 ml of *acetate buffer pH 6.0*. Extract with successive quantities of 20, 20 and 10 ml of a 0.5 per cent w/v solution of *8-hydroxyquinoline* in *chloroform* and dilute the combined extracts to 50 ml with *chloroform*. Use as the standard solution a mixture of 2 ml of *aluminium standard solution (2 ppm Al)*, 10 ml of *acetate buffer pH 6.0* and 98 ml of *water* treated in the same manner and as the blank solution a mixture of 10 ml of *acetate buffer pH 6.0* and 100 ml of *water* treated in the same manner. Measure the fluorescence of the test and standard solutions (2.4.5), using an excitation wavelength of about 392 nm and emission wavelength of about 518 nm and setting the instrument to zero with the blank solution in each case. The fluorescence of the test is not greater than that of the standard solution (1 ppm).

Potassium chloride intended for use in the manufacture of Parenteral Preparations or for the preparation of haemodialysis solution complies with the following additional requirement.

Sodium. Not more than 0.1 per cent, determined by atomic absorption spectrophotometry (2.4.2), using a 1.0 per cent w/v solution and measuring at 589 nm. Use *sodium solution AAS*, suitably diluted with *water*, for the standard solutions.

Storage. Store protected from moisture.

Labelling. The label states whether or not the material is suitable for use in the manufacture of Parenteral Preparations or for the preparation of haemodialysis or dialysis solutions.

Potassium Chloride for Injection

Potassium Chloride for Injection Concentrate

Potassium Chloride for Injection is a sterile solution of Potassium Chloride in Water for Injection.

Potassium Chloride for Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of KCl.

Identification

It gives the reaction of potassium salts (2.3.1) and chlorides (2.3.1).

Tests

pH (2.4.24). 4.0 to 8.0.

Bacterial endotoxins (2.2.3). Not more than 8.8 Endotoxin Units per mEq.

Other tests. Comply with the tests stated under Parenteral Preparations (Concentrate for Injections).

Assay. Determine by atomic absorption spectrophotometery (2.4.2), equipped with a potassium hollow-cathode lamp and an air–acetylene flame.

Potassium stock solution. Dissolve 190.7 mg of potassium chloride, previously dried at 105° for 2 hours in *water* and dilute to 1000 ml with *water*. Dilute 100.0 ml of the solution to 1000 ml with *water*. This solution contains 10 µg of potassium (equivalent to 19.07 µg of potassium chloride) per ml.

Reference solutions. To separate 100-ml volumetric flasks transfer 10.0, 15.0, and 20.0 ml, respectively, of *potassium stock solution*. To each flask, add 2.0 ml of 20 per cent w/v *sodium chloride solution* and 1.0 ml of *hydrochloric acid*, dilute to volume with *water*. The reference solutions contain 1.0 µg, 1.5 µg and 2.0 µg of potassium per ml respectively.

Test solution. Dilute a volume of concentrate containing 600 mg of *potassium chloride* to 500 ml with *water*. Dilute 5.0 ml of the solution to 100 ml with *water*. To 5.0 ml of the solution, add 2.0 ml of 20 per cent w/v *sodium chloride solution* and 1.0 ml of *hydrochloric acid*, dilute to 100 ml with *water*.

Determine the absorbances of the reference solutions and the test solution at the potassium emission line of 766.5 nm. Plot the absorbance of the reference solution versus concentration, in µg per ml, of potassium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration in µg per ml of potassium in the test solution.

Calculate the quantity, in mg, of KCl in the portion of concentrate taken by the formula:

$$200C(1.907)$$

where C is the concentration, in µg per ml, of potassium in the test solution, and 1.907 is the ratio of the molecular weight of potassium chloride to the atomic weight of potassium.

Storage. Store in single-dose or in multiple-dose containers, preferably of Type I or Type II glass.

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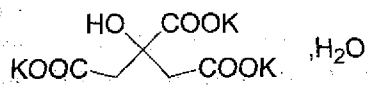
Labelling. The label states the potassium chloride content in terms of weight and of milliequivalents in a given volume. Label the Concentrate to indicate that it is to be diluted to appropriate strength with water or other suitable fluid prior to administration. Immediately following the name, the label bears the boxed warning:

Concentrate must be diluted before use. The cap of the container and the overseal of the cap must be black, and both bear the words: "Must Be Diluted" in readily legible type, in a colour that stands out from its background or the overseal may be of a clear plastic material through which the black cap is visible and the printing is readily legible.

When the nature of the container-closure system prevents compliance, the design shall follow the intent of this requirement as closely as possible, the black colour being used beneath the words "Must be Diluted," which are so placed that words are readily visible as the contents of the container are being removed. Ampules shall be identified by a black band or a series of black bands above the constriction. The label states also the total osmolar concentration in mOsmol per l. Where the contents are less than 100 ml, the label alternatively may state the total osmolar concentration in mOsmol per ml.

Potassium Citrate



$C_6H_5K_3O_7,H_2O$ Mol. Wt. 324.4

Potassium citrate is tripotassium 2-hydroxypropane-1,2,3-tricarboxylate monohydrate.

Potassium Citrate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_6H_5K_3O_7$, calculated on the anhydrous basis.

Category. Systemic alkaliniser.

Description. White, granular crystals or a crystalline powder; hygroscopic.

Identification

A. Dissolve 10 g in 100 ml of *carbon dioxide-free water* prepared from *distilled water* (solution A). 0.5 ml of solution A gives the reactions of potassium salts (2.3.1).

B. To 1 ml of solution A add 4 ml of *water*; the solution gives the reactions of citrates (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. To 10 ml of solution A add 0.1 ml of *dilute phenolphthalein solution*; not more than 0.2 ml of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the solution.

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water* and add 15 ml of *stannated hydrochloric acid*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method A (10 ppm).

Sodium. Not more than 0.3 per cent, determined by atomic absorption spectrophotometry (2.4.2), Method II, measuring at 589 nm. Prepare the test solution by addition of 1 ml of *2 M hydrochloric acid* to 10.0 ml of solution A and diluting to 100.0 ml with *distilled water*. Use *sodium solution AAS*, suitably diluted with *distilled water*, for the standard solutions.

Chlorides (2.3.12). Dilute 25.0 ml of solution A to 35 ml with *water*. The resulting solution complies with the limit test for chlorides (100 ppm).

Oxalate. Dissolve 0.5 g in 4 ml of *water*, add 3 ml of *hydrochloric acid* and 1 g of *granulated zinc* and heat on a water-bath for 1 minute. Allow to stand for 2 minutes, decant into 0.25 ml of a 1 per cent w/v solution of *phenylhydrazine hydrochloride*, heat to boiling, cool rapidly, add an equal volume of *hydrochloric acid* and 0.25 ml of *potassium ferricyanide solution*, shake and allow to stand for 30 minutes. Any pink colour produced is not more intense than that obtained by treating 4.0 ml of a 0.005 per cent w/v solution of *oxalic acid* at the same time and in the same manner (300 ppm, calculated as anhydrous oxalic acid).

Sulphates (2.3.17). To 10.0 ml of solution A add 2 ml of *7 M hydrochloric acid* and dilute to 15 ml with *distilled water*; the solution complies with the limit test for sulphates (150 ppm).

Readily carbonisable substances. Heat 0.20 g, in powder, with 10 ml of *sulphuric acid (96 per cent w/w)* in a water-bath at 90° ± 1° for 60 minutes and cool rapidly. The solution is not more intensely coloured than reference solution YS2 or GYS2 (2.4.1).

Water (2.3.43). 4.0 to 7.0 per cent, determined on 0.5 g, titration being done after stirring for 15 minutes subsequent to addition of the substance under examination.

Assay. Dissolve 0.2 g in 20 ml of *anhydrous glacial acetic* acid, heat to about 50°, allow to cool. Titrate with *0.1 M perchloric acid*, using 0.25 ml of *1-naphtholbenzein solution* as indicator and titrating until a green colour is obtained. Carry out a blank titration.

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1 ml of *0.1 M perchloric acid* is equivalent to 0.01021 g of $C_6H_5K_3O_7$.

Storage. Store protected from moisture.

Potassium Iodide

KI Mol. Wt. 166.0

Potassium Iodide contains not less than 99.0 per cent and not more than 100.5 per cent of KI, calculated on the dried basis.

Category. Antithyroid; antifungal; expectorant.

Description. Colourless crystals or a white powder.

Identification

Dissolve 10 g in 100 ml of *carbon dioxide-free water* (solution A). The solution gives the reactions of potassium salts, and of iodides (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Alkalinity. To 10 ml of solution A add 0.2 ml of *0.01 M sulphuric acid*; no colour is produced on addition of a drop of *phenolphthalein solution*.

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water* and 12 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method A (10 ppm).

Iron (2.3.14). 20 ml of solution A complies with the limit test for iron (20 ppm).

Barium. Dissolve 0.5 g in 10 ml of *water* and add 1 ml of *dilute sulphuric acid*; no turbidity develops within one minute.

Cyanide. Warm 5 ml of Solution A, add one drop of *ferrous sulphate solution* and 0.5 ml of *sodium hydroxide solution* and acidify with *hydrochloric acid*; no blue colour is produced.

Iodate. Dissolve 0.5 g in 10 ml of *carbon dioxide-free water* and add 0.15 ml of *dilute sulphuric acid* and a drop of *iodide-free starch solution*; no blue colour is produced within 2 minutes.

Sulphates (2.3.17). 1.0 g dissolved in 15 ml of *water* complies with the limit test for sulphates (150 ppm).

Thiosulphate. Dissolve 1 g in 10 ml of *water*, add 0.1 ml of *starch solution* and 0.1 ml of *0.005 M iodine*; a blue colour is produced.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g of the powdered substance by drying in an oven at 105° for 3 hours.

Assay. Dissolve 0.35 g in about 10 ml of *water*, add 35 ml of *hydrochloric acid* and 5 ml of *chloroform*. Titrate with *0.05 M potassium iodate* until the purple colour of iodine disappears from the chloroform. Add the last portion of the iodate solution dropwise and agitate vigorously and continuously. Allow to stand for 5 minutes. If any colour develops in the chloroform layer continue the titration until the chloroform is decolorised.

1 ml of *0.05 M potassium iodate* is equivalent to 0.0166 g of KI.

Storage. Store protected from light and moisture.

Potassium Sorbate

$CH_3CH=CHCH=CHCOOK$

$C_6H_7KO_2$ Mol. Wt.150.2

Potassium Sorbate is potassium 2,4-hexadienoate.

Potassium Sorbate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_6H_7KO_2$, calculated on the dried basis.

Category. Food preservative.

Description. A white or almost white powder or granules.

Identification

Test A may be omitted if tests B and C are carried out. Test B may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *potassium sorbate IPRS* or with the reference spectrum of potassium sorbate.

B. When examined in the range 230 nm to 350 nm (2.4.7), a 0.02 per cent w/v solution in *water*, dilute 1.0 ml of the solution to 100.0 ml with *0.1 M hydrochloric acid* shows an absorption maximum at about 264 nm and the specific absorbance at 264 nm is 1650 to 1900.

C. Dissolve 0.2 g in 2 ml of *water* and add 2 ml of *dilute acetic acid*, filter. The solution gives reaction (B) of potassium salts (2.3.1).

Tests

Appearance of solution. A 5 per cent w/v solution in *carbon dioxide-free water* (solution A) is clear (2.4.1) and not more intensely coloured than reference solution YS5 (2.4.1).

Acidity or alkalinity. To 20 ml of solution A, add 0.1 ml of *phenolphthalein solution*. Titrate with *0.1 M sodium*

hydroxide or *0.1 M hydrochloric acid*. Not more than 0.25 ml is required to change the colour of the indicator.

Aldehydes. Not more than 0.15 per cent, determined by dissolving 1.0 g in a mixture of 50 ml of *2-propanol* and 30 ml of *water*, adjusted to pH 4.0 with *1 M hydrochloric acid* and dilute to 100 ml with *water*. To 10 ml of the solution add 1 ml of *decolorised fuchsin solution* and allow to stand for 30 minutes. Any colour produced is not more intense than that obtained in a solution prepared simultaneously by adding by 1 ml of decolorised *fuchsin solution* to a mixture of 1.5 ml of *acetaldehyde standard solution (100 ppm C_2H_4O)*, 4 ml of *2-propanol* and 4.5 ml of *water*.

Heavy metals (2.3.13). Weigh in a silica crucible 2 g of the substance under examination, mix with 0.5 g of *magnesium oxide*. Ignite to dull redness until a homogeneous white or greyish-white mass is obtained. After 30 minutes of ignition if mixture remains coloured, allow to cool, mix using a fine glass rod and repeat the ignition. If necessary repeat the operation. Heat at 800° for about 1 hour. Dissolve the residue in 5 ml of a mixture of equal volumes of *hydrochloric acid* and *water*. Add 0.1 ml *of phenolphthalein solution* and then *concentrated ammonia* until a pink colour is obtained. Cool, add *glacial acetic acid* until the solution is decolourised and add 0.5 ml in excess. Filter if necessary and wash the filter. Dilute to 20 ml with *water*, 12 ml of the resulting solution complies with the limit test for heavy metals, Method D (10 ppm). Use lead standard solution (10 ppm pb), diluting 1 ml standard solution to 10 ml with a mixture of equal volumes of *hydrochloric acid* and *water*.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

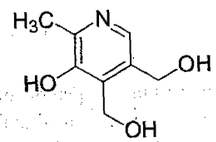
Assay. Dissolve 0.12 g in 20 ml of *anhydrous acetic acid*. Titrate with *0.1 M perchloric acid* using 0.1 ml of *crystal violet solution* as indicator until the colour changes from violet to bluish-green. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01502 g of $C_6H_7KO_2$.

Storage. Store protected from light.

Pyridoxine Hydrochloride

Vitamin B_6



, HCl

$C_8H_{11}NO_3,HCl$ Mol. Wt. 205.6

Pyridoxine Hydrochloride is 5-hydroxy-6-methylpyridine-3,4-dimethanol hydrochloride.

Pyridoxine Hydrochloride contains not less than 99.0 per cent and not more than 101.0 per cent of $C_8H_{11}NO_3,HCl$, calculated on the dried basis.

Category. B group vitamin; sideroblastic anaemia therapy; anti-isoniazid neuropathy.

Description. A white or almost white, crystalline powder.

Identification

Test A may be omitted if tests B, C and D are carried out. Tests B may be omitted if tests A, C and D are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *pyridoxine hydrochloride IPRS* or with the reference spectrum of pyridoxine hydrochloride.

B. When examined in the range 250 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *0.1 M hydrochloric acid* shows an absorption maximum at 288 nm to 296 nm; absorbance at the maximum, 0.420 to 0.445. A solution prepared by diluting 1 ml of a 0.1 per cent w/v solution in *0.1 M hydrochloric acid* to 100 ml with *0.025 M standard phosphate buffer* shows absorption maxima at 248 nm to 256 nm and at 320 nm to 327 nm; absorbances at the maxima, 0.175 to 0.195 and 0.345 to 0.365, respectively.

C. In the test for Related substances, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution (a).

D. A 5.0 per cent w/v solution gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution is clear (2.4.1), and not more intensely coloured than reference solution YS7 (2.4.1).

pH (2.4.24). 2.4 to 3.0, determined in a 5.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 25 mg of the substance under examination in *water* and dilute to 10.0 ml with *water*.

Reference solution (a). A 0.00025 per cent w/v solution of *pyridoxine hydrochloride IPRS* in *water*.

Reference solution (b). A 0.005 per cent w/v solution each of *pyridoxine impurity A IPRS* and pyridoxine impurity B in *water*.

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Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with base-deactivated end-capped octadecylsilane bonded to porous silica (5 µm),
- mobile phase: dissolve 2.72 g of *potassium dihydrogen phosphate* in 900 ml of *water*, adjusted to pH 3.0 with *dilute orthophosphoric acid* and dilute to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 5 µl.

Name	Relative retention time	Correction factor
Pyridoxine (Retention time: about 12 minutes)	1.0	—
Pyridoxine impurity A[1]	1.7	—
Pyridoxine impurity B[2]	1.9	1.5

[1]6-methyl-1,3-dihydrofuro[3,4-*c*]pyridin-7-ol,

[2]5-(hydroxymethyl)-2,4-dimethylpyridin-3-ol.

For identification of impurities, use the chromatogram obtained with reference solution (b) to identify the peaks due to pyridoxine impurities A and B.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to pyridoxine impurities A and B is not less than 1.5.

Inject reference solution (a) and the test solution. Run the chromatogram 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any secondary peak corresponding to pyridoxine impurity B is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of areas of all the secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 12 ml of a 5.0 per cent w/v solution complies with the limit test for heavy metals, Method D (20 ppm). Use *lead standard solution (1 ppm Pb)* to prepare the standard.

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.15 g in a mixture of 5 ml of *anhydrous glacial acetic acid* and 6 ml of *mercuric acetate solution*. Titrate with *0.1 M perchloric acid*, using *crystal violet solution* as indicator, until a green colour is produced. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02056 g of $C_8H_{11}NO_3$, HCl.

Storage. Store protected from moisture.

Pyridoxine Tablets

Pyridoxine Hydrochloride Tablets

Pyridoxine Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of pyridoxine hydrochloride, $C_8H_{11}NO_3$, HCl.

Usual strength. 5 mg.

Identification

A. Shake a quantity of the powdered tablets containing 20 mg of Pyridoxine Hydrochloride with 50 ml of *0.025 M standard phosphate buffer* for 15 minutes and dilute to 100 ml with the same solvent. Mix, filter and dilute 5 ml of the filtrate to 100 ml with the same solvent. When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution exhibits two maxima, at about 254 nm and 324 nm.

B. Triturate a quantity of the powdered tablets containing 20 mg of Pyridoxine Hydrochloride with 50 ml of *water* and allow to stand for 20 minutes. To 1 ml of the supernatant liquid add 10 ml of a 5 per cent w/v solution of *sodium acetate*, 1 ml of *water* and 1 ml of a 0.5 per cent w/v solution of *2,6-dichloroquinone-4-chloroimide* in *ethanol (95 per cent)* and shake; a blue colour is produced which fades rapidly and becomes brown. Repeat the operation but adding 1 ml of a 0.3 per cent w/v solution of *boric acid* in place of 1 ml of *water*; no blue colour is produced.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),

Medium. 900 ml of *water*,

Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a suitable quantity of *pyridoxine hydrochloride IPRS* in the dissolution medium to obtain a solution of a known concentration similar to the expected concentration of the test solution.

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Chromatographic system
- a stainless steel column 30 cm × 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 73 volumes of 0.14 per cent w/v solution of *sodium 1-hexane sulphonate* in *water*, 27 volumes of *methanol* and 1 volume of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_8H_{11}NO_3$,HCl in the medium.

Q. Not less than 75 per cent of the stated amount of $C_8H_{11}NO_3$,HCl.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 65 volumes of *acetone*, 13 volumes of *dichloromethane*, 13 volumes of *tetrahydrofuran* and 9 volumes of *strong ammonia solution*.

Test solution. Shake a quantity of the powdered tablets containing 40 mg of Pyridoxine Hydrochloride with 10.0 ml of *water* for 15 minutes, filter and use the filtrate.

Reference solution. Dilute 1.0 ml of the test solution to 200.0 ml with *water*.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and spray with a 5 per cent w/v solution of *sodium carbonate* in a mixture of 70 volumes of *water* and 30 volumes of *ethanol (95 per cent)*. Dry it in a current of air, spray with a 0.1 per cent w/v solution of *2,6-dichloroquinone-4-chloroimide* in *ethanol (95 per cent)* and examine immediately. Any secondary spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Uniformity of content. Complies with the test stated under Tablets.

Powder one tablet, add 50 ml of *0.1 M hydrochloric acid* and heat on a water-bath for 15 minutes, swirling occasionally. Cool, dilute to 100.0 ml with *0.1 M hydrochloric acid*, filter, discarding the first 20 ml of the filtrate. If necessary, dilute quantitatively and stepwise with *0.1 M hydrochloric acid* to produce a solution containing 10 µg of the pyridoxine hydrochloride per ml and measure the absorbance of the resulting solution at the maximum at about 290 nm (2.4.7). Calculate the content of $C_8H_{11}NO_3$, HCl taking 430 as the specific absorbance at 290 nm.

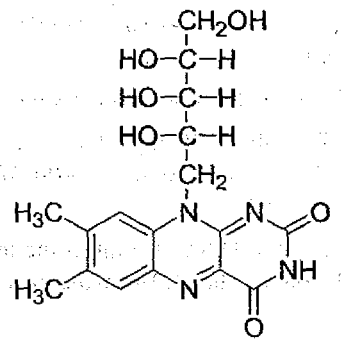
Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 20 mg of Pyridoxine Hydrochloride, add 50 ml of *0.1 M hydrochloric acid* and heat on a water-bath for 15 minutes, swirling occasionally. Cool, dilute to 100.0 ml with *0.1 M hydrochloric acid* and filter, discarding the first 20 ml of the filtrate. Dilute 5.0 ml of the filtrate to 100.0 ml with *0.1 M hydrochloric acid* and measure the absorbance of the resulting solution at the maximum at about 290 nm (2.4.7). Calculate the content of $C_8H_{11}NO_3$, HCl taking 430 as the specific absorbance at 290 nm.

Storage. Store protected from light and moisture.

Riboflavin

Lactoflavin; Vitamin B_2



$C_{17}H_{20}N_4O_6$ Mol. Wt. 376.4

Riboflavin is 3,10-dihydro-7,8-dimethyl-10-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl]benzo[*g*]pteridine-2,4-dione.

Riboflavin contains not less than 98.0 per cent and not more than 101.0 per cent of $C_{17}H_{20}N_4O_6$, calculated on the dried basis.

Category. B-group vitamin.

Description. A yellow to orange-yellow, crystalline powder.

Identification

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *riboflavin IPRS* or with the reference spectrum of riboflavin.

B. Dissolve about 1 mg in 100 ml of *water*. The solution has a pale greenish yellow colour by transmitted light and an intense yellowish green fluorescence by reflected light, which disappears on addition of mineral acids or alkalis.

Tests

pH (2.4.24). 5.5 to 7.2, determined in a saturated solution.

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Specific optical rotation (2.4.22). –135° to –115°, determined in a 0.5 per cent w/v solution in carbonate-free *0.05 M sodium hydroxide*. Measure the angle of rotation within 30 minutes of preparing the solution.

Light absorption (2.4.7). Dilute a suitable volume of the final solution obtained in the Assay with an equal volume of *water*.

When examined in the range 210 nm to 460 nm, the resulting solution exhibits maxima at about 223 nm, 267 nm, 373 nm and 444 nm; the ratio of the absorbance at the maximum at about 373 nm to that at about 267 nm, 0.31 to 0.33 and the ratio of the absorbance at the maximum at about 444 nm to that at about 267 nm, 0.36 to 0.39.

Lumiflavine. Shake 25 mg with 10 ml of *chloroform* for 5 minutes and filter. The filtrate is not more intensely coloured than reference solution BYS6 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Prepare the solutions immediately before use and protect from light.

Solution A. 1.36 per cent w/v solution of *sodium acetate*.

Test solution. Dissolve 120 mg of the substance under examination in 10 ml of *0.1 M sodium hydroxide* and dilute to 100.0 ml with solution A.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with solution A. Dilute 1.0 ml of the solution to 100.0 ml with solution A.

Reference solution (b). Dissolve 10 mg of the substance under examination in 1 ml of *0.5 M sodium hydroxide*, expose to daylight for 90 minutes. Add 0.5 ml *acetic acid* and dilute to 100.0 ml with *water* (In order to generate impurity A and B).

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a 0.1 per cent v/v of *orthophosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 267 µm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
5	90	10
20	80	20
25	80	20
35	50	50
45	50	50
50	90	10

Name	Relative retention time	Correction factor
Riboflavin impurity C[1]	0.2	2.3
Riboflavin impurity D[2]	0.5	1.4
Riboflavin (Retention time: about 16 minutes)	1.0	—
Riboflavin impurity A[3]	1.4	0.7
Riboflavin impurity B[4]	1.9	1.4

[1]6,7-dimethyl-8-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl]pteridine-2,4(3*H*,8*H*)-dione,

[2]8-(hydroxymethyl)-7-methyl-10-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl]benzo[g]pteridine-2,4(3*H*,10*H*)-dione,

[3]7,8,10-trimethylbenzo[g]pteridine-2,4(3*H*,10*H*)-dione(lumiflavine),

[4]7,8-dimethylbenzo[g]pteridine-2,4(1*H*,3*H*)-dione.

Inject reference solution (b). The test is not valid unless the resolution between the peak due to riboflavin impurity A and riboflavin impurity B is not less than 5.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to riboflavin impurity A is not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.025 per cent), the area of any peak corresponding to riboflavin impurity B, riboflavin impurity C, riboflavin impurity D, each of, is not more than twice the area of principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Ignore any peak,other than riboflavin impurity A,with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.5 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. *Carry out the procedure in subdued light.*

Weigh 65 mg and transfer to an amber-glass 500-ml volumetric flask, suspend in 5 ml of *water*, ensuring that it is completely wetted. Dissolve in 5 ml of *2 M sodium hydroxide*. As soon as dissolution is complete add 100 ml of *water* and 2.5 ml of *glacial acetic acid* and dilute to 500.0 ml with *water*. To 20.0 ml of the solution add 3.5 ml of a 1.4 per cent w/v solution of *sodium acetate* and dilute to 200.0 ml with *water*. Measure the absorbance of the resulting solution at the maximum at about 444 nm (2.4.7).

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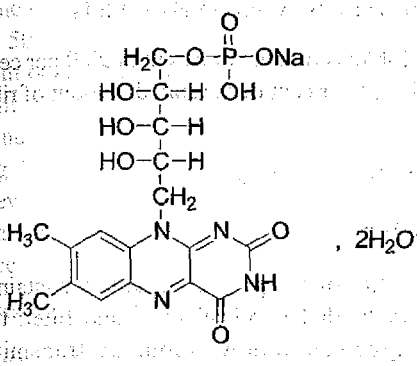
Calculate the content of $C_{17}H_{20}N_4O_6$ taking 328 as the specific absorbance at 444 nm.

Storage. Store protected from light and moisture.

Riboflavin Sodium Phosphate

Riboflavine-5-phosphate (Sodium Salt); Vitamin B_2 Sodium Phosphate



$C_{17}H_{20}N_4NaO_9P, 2H_2O$ Mol. Wt. 514.4

Riboflavin Sodium Phosphate is monosodium 3,10-dihydro-7,8-dimethyl-10-[(2*S*,3*S*,4*R*)-2,3,4-trihydroxypentyl] benzo[*g*]pteridine-2,4-dione 5-phosphate dihydrate.

Riboflavin Sodium Phosphate contains the equivalent of not less than 73.0 per cent and not more than 79.0 per cent of $C_{17}H_{20}N_4O_6$, calculated on the dried basis.

Category. B-group vitamin.

Description. A yellow to orange-yellow, crystalline powder; hygroscopic.

Identification

A. When examined in the range 230 nm to 360 nm (2.4.7), a 0.001 per cent w/v solution in *phosphate buffer pH 7.0* shows an absorption maximum at about 267 nm; absorbance at about 267 nm, 0.58 to 0.64.

B. Dissolve about 10 mg in sufficient *2 M sodium hydroxide* to produce 100 ml, expose 1 ml to ultraviolet light at 254 nm for 5 minutes, add sufficient *5 M acetic acid* to make the solution acidic to *litmus paper* and shake the mixture with 2 ml of *dichloromethane*; the lower layer exhibits a yellow fluorescence.

C. To 0.5 g add 10 ml of *nitric acid*, evaporate the mixture to dryness on a water-bath, ignite the residue until the carbon is removed, dissolve the final residue in 5 ml of *water* and filter. The filtrate gives the reactions of sodium salts and reaction (B) of phosphates (2.3.1).

Tests

pH (2.4.24). 4.0 to 6.3, determined in a 2.0 per cent w/v solution.

Specific optical rotation (2.4.22). +38.0° to +42.0°, determined in a 1.5 per cent w/v solution in *5 M hydrochloric acid*.

Lumiflavine. Shake 35 mg with 10 ml of *dichloromethane* for 5 minutes and filter. The filtrate is not more intensely coloured than reference solution BYS6 (2.4.1).

Related substances. Determine by liquid chromatography (2.4.14).

NOTE — Protect the solutions from actinic light.

Test solution. Dissolve 100 mg of the substance under examination in 50 ml of *water* and dilute to 100.0 ml with the mobile phase. Dilute 8.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 60 mg of *riboflavin IPRS* in 1 ml of *hydrochloric acid* and dilute to 250.0 ml with *water*. Dilute 4.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 100 mg of *riboflavin sodium phosphate IPRS* in 50 ml of *water* and dilute to 100.0 ml with the mobile phase. Dilute 8.0 ml of the solution to 50.0 ml with the mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 85 volumes of 0.73 per cent w/v solution of *potassium dihydrogen orthophosphate* and 15 volumes of *methanol*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 266 nm,
- injection volume: 100 µl.

Name	Relative retention time
Riboflavin impurity A[1]	0.2
Riboflavin impurity B[2]	0.3
Riboflavinimpurity C[3]	0.5
Riboflavin 3′-monophosphate	0.7
Riboflavin 4′-monophosphate	0.9
Riboflavin 5′-monophosphate (Retention time: about 20 minutes)	1.0
Riboflavin impurity D[4]	2.0

[1]riboflavin 3′, 4′-diphosphate,
[2]riboflavin 3′, 5′-diphosphate,
[3]riboflavin 4′, 5′-diphosphate,
[4]riboflavin.

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Inject reference solution (b). The test is not valid unless the resolution between the peak due to riboflavin 4′-monophosphate and riboflavin 5′-monophosphate is not less than 1.5.

Calculate the percentage content of free riboflavin (impurity D) and of riboflavin in the form of the diphosphates of riboflavin (impurities A,B,C) from the areas of the peaks in the chromatogram obtained with the test solution and amount of free riboflavin in reference solution (a).

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to riboflavin impurity D is not more than the area of principal peak in the chromatogram obtained with reference solution (a) (6.0 per cent) and the sum of the areas of impurity A, B and C is not more than the area of the principal peak the chromatogram obtained with reference solution (a) (6.0 per cent).

Inorganic phosphate. Not more than 1.5 per cent, determined by the following method. Dissolve 0.1 g in sufficient *water* to produce 100 ml. Dilute 5 ml with 10 ml of *water* and add 5 ml of *buffered cupric sulphate solution pH 4.0*, 2 ml of a 3 per cent w/v solution of *ammonium molybdate*, 1 ml of a freshly prepared solution containing 2 per cent w/v of *4-methyl-aminophenol sulphate* and 5 per cent w/v of *sodium meta-bisulphite* and 1 ml of a 3 per cent v/v solution of *perchloric acid*. Add sufficient *water* to produce 25 ml, mix and measure the absorbance of the resulting solution at the maximum at about 800 nm (2.4.7), within 15 minutes of its preparation, using as the blank a solution prepared in the same manner but omitting the substance under examination. The absorbance is not greater than that produced by repeating the operation using a solution prepared in the same manner using 15 ml of *phosphate standard solution (5 ppm PO_4)* and beginning at the words "add 5 ml of *buffered cupric sulphate solution pH 4.0*,"

Heavy metals (2.3.13). To 2.0 g in a silica crucible add 2 ml of *nitric acid* dropwise followed by 0.25 ml of *sulphuric acid*. Heat cautiously until white fumes are evolved and ignite. Extract the cooled residue with two quantities, each of 2 ml, of *hydrochloric acid* and evaporate the extracts to dryness. Dissolve the residue in 2 ml of *2 M acetic acid* and dilute to 20 ml with *water*. 12 ml of the solution complies with the limit test for heavy metals, Method D (10 ppm). Use 1.0 ml of *lead standard solution (10 ppm Pb)* to prepare the standard.

Loss on drying (2.4.19). Not more than 8.0 per cent, determined on 0.5 g by drying in an oven at 100° at a pressure not exceeding 0.7 kPa for 5 hours.

Assay. *Carry out the procedure protected from light.*

Dissolve 0.1 g in 150 ml of *water*, add 2 ml of *glacial acetic acid* and dilute to 1000.0 ml with *water*. To 10.0 ml add 3.5 ml of a 1.4 per cent w/v solution of *sodium acetate*, dilute to 50.0 ml with *water* and measure the absorbance of the resulting solution at the maximum at about 444 nm (2.4.7). Calculate the percentage content of $C_{17}H_{20}N_4O_6$ taking 328 as the specific absorbance at 444 nm.

Storage. Store protected from light and moisture.

Riboflavin Tablets

Lactoflavin Tablets; Vitamin B_2 Tablets

Riboflavin Tablets contain not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of riboflavine, $C_{17}H_{20}N_4O_6$.

Usual strengths. 2 mg; 5 mg.

Identification

Shake a quantity of the powdered tablets containing 1 mg of Riboflavine with 100 ml of *water* and filter; the filtrate has a pale greenish yellow colour by transmitted light and an intense yellowish green fluorescence by reflected light, which disappears on addition of mineral acids or alkalis.

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a suitable quantity of *riboflavin IPRS* in the dissolution medium to obtain a solution of a known concentration similar to the expected concentration of the test solution.

Chromatographic system
- a stainless steel column 30 cm × 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 73 volumes of 0.14 per cent w/v solution of *sodium 1-hexane sulphonate* in *water*, 27 volumes of *methanol* and 1 volume of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

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Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of $C_{17}H_{20}N_4O_6$ in the medium.

Q. Not less than 75 per cent of the stated amount of $C_{17}H_{20}N_4O_6$.

Uniformity of content. Complies with the test stated under Tablets.

Powder one tablet, add a mixture of 2.5 ml of *glacial acetic acid* and 50 ml of *water* and heat on a water-bath for 1 hour with occasional stirring. Dilute with 50 ml of *water*, add 15 ml of *1 M sodium hydroxide* with continuous stirring and then sufficient *water* to produce a solution containing 10 µg of Riboflavine per ml. Filter and discard the first few ml of the filtrate. On the clear filtrate carry out the Assay beginning at the words "Measure the absorbance....".

Other tests. Comply with the tests stated under Tablets.

Assay. *Carry out the procedure in subdued light.*

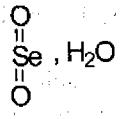
Weigh and powder 20 tablets. Weigh a quantity of the powder containing about 10 mg of Riboflavine, add a mixture of 5 ml of *glacial acetic acid* and 100 ml of *water* and heat on a water-bath for 1 hour with occasional shaking. Dilute with 50 ml of *water*, cool, add 30 ml of *1 M sodium hydroxide* with continuous stirring. Add sufficient *water* to produce 1000.0 ml, mix and filter, discarding the first few ml of the filtrate. Measure the absorbance of the filtrate at the maximum at about 444 nm (2.4.7).

Calculate the content of $C_{17}H_{20}N_4O_6$ taking 328 as the specific absorbance at 444 nm.

Storage. Store protected from light and moisture.

Selenious Acid

Selenium Dioxide monohydrate



H_2SeO_3 Mol. Wt. 129.0

Selenious Acid contains not less than 93.0 per cent and not more than 101.0 per cent of H_2SeO_3.

Category. Oxidizing agent.

Description. A white crystalline powder.

Identification

A. Dissolve 50 mg of substances under examination in 5 ml of *water*, add 100 mg of *sodium bicarbonate* and mix, Gas bubbles develop.

B. Dissolve 50 mg of substances under examination in 5 ml of *0.1 M hydrochloride acid*, add 50 mg of *stannous chloride*. A curdy tan-orange precipitate is formed.

Tests

Insoluble matter. Dissolve 1 g in 5 ml of *water*; it dissolves completely and the solution is clear.

Selenate and sulphate. Dissolve 0.5 g in 10 ml of *water*. Add 0.1 ml of *hydrochloride acid*, 1 ml of *barium chloride*, and mix. No turbidity or precipitate is formed in 10 minutes.

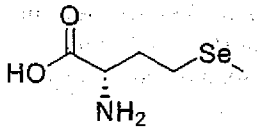
Sulphated ash (2.3.18). Not more than 0.01 per cent, determined on 10.0 g.

Assay. Dissolve 100 mg of substance under examination in 50 ml of *water* in a suitable glass-stopper flask. Add 10 ml of 30 per cent w/v solution of *potassium iodide* and 5 ml of *hydrochloric acid*, and mix. Insert the stopper in the flask, and allow to stand for 10 minutes. Add 50 ml of *water* and 3 ml of *starch solution*. Titrate with *0.1 M sodium thiosulphate* until the solution is colorless, and then titrate with *0.05 M iodine* to a blue endpoint. Subtract the volume of *0.05 M iodine* from the volume of *0.1 M sodium thiosulphate* to obtain the volume of *0.1 M sodium thiosulphate* equivalent to selenious acid.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 0.003225 g of H_2SeO_3.

Storage. Store protected from moisture.

Selenomethionine



$C_5H_{11}NO_2Se$ Mol. Wt. 196.1

Selenomethionine is Butanoic acid, 2-amino-4-(methylseleno)-, (S)-.

Selenomethionine contains not less than 97.0 per cent and not more than 103.0 per cent of $C_5H_{11}NO_2Se$ and contains not less than 39.0 per cent and not more than 41.0 per cent of selenium calculated on as is basis.

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Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *selenomethionine IPRS* or with the reference spectrum of selenomethionine.

Tests

Specific optical rotation (2.4.22). + 17.0° to + 19.5°, determined in 1.0 per cent w/v solution in *1M hydrochloric acid*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 40 volumes of *butanol*, 10 volumes of *water* and 10 volumes of *glacial acetic acid*.

Test solution. Dissolve 50 mg of the substance under examination in 2 ml of *water*, warm to dissolve if necessary and dilute to 10.0 ml with *methanol*.

Reference solution (a). Dissolve 50 mg of *selenomethionine IPRS* in 2 ml of *water*, warm to dissolve if necessary and dilute to volume with *methanol*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *methanol*.

Apply the plate 10 µl of each solution. After development, dry the plate in air and spray with 0.2 per cent w/v solution of *ninhydrin* in *ethanol* and heat at 100° for about 10 minutes. The R_f value of the principal spot of the test solution corresponds to that of reference solution (a) and any secondary spot is not larger or more intense than the spot in the chromatogram obtained with reference solution (b).

Sodium. Not more than 0.1 per cent.

Determine by atomic absorption spectrometry (2.4.2), using Sodium hollow-cathode lamp at 589 nm.

Test solution. Dissolve 100 mg of the substance under examination in 2.0 ml of 20 per cent w/v solution of *potassium chloride* and 1.0 ml of *hydrochloric acid*, dilute to 100.0 ml with *water*.

Reference solution (a). Dissolve a quantity of *sodium chloride* (previously dried at 105° for 2 hours) in *water* and dilute with *water* to obtain a solution containing 0.001 per cent w/v of sodium.

Reference solution (b). Transfer 2.0 ml, 5.0 ml and 10.0 ml of reference solution (a) to separate add 100-ml volumetric flask. To each flask, add 2.0 ml of 20 per cent w/v solution of *potassium chloride* and 1.0 ml of *hydrochloric acid* and dilute to volume with *water*.

Inject reference solution (b) and the test solution. Determine the absorbance of the solution. Plot the absorbance of the reference solution versus their concentration and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration of Na in the test solution.

Calculate the percentage of Na in the selenomethionine.

Selenium. Determine by atomic absorption spectrometry (2.4.2), using Selenium hollow-cathode lamp at 196 nm.

NOTE — Selenium is toxic; handle it with care.

Test solution. Dissolve 12.5 mg of the substance under examination in *water* and dilute to 100.0 ml with *water*.

Reference solution (a). Dissolve 1.0 g of metallic selenium in a minimum volume of *nitric acid*. Evaporate to dryness, add 2 ml of *water*, and evaporate to dryness. Repeat the addition of *water* and evaporation to dryness three times. Dissolve the residue in *3 M hydrochloric acid*, transfer to a 1000-ml volumetric flask, and dilute with *3 M hydrochloric acid* to volume (This solution contains 0.1 per cent w/v of selenium).

Reference solution (b). Transfer 2.0 ml, 5.0 ml and 10.0 ml of reference solution (a) to separate 100-ml volumetric flasks and dilute to volume with *water*.

Inject reference solution (b) and the test solution. Determine the absorbance of the solution. Plot the absorbance of the reference solution versus their concentration and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration of Se in the test solution.

Calculate the percentage of Se in the selenomethionine.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 16 mg of the substance under examination in the mobile phase, with the aid of ultrasound and dilute to 100.0 ml with the mobile phase.

Reference solution (a). A solution containing 0.016 per cent w/v of *selenomethionine IPRS* and 0.08 per cent w/v *L-methionine IPRS* in the mobile phase.

Reference solution (b). A 0.016 per cent w/v solution of *selenomethionine IPRS* in the mobile phase.

Chromatographic system

- a stainless steel column of 25 cm x 4.6 mm, packed with end-capped octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a buffer solution prepared by dissolving 6.8 g of *monobasic potassium phosphate* in 1000 ml of *water*, adjusted to pH 2.75 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

The relative retention time for methionine with reference to selenomethionine is about 0.8.

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Inject reference solution (a) and (b). The test is not valid unless the resolution between the peaks due to methionine and selenomethionine is not less than 3.0, the tailing factor for selenomethionine peak is not more than 2.0 in the chromatogram obtained with reference solution (a) and the relative standard deviation for replicate injections is not more than 2.0 per cent in the chromatogram obtained with reference solution (b)

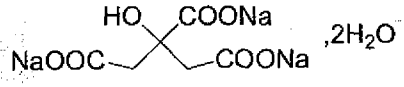
Inject reference solution (b) and the test solution.

Calculate the content of $C_5H_{11}NO_2Se$.

Storage. Store protected from moisture.

Sodium Citrate

Trisodium Citrate



$C_6H_5Na_3O_7,2H_2O$ Mol. Wt. 294.1

Sodium Citrate is trisodium 2-hydroxypropane-1,2,3-tricarboxylate dihydrate.

Sodium Citrate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_6H_5Na_3O_7$, calculated on the anhydrous basis.

Category. Systemic alkalinising agent.

Description. White, granular crystals or a white, crystalline powder.

Identification

A 10.0 per cent w/v solution in *carbon dioxide-free water* (solution A) gives the reactions of sodium salts and reactions of citrates (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

Acidity or alkalinity. Titrate 20 ml of solution A with *0.05 M sulphuric acid* or *0.1 M sodium hydroxide* using *thymol blue solution* as indicator; not more than 0.5 ml of *0.05 M sulphuric acid* or *0.1 M sodium hydroxide* is required.

Arsenic (2.3.10). Dissolve 5.0 g in 50 ml of *water* and add 15 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (2 ppm).

Heavy metals (2.3.13). Dissolve 2.0 g in 10 ml of *water*, 5 ml of *dilute hydrochloric acid* and sufficient *water* to produce 25 ml. The solution complies with the limit test for heavy metals, Method A (10 ppm).

Chlorides (2.3.12). 25 ml of solution A complies with the limit test for chlorides (100 ppm).

Oxalate. Dissolve 0.5 g in 4 ml of *water*, add 3 ml of *hydrochloric acid* and 1 g of *zinc*, in granules, and heat on a water-bath for 1 minute. Allow to stand for 2 minutes, decant the liquid into a test-tube containing 0.25 ml of a 1 per cent w/v solution of *phenylhydrazine hydrochloride* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid* and 0.25 ml of *potassium ferricyanide solution*. Shake and allow to stand for 30 minutes. Any pink colour produced is not more intense than that obtained by treating at the same time and in the same manner 4 ml of a 0.005 per cent w/v solution of *oxalic acid* (300 ppm, calculated as anhydrous oxalic acid).

Sulphates (2.3.17). To 10 ml of solution A add 2 ml of *7 M hydrochloric acid* and dilute to 15 ml with *distilled water*; the resulting solution complies with the limit test for sulphates (150 ppm).

Tartrates. To a solution of 1 g in 2 ml of *water* in a test-tube, add 1 ml of a 10 per cent w/v solution of *potassium acetate* and 1 ml of *6 M acetic acid*. Scratch the walls of the test-tube with a glass rod; no crystalline precipitate is formed.

Readily carbonisable substances. Heat 0.2 g, in powder, with 10 ml of *sulphuric acid (96 per cent w/w)* in a water-bath at 90° ± 1° for 1 hour and cool rapidly. The solution is not more intensely coloured than reference solution YS2 or GYS2 (2.4.1).

Water (2.3.43). 11.0 to 13.0 per cent, determined on 0.3 g, using a mixture of 20 ml of *methanol*, 30 ml of *formamide* and 5 g of *salicylic acid*.

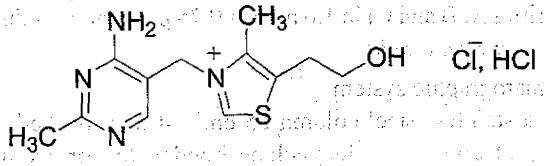
Assay. Dissolve 0.15 g in 20 ml of *anhydrous glacial acetic acid*, warming to about 50°. Allow to cool. Titrate with *0.1 M perchloric acid*, using 0.25 ml of *1-naphtholbenzein solution* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.008602 g of $C_6H_5Na_3O_7$.

Storage. Store protected from light.

Thiamine Hydrochloride

Aneurine Hydrochloride; Vitamin B_1



$C_{12}H_{17}ClN_4OS,HCl$ Mol. Wt. 337.3

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Thiamine Hydrochloride is 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium chloride hydrochloride.

Thiamine Hydrochloride contains not less than 98.5 per cent and not more than 101.5 per cent of $C_{12}H_{17}ClN_4OS,HCl$, calculated on the dried basis.

Category. B-group vitamin.

Description. A white or almost white, crystalline powder or small colourless crystals.

Identification

Test A may be omitted if tests B and C are carried out. Test B may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *thiamine hydrochloride IPRS* or with the reference spectrum of thiamine hydrochloride.

B. Dissolve about 20 mg in 10 ml of *water*, add 1 ml of *2 M acetic acid* and 1.6 ml of *1 M sodium hydroxide*, heat on a water-bath for 30 minutes and allow to cool. Add 5 ml of *2 M sodium hydroxide*, 10 ml of *potassium ferricyanide solution* and 10 ml of *1-butanol* and shake vigorously for 2 minutes. The upper layer exhibits an intense light blue fluorescence, particularly under ultraviolet light at 365 nm. Repeat the test but adding 0.9 ml of *1 M sodium hydroxide* and 0.2 g of *sodium sulphite* in place of the 1.6 ml of *1 M sodium hydroxide*; practically no fluorescence is produced.

C. It gives reaction (A) of chlorides (2.3.1).

Tests

Appearance of solution. A 5.0 per cent w/v solution is clear (2.4.1), and not more intensely coloured than reference solution YS7 or GYS6 (2.4.1).

pH (2.4.24). 2.7 to 3.3, determined in a 2.5 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.35 g of the substance under examination in 15.0 ml of 5 per cent v/v solution of *glacial acetic acid* and dilute to 100.0 ml with *water*.

Reference solution (a). A 0.00035 per cent w/v solution of *thiamine hydrochloride IPRS* in *water*.

Reference solution (b). Dissolve the contents of a vial of *thiamine for system suitability IPRS* (containing thiamine impurities A, B and C) in 1.0 ml of a 0.75 per cent v/v solution of *glacial acetic acid*.

Chromatographic system

- a stainless steel column 25 cm × 4 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: A. a 0.38 per cent w/v solution of *sodium hexanesulphonate*, adjusted to pH 3.1 with *orthophosphoric acid*,
  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 248 nm,
- injection volume: 25 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
2	90	10
27	70	30
35	50	50
42	50	50
45	90	10

Name	Relative retention time
Thiamine impurity A[1]	0.3
Thiamine impurity B[2]	0.9
Thiamine (Retention time: about 30 minutes)	1.0
Thiamine impurity C[3]	1.2

[1]2-[3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium-5yl]ethyl sulfate (thiamine sulfate ester),

[2]3-[(4-aminopyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-,3-thiazol-3-ium (desmethylthiamine),

[3]3-[(4-amino-2-methylpyrimidin-5-yl)methyl-5-(2-chloroethyl)-4-methyl-1,3-thiazol-3-ium (chlorothiamine).

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to thiamine impurity B and thiamine is not less than 3.0 and the resolution between the peaks due to thiamine and thiamine impurity C is not less than 2.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to thiamine impurity B is not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent), the area of any peak corresponding to thiamine impurity A and thiamine impurity C, each of, is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per

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cent). Ignore any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method A (20 ppm).

Nitrates. To 2 ml of a 2.0 per cent w/v solution add 2 ml of *sulphuric acid*, cool and superimpose 2 ml of *ferrous sulphate solution*; no brown ring is produced at the junction of the two layers.

Sulphates (2.3.17). 0.5 g complies with the limit test for sulphates (300 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Dissolve 0.15 g in 5 ml of *anhydrous formic acid*, add 65 ml of *anhydrous glacial acetic acid* and 10 ml of *mercuric acetate solution*, with stirring. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01686 g of $C_{12}H_{17}ClN_4OS,HCl$.

Storage. Store protected from light and moisture, non-metallic containers.

Thiamine Injection

Thiamine Hydrochloride Injection; Aneurine Hydrochloride Injection; Vitamin B_1 Injection

Thiamine Injection is a sterile solution of Thiamine Hydrochloride in Water for Injection.

Thiamine Injection contains not less than 95.0 per cent and not more than 110.0 per cent of the stated amount of thiamine hydrochloride, $C_{12}H_{17}ClN_4OS,HCl$.

Usual strength. 100 mg per ml.

Identification

A. To a volume containing 20 mg of Thiamine Hydrochloride in 10 ml of *water*, add 1 ml of *2 M acetic acid* and 1.6 ml of *1 M sodium hydroxide*, heat on a water-bath for 30 minutes and allow to cool. Add 5 ml of *2 M sodium hydroxide*, 10 ml of *potassium ferricyanide solution* and 10 ml of *1-butanol* and shake vigorously for 2 minutes. The upper layer exhibits an intense light blue fluorescence, particularly under ultraviolet light at 365 nm. Repeat the test but adding 0.9 ml of *1 M sodium hydroxide* and 0.2 g of *sodium sulphite* in place of the 1.6 ml of *1 M sodium hydroxide*; practically no fluorescence is produced.

B. To a mixture of 0.1 ml of *nitrobenzene* and 0.2 ml of *sulphuric acid* add a volume containing 5 mg of Thiamine Hydrochloride. Allow to stand for 10 minutes, cool in ice and add slowly with stirring 5 ml of *water* followed by 5 ml of *10 M sodium hydroxide*. Add 5 ml of *acetone* and allow to stand; no violet colour is produced in the upper layer.

Tests

pH (2.4.24). 2.5 to 4.5.

Bacterial endotoxins (2.2.3). Not more than 3.5 Endotoxin Units per mg of thiamine hydrochloride.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dilute a volume of the injection containing about 0.1 g of Thiamine Hydrochloride to 100.0 ml with *0.1 M hydrochloric acid* and further dilute 5.0 ml to 100.0 ml with *water*.

Reference solution. A 0.005 per cent w/v solution of *thiamine mononitrate IPRS* in *0.005 M hydrochloric acid*.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a solution prepared by dissolving 1 g of *sodium heptanesulphonate* in a mixture of 180 ml of *methanol* and 10 ml of *triethylamine*, diluting to 1000 ml with *water* and adjusted to pH 3.2 with *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 244 nm,
- injection volume: 20 µl.

Calculate the content of $C_{12}H_{17}ClN_4OS,HCl$ in the injection.

1 mg of $C_{12}H_{17}N_5O_4S$ is equivalent to 1.030 mg of $C_{12}H_{17}ClN_4OS,HCl$.

Storage. Store protected from light.

Thiamine Tablets

Thiamine Hydrochloride Tablets; Aneurine Hydrochloride Tablets; Vitamin B_1 Tablets

Thiamine Tablets contain not less than 92.5 per cent and not more than 110.0 per cent of the stated amount of thiamine hydrochloride, $C_{12}H_{17}ClN_4OS,HCl$.

Usual strengths. 5 mg; 10 mg; 50 mg.

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Identification

A. Dissolve a quantity of the powdered tablets containing 20 mg of Thiamine Hydrochloride as completely as possible in 10 ml of *water* and 2 ml of *1 M acetic acid* and filter. Add 5 ml of *2 M sodium hydroxide*, 10 ml of *potassium ferricyanide solution* and 10 ml of *1-butanol* and shake vigorously for 2 minutes. The upper layer exhibits an intense light blue fluorescence, particularly under ultraviolet light at 365 nm. Repeat the test but adding 0.9 ml of *1 M sodium hydroxide* and 0.2 g of *sodium sulphite* in place of the 1.6 ml of *1 M sodium hydroxide*; practically no fluorescence is produced.

B. To a mixture of 0.1 ml of *nitrobenzene* and 0.2 ml of *sulphuric acid* add the powdered tablets containing 5 mg of Thiamine Hydrochloride. Allow to stand for 10 minutes, cool in ice and add slowly with stirring 5 ml of *water* followed by 5 ml of *10 M sodium hydroxide*. Add 5 ml of *acetone* and allow to stand; no violet colour is produced in the upper layer.

C. The powdered tablets give the reactions of chlorides (2.3.1).

Tests

Dissolution (2.5.2).

Apparatus No. 2 (Paddle),
Medium. 900 ml of *water*,
Speed and time. 50 rpm and 45 minutes.

Withdraw a suitable volume of the medium and filter.

Determine by liquid chromatography (2.4.14).

Test solution. Use the filtrate, dilute if necessary, with the dissolution medium.

Reference solution. Dissolve a suitable quantity of *thiamine hydrochloride IPRS* in the dissolution medium, to obtained a solution having known concentration similar to the test solution.

Chromatographic system
- a stainless steel column 30 cm × 3.9 mm, packed with octadecylsilane bonded to porus silica (5 µm),
- mobile phase: a 0.14 per cent w/v solution of *sodium 1-hexanesulphonate* in a mixture of 27 volumes of *methanol*, 73 volumes of *water* and 1 volume of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless and the relative standard deviation for replicate injections is not more than 3.0 per cent.

Inject the reference solution and the test solution.

Calculate the total content of $C_{12}H_{17}ClN_4OS,HCl$.

Q. Not less than 75 per cent of the stated amount of $C_{12}H_{17}ClN_4OS,HCl$.

Uniformity of content. Complies with the test stated under Tablets.

Finely crush one tablet, add 20 ml of *ethanol (95 per cent)*, stir the mixture for 30 minutes and centrifuge. Repeat the extraction with three further quantities, each of 15 ml, of *ethanol (95 per cent)*. Combine the extracts, add sufficient *ethanol (95 per cent)* to produce 100.0 ml and mix. Dilute a suitable volume of the resulting solution containing 1 mg of Thiamine Hydrochloride with sufficient *ethanol (95 per cent)* to produce 100.0 ml. Measure the absorbance of the resulting solution at the maximum at about 233 nm (2.4.7).

Calculate the content of $C_{12}H_{17}ClN_4OS$, HCl in the tablet taking 380 as the specific absorbance at 233 nm.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. For tablets containing less than 10 mg of Thiamine Hydrochloride, add 5 ml of *0.1 M hydrochloric acid* and 50 ml of *water* to a quantity of the powdered tablets containing 6 mg of Thiamine Hydrochloride, shake for 20 minutes, dilute to 100.0 ml with *water* and filter. For tablets containing 10 mg or more of Thiamine Hydrochloride, add 50 ml of *0.1 M hydrochloric acid* and 500 ml of *water* to a quantity of the powdered tablets containing 60 mg of Thiamine Hydrochloride, shake for 20 minutes, dilute to 1000.0 ml with *water* and filter.

Reference solution. A 0.006 per cent w/v solution of *thiamine mononitrate IPRS* in *0.005 M hydrochloric acid*.

Chromatographic system
- a stainless steel column 10 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a solution prepared by dissolving 1 g of *sodium heptanesulphonate* in a mixture of 180 ml of *methanol* and 10 ml of *triethylamine*, diluting to 1000 ml with *water* and adjusted to pH 3.2 with *orthophosphoric acid*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 244 nm,
- injection volume: 20 µl.

Calculate the content of $C_{12}H_{17}ClN_4OS,HCl$ in the tablets.

1 mg of $C_{12}H_{17}N_5O_4S$ is equivalent to 1.030 mg of $C_{12}H_{17}ClN_4OS$, HCl.

Storage. Store protected from light and moisture in non-metallic containers.

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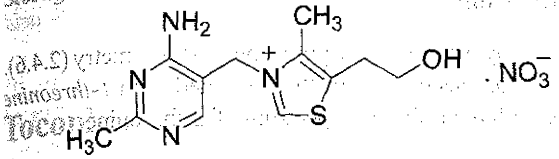
Thiamine Mononitrate

Thiamine Nitrate



$C_{12}H_{17}N_5O_4S$ Mol. Wt. 327.4

Thiamine Mononitrate is 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium nitrate.

Thiamine Mononitrate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{12}H_{17}N_5O_4S$, calculated on the dried basis.

Category. B-group vitamin.

Description. A white or almost white, crystalline powder or small, colourless crystals.

Identification

Test A may be omitted if tests B and C are carried out. Test B may be omitted if tests A and C are carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *thiamine mononitrate IPRS* or with the reference spectrum of thiamine mononitrate.

B. Dissolve about 20 mg in 10 ml of *water*, add 1 ml of *2 M acetic acid* and 1.6 ml of *1 M sodium hydroxide*, heat on a water-bath for 30 minutes and allow to cool. Add 5 ml of *2 M sodium hydroxide*, 10 ml of *potassium ferricyanide solution* and 10 ml of *1-butanol* and shake vigorously for 2 minutes. The upper layer exhibits an intense light blue fluorescence, particularly under ultraviolet light at 365 nm. Repeat the test but adding 0.9 ml of *1 M sodium hydroxide* and 0.2 g of *sodium sulphite* in place of the 1.6 ml of *1 M sodium hydroxide*; practically no fluorescence is produced.

C. It gives reaction (A) of nitrates (2.3.1).

Tests

Appearance of solution. A 2.0 per cent w/v solution in *carbon dioxide-free water* is clear (2.4.1), and not more intensely coloured than reference solution YS7 (2.4.1).

pH (2.4.24). 6.8 to 7.6, determined in a 2.0 per cent w/v solution.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 0.35 g of the substance under examination in 15.0 ml of 5 per cent v/v solution of *glacial acetic acid* and dilute to 100.0 ml with *water*.

Reference solution (a). Dissolve 35 mg of *thiamine mononitrile IPRS* in 15.0 ml of 5 per cent v/v solution of *glacial acetic acid* and dilute to 100.0 ml with *water*. Dilute 1.0 ml of the solution to 100.0 ml with *water*.

Reference solution (b). Dissolve the contents of a vial of *thiamine for system suitability IPRS* (containing thiamine impurities A, B and C) in 1.0 ml of a 0.75 per cent v/v solution of *glacial acetic acid*.

Chromatographic system

- a stainless steel column 25 cm × 4 mm, packed with endcapped octadecylsilane bonded to porous silica (5 µm),
- column temperature: 45°,
- mobile phase: A. a 0.38 per cent w/v solution of *sodium hexanesulphonate*, adjusted to pH 3.1 with *orthophosphoric acid*,
  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 248 nm,
- injection volume: 25 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
2	90	10
27	70	30
35	50	50
42	50	50
45	90	10

Name	Relative retention time
Thiamine impurity A[1]	0.3
Thiamine impurity B[2]	0.9
Thiamine (Retention time: about 30 minutes)	1.0
Thiamine impurity C[3]	1.2

[1]2-[3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium-5yl]ethyl sulfate (thiamine sulfate ester),

[2]3-[(4-aminopyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-,3-thiazol-3-ium (desmethylthiamine),

[3]3-[(4-amino-2-methylpyrimidin-5-yl)methyl-5-(2-chloroethyl)-4-methyl-1,3-thiazol-3-ium (chlorothiamine).

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to thiamine impurity B and thiamine is not less than 3.0 and the resolution between the peaks due to thiamine and thiamine impurity C is not less than 2.0.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the area of any

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peak corresponding to thiamine impurity B is not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent), the area of any peak corresponding to thiamine impurity C is not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent), the area of any peak corresponding to thiamine impurity A is not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent), the area of any other secondary peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the secondary peaks is not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Ignore the peak due to nitrate ion at relative retention time of about 0.05 and any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method A (20 ppm).

Chlorides (2.3.12). 1.0 g complies with the limit test for chlorides (250 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105°.

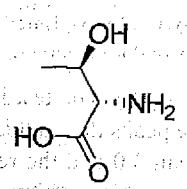
Assay. Dissolve 0.15 g in 5 ml of *anhydrous formic acid*, add 70 ml of *acetic anhydride*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01637 g of $C_{12}H_{17}N_5O_4S$.

Storage. Store protected from light and moisture, non-metallic containers.

Threonine

L-Threonine



$C_4H_9NO_3$ Mol. Wt. 119.1

Threonine contains not less than 98.5 per cent and not more than 101.5 per cent of $C_4H_9NO_3$, calculated on the dried basis.

Category. Amino acid.

Description. A white crystals.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *L-threonine IPRS* or with the reference spectrum of *L*-threonine.

Tests

pH (2.4.24). 5.0 to 6.5, determined in a 5 per cent w/v solution.

Specific optical rotation (2.4.22). −29.1° to −26.7°, determined in a 6 per cent w/v solution in *water*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 30 volumes of *butyl alcohol*, 10 volumes of *glacial acetic acid* and 10 volumes of *water*.

Test solution. Dissolve 100 mg of the substance under examination in 10.0 ml of *2 M hydrochloric acid*.

Reference solution (a). A 0.005 per cent w/v solution of *L-threonine IPRS* in 1 per cent v/v solution of *hydrochloric acid*.

Reference solution (b). A 0.04 per cent w/v solution each of *L- threonine IPRS* and *L-proline IPRS* in 1 per cent v/v solution of *hydrochloric acid*.

Apply to the plate 5 µl of each solution. Dry the plate in air and spray the plate with a 0.2 per cent w/v solution of *ninhydrin* in a mixture of 95 volumes of *butyl alcohol* and 5 volumes of *2 M acetic acid* and heat the plate at 105° for 15 minutes. Cool and examine in daylight. Any secondary spot in the chromatogram obtained with the test solution is not larger or more intense than the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

Iron (2.3.14). 1.33 g complies with the limit test for iron (30 ppm).

Chlorides (2.3.12). 0.5 g complies with the limit test for chlorides (500 ppm).

Sulphates (2.3.17). 0.5 g complies with the limit test for sulphates (300 ppm).

Sulphated ash (2.3.18). Not more than 0.4 per cent.

Loss on drying (2.4.19). Not more than 0.2 per cent, drying in an oven at 105° for 3 hours.

Assay. Dissolve 110 mg of the substance under examination, in a mixture of 3 ml of *formic acid* and 50 ml of *glacial acetic acid* and titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

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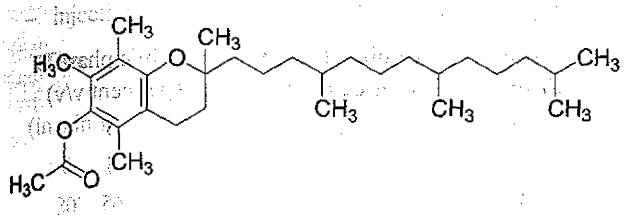
1 ml of *0.1 M perchloric acid* is equivalent to 0.01191 g of $C_7H_9NO_3$.

Storage. Store protected from moisture.

Tocopheryl Acetate

α-Tocopheryl Acetate; α-Tocopherol Acetate; Vitamin E Acetate



$C_{31}H_{52}O_3$ Mol. Wt. 472.8

Tocopheryl Acetate is (2*RS*,4'*RS*,8'*RS*)-6-acetoxy-2,5,7,8-tetramethyl-2-(4', 8', 12'-trimethyltri-decyl)chroman(*all-rac*-α-tocopherol acetate).

Tocopheryl Acetate contains not less than 96.0 per cent and not more than 102.0 per cent of $C_{31}H_{52}O_3$.

Category. Vitamin E supplement.

Description. A clear, slightly greenish yellow, viscous, oily liquid.

Identification

Test A may be omitted if tests B and C are carried out. Tests B and C may be omitted if test A is carried out.

A. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *α-tocopheryl acetate IPRS*.

B. When examined in the range 230 nm to 360 nm (2.4.7), a 0.01 per cent w/v solution in *ethanol* exhibits a maximum at about 284 nm, a shoulder at about 278 nm and a minimum at about 254 nm.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel HF254*.

Mobile phase. A mixture of 80 volumes of *cyclohexane* and 20 volumes of *ether*.

Test solution (a). Dissolve 0.5 g of the substance under examination in 100 ml of *cyclohexane*.

Test solution (b). Dissolve 10 mg of the substance under examination in 2 ml of *5 M ethanolic sulphuric acid*, heat on a water-bath for 5 minutes, cool, add 2 ml of *water* and 2 ml of *cyclohexane* and shake for 1 minute; use the upper layer.

Reference solution (a). A 0.5 per cent w/v solution of *α-tocopheryl acetate IPRS* in *cyclohexane*.

Reference solution (b). Prepare in the same manner as test solution (b) but using 10 mg of *α-tocopheryl acetate IPRS* in place of the substance under examination.

Apply to the plate 10 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (a) corresponds to that in the chromatogram obtained with reference solution (a). There are two spots in each of the chromatograms obtained with test solution (b) and reference solution (b). The spots of higher R_f value are due to α-tocopheryl acetate and correspond to that in the chromatogram obtained with reference solution (a). The spots of lower R_f value are due to α-tocopheryl. Spray the plate with a mixture of 1 volume of *hydrochloric acid*, 4 volumes of a 0.25 per cent w/v solution of *ferric chloride* in *ethanol (95 per cent)* and 4 volumes of a 1.0 per cent w/v solution of *1,10-phenanthroline hydrochloride* in *ethanol (95 per cent)*. In the chromatograms obtained with test solution (b) and reference solution (b) the spot of lower R_f value α-tocopheryl is orange.

Tests

Refractive index (2.4.27). 1.494 to 1.498, determined at 20°.

Acid value (2.3.23). Not more than 2.0, determined on 2.0 g.

Free tocopherol. Not more than 2.0 per cent, determined by the following method. Weigh 0.5 g, dissolve in 100 ml of *0.25 M ethanolic sulphuric acid*, add 20 ml of *water* and 0.1 ml of a 0.25 per cent w/v solution of *diphenylamine* in *sulphuric acid* and titrate with *0.01 M ceric ammonium nitrate* until a blue colour is produced that persists for at least 5 seconds. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of *ceric ammonium nitrate* required.

1 ml of *0.01 M ceric ammonium nitrate* is equivalent to 0.002154 g of tocopherol.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Assay. Dissolve 0.3 g in 25 ml of *ethanol (95 per cent)*, add 20 ml of *2.5 M ethanolic sulphuric acid* and heat on a water-bath under a reflux condenser for 3 hours. Cool, transfer the solution quantitatively to a 200-ml volumetric flask, rinse the apparatus with *ethanol (95 per cent)* and add the rinsings to the flask. Make up to volume with *ethanol (95 per cent)* and mix. To 25.0 ml of the resulting solution in a flask add 25 ml of *0.25 M ethanolic sulphuric acid*, 10 ml of *water* and titrate with *0.01 M ceric ammonium nitrate* using 0.1 ml of

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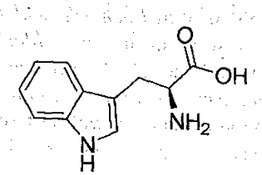
diphenylamine as indicator, until a blue colour persisting for at least 5 seconds is obtained. Repeat the operation without the substance under examination. The difference between the titrations represents the amount of *ceric ammonium nitrate* required.

1 ml of *0.01 M ceric ammonium nitrate* is equivalent to 0.002364 g of $C_{31}H_{52}O_3$.

Storage. Store protected from light and moisture.

Tryptophan

L-Tryptophan



$C_{11}H_{12}N_2O_2$ Mol. Wt. 204.2

Tryptophan contains not less than 98.5 per cent and not more than 101.5 per cent of $C_{11}H_{12}N_2O_2$, calculated on the dried basis.

Category. Amino acid

Description. A white to slightly yellowish-white crystals.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *L-tryptophan IPRS* or with the reference spectrum of *L*-tryptophan.

Tests

pH (2.4.24). 5.5 to 7.0, determined in 1 per cent w/v solution.

Specific optical rotation (2.4.22). –32.8° to –29.4°, determined in 1.0 per cent w/v solution in *water*, heat gently to dissolve.

Related substances. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in 10.0 ml of *water*.

Reference solution (a). A solution containing 0.0001 per cent w/v, each of, *tryptophan related compound A IPRS* and *tryptophan related compound B IPRS* in *water*.

Reference solution (b). A 0.0001 per cent w/v solution of *tryptophan related compound B IPRS* in *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. 0.1 per cent v/v of *trifluoroacetic acid* in *water*,

B. 0.1 per cent v/v solution of *trifluoroacetic acid* in a mixture of 80 volumes of *acetonitrile* and 20 volumes of *water*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
2	95	5
37	35	65
42	0	100
47	0	100
50	95	5
60	95	5

Inject reference solution (b): The test is not valid unless the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, the sum of the areas of all the secondary peaks, eluting prior to tryptophan peak is not more than the area of the peak due to tryptophan related compound B in the chromatogram obtained with reference solution (a) (0.01 per cent) and the sum of the areas of all the secondary peaks, eluting after the tryptophan peak other than tryptophan related compound B peak is not more than 3 times the area of the peak due to tryptophan related compound B in the chromatogram obtained with reference solution (a) (0.03 per cent).

NOTE — If a peak for tryptophan related compound A is observed in the test solution, then perform the test for Tryptophan related compound A.

Tryptophan related compound A. Not more than 10 ppm.

Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in 10.0 ml of *water*.

Reference solution. A 0.001 per cent w/v solution of *tryptophan related compound A IPRS* in *water*. Dilute 1.0 ml of the solution to 100.0 ml with *water*.

Chromatographic system

- a stainless steel column 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm),

- mobile phase: A. a mixture of 90 volumes of *0.018 M monobasic sodium phosphate*, adjusted to pH 2.5 and 10 volumes of *acetonitrile*,

B. a mixture of 50 volumes of *0.01 M monobasic sodium phosphate*, adjusted to pH 2.5 and 50 volumes of *acetonitrile*,

C. a mixture of 70 volumes of *acetonitrile* and 30 volumes of *water*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 216 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)	Mobile phase C (per cent v/v)
0	100	0	0
30	44	56	0
30.1	0	0	100
45	0	0	100
45.1	100	0	0
60	100	0	0

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 5.0 per cent.

Inject the reference solution and the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to tryptophan related compound A is not more than the area of the principal peak in the chromatogram obtained with the reference solution.

Iron (2.3.14). 1.33 g complies with the limit test for iron (30 ppm).

Chlorides (2.3.12). 0.5 g complies with the limit test for chlorides (500 ppm).

Sulphates (2.3.17). 0.5 g complies with the limit test for sulphates (300 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.3 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

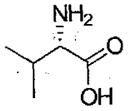
Assay. Dissolve 0.2 g of the substance under examination, in a mixture of 3 ml of *formic acid* and 50 ml of *glacial acetic acid* and titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.02042 g of $C_{11}H_{12}N_2O_2$.

Storage. Store protected from moisture.

Valine

L-Valine



$C_5H_{11}NO_2$ Mol. Wt. 117.2

Valine contains not less than 98.5 per cent and not more than 101.5 per cent of $C_5H_{11}NO_2$ calculated on the dried basis.

Category. Amino acid

Description. A white crystals.

Identification

Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *L-valine IPRS* or with the reference spectrum of valine.

Tests

pH (2.4.24). 5.5 to 7.0, determined in 5.0 per cent w/v solution.

Specific optical rotation (2.4.22). +26.6° to +28.8°, determined in 8.0 per cent w/v solution in *6 M hydrochloric acid*.

Related substances. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 30 volumes of *butyl alcohol*, 10 volumes of *water* and 10 volumes of *glacial acetic acid*.

Test solution. Dissolve 100 mg of the substance under examination in *2 M hydrochloric acid* and dilute to 10.0 ml of *2 M hydrochloric acid*.

Reference solution (a). A 0.005 per cent w/v solution of *L-valine IPRS* in *0.1M hydrochloric acid*.

Reference solution (b). A solution containing 0.04 per cent w/v, each of, *L-valine IPRS* and *L-phenylalanine IPRS* in *0.1 M hydrochloric acid*.

Apply the plate 5µl of each solution. Dry the plate in air and spray with 0.2 per cent w/v solution of *ninhydrin* in a mixture of 95 volumes of *butyl alcohol* and 5 volumes *2 M acetic acid* and heat at 100° to 105° for about 15 minutes. Examine the plate under day light. Any spot in the chromatogram obtained with the test solution is not large or more intense than the spot in the chromatogram obtained with reference solution (a). The chromatogram obtained with reference solution (b) shows two clearly separated spots.

Iron (2.3.14). 1.33 g complies with the limit test for iron (30 ppm).

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Chlorides (2.3.12). 0.5 g complies with the limit test for chlorides (500 ppm).

Sulphates (2.3.17). 0.5 g complies with the limit test for sulphates (300 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Loss on drying (2.4.19). Not more than 0.3 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Assay. Dissolve 110 mg of substance under examination in a mixture of 3 ml of *formic acid* and 50 ml of *glacial acetic acid*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.4.25). Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 0.01172 g of $C_5H_{11}NO_2$.

Storage. Store protected from moisture.

Vitamin A Capsules

Retinol Capsules

Vitamin A Capsules contain Vitamin A concentrate oil. The capsule contains not less than 90.0 per cent and not more than 120.0 per cent of the stated amount of vitamin A.

Usual strengths. 10,000 IU; 25,000 IU.

Identification

Tests C and D may be omitted if tests A and B are carried out. Tests B and D may be omitted if tests A and C are carried out.

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 12 volumes of *cyclohexane* and 1 volume of *ether*.

Test solution. Mix a quantity of the capsule containing about 50,000 IU of vitamin A in 10 ml of *cyclohexane*.

Reference solution (a). Dissolve about 50,000 IU of *retinol acetate IPRS* in 10 ml of *cyclohexane*.

Reference solution (b). Dissolve about 50,000 IU of *retinol propionate IPRS* in 10 ml of *cyclohexane*.

Reference solution (c). Dissolve about 50,000 IU of *retinol palmitate IPRS* in 10 ml of *cyclohexane*.

Apply to the plate 2 µl of each solution. After development, dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solutions (a), (b) and (c).

B. Dissolve a small quantity of retinol in about 1 ml of *dichloromethane* and add 5 ml of *antimony trichloride*; a blue colour is immediately produced which turns gradually to violet-red.

C. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

D. To a quantity of retinol containing about 50,000 IU of vitamin A, add 100 ml of *ethanol (95 per cent)*. Dilute 1 ml of the solution to 50 ml with a mixture of 100 volumes of *ethanol (95 per cent)* and 1 volume of *5 M hydrochloric acid*. Immediately after preparation measure the absorbance in the range 300 to 400 nm (2.4.7). The solution exhibits a single maximum at about 326 nm. Heat the solution in a water-bath for 30 seconds and cool rapidly. The absorption spectrum of the resulting solution, when observed between 300 and 400 nm, exhibits a shoulder at about 332 nm and maxima at about 348, 367 and 389 nm.

Tests

Other tests. Comply with the tests stated under Capsules.

Assay. Determine by liquid chromatography (2.4.14).

NOTE — Carry out the assay as rapidly as possible, avoiding exposure to actinic light and oxidising agents, and maintaining whenever possible an atmosphere of nitrogen.

Test solution. Weigh and mix the contents of 20 capsules. Disperse a quantity of the mixed content of capsules containing about 1,00,000 IU of vitamin A, into a 100-ml volumetric flask. Dissolve immediately in 5.0 ml of *n-pentane*, add 40 ml of *0.1 M tetrabutylammonium hydroxide* in *2-propanol*. Swirl gently and let the mixture react for 10 minutes at 60° to 65° for hydrolysis, swirling occasionally. Allow to cool to room temperature, dilute to volume with *2-propanol* containing about 0.1 per cent w/v solution of *butylated hydroxytoluene*. Dilute 5.0 ml of the solution to 50.0 ml with *2-propanol*.

Reference solution (a). Weigh a quantity of *retinol IPRS* or *retinol palmitate IPRS* containing about 1, 00,000 IU of vitamin A, into a 100-ml volumetric flask. Dissolve immediately in 5.0 ml of *n-pentane*, add 40 ml of *0.1 M tetrabutylammonium hydroxide* in *2-propanol*. Swirl gently and let the mixture react for 10 minutes at 60° to 65° for hydrolysis, swirling occasionally. Allow to cool to room temperature, dilute to volume with *2-propanol* containing about 0.1 per cent w/v solution of *butylated hydroxytoluene*. Dilute 5.0 ml of the solution to 50.0 ml with *2-propanol*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecysilane groups bonded to porous silica (5 µm),

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— mobile phase: a mixture of 95 volumes of the *methanol* and 5 volumes of *water*,
— flow rate: 1 ml per minute,
— spectrophotometer set at 325 nm,
— injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent. Run the chromatograms for 1.5 times the retention time of retinol.

Inject the reference solution and the test solution.

Calculate the content of vitamin A in the capsules.

Storage. Store protected from heat, light and moisture.

Labelling. The labelling state the name of the retinol ester or esters present, the proportion of Vitamin A expressed in International Units (IU), and the names and proportions of any stabilizing agents added.

Vitamin A Concentrate Oil

Synthetic Vitamin A Concentrate (Oily Form); Synthetic Retinol Concentrate (Oily Form).

Vitamin A Concentrate Oil consists of an ester or a mixture of esters of retinol (as acetate, propionate or palmitate) prepared by synthesis. It may be diluted with a suitable vegetable oil. It may contain suitable stabilising agents such as antioxidants.

Vitamin A Concentrate Oil contains not less than 500,000 Units of Vitamin A per g, and not less than 95.0 per cent and not more than 110.0 per cent of the stated number of Units of Vitamin A per g.

Category. Antixerophthalmic vitamin.

Description. A yellow to brownish yellow, oily liquid.

Identification

A. When examined in the range 230 nm to 360 nm (2.4.7), a solution in *2-propanol* containing 10 to 15 Units per ml shows an absorption maximum at about 325 nm to 327 nm.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 80 volumes of *cyclohexane* and 20 volumes of *ether*.

Test solution. Prepare a solution containing 5 Units per µl of the substance under examination in *cyclohexane*.

Reference solution (a). Prepare a solution containing 5 Units per µl of *retinyl acetate IPRS* in *cyclohexane*.

Reference solution (b). Prepare a solution containing 5 Units per µl of *retinyl propionate IPRS* in *cyclohexane*.

Reference solution (c). Prepare a solution containing 5 Units per µl of *retinyl palmitate IPRS* in *cyclohexane*.

Apply to the plate 2 µl of each solution. After development, dry the plate in air, spray with *antimony trichloride solution*. The principal spot or spots in the chromatogram obtained with the test solution correspond to one or more of the spots in the chromatograms obtained with reference solutions (a), (b) and (c).

C. Dissolve a quantity containing 10 to 15 Units in 1 ml of *chloroform* and add 5 ml of *antimony trichloride solution*; a transient bright blue colour is produced immediately.

Tests

Acid value (2.3.23). Not more than 2.0, determined on 2.0 g.

Peroxides. Add 0.3 g to 25.0 ml of a mixture of 6 volumes of *toluene* and 4 volumes of *methanol* (solution A). Add in a test-tube, in the following order, mixing after each addition, 0.3 ml of a 1.8 per cent w/v solution of *ammonium thiocyanate*, 10.0 ml of *methanol*, 0.3 ml of *ferrous sulphate solution* and 15.0 ml of *toluene* and add 1.0 ml of solution A. The colour produced after 5 minutes is not more intense than that obtained in a solution prepared at the same time and in the same manner but using a solution prepared in the following manner in place of solution A. Add 1.0 ml of a 27.0 per cent w/v solution of *ferric chloride hexahydrate* to 99 ml of a mixture of 6 volumes of *toluene* and 4 volumes of *methanol* and dilute 2.0 ml to 100.0 ml with the same solvent mixture.

Assay. Carry out the assay of vitamin A, Method A (2.3.41).

Storage. Store protected from light, in well-filled containers. Once the container has been opened, its contents should be used as soon as possible; any part of the contents not used at once should be protected by an atmosphere of an inert gas.

Labelling. The label states (1) the number of Units of Vitamin A per g; (2) the name of the ester or esters; (3) the name(s) of any added stabilising agent(s); (4) the method of restoring the solution if partial crystallisation has occurred.

Vitamin A Concentrate Powder

Synthetic Vitamin A Concentrate (Powder Form); Synthetic Retinol Concentrate (Powder Form).

Vitamin A Concentrate Powder consists of an ester or a mixture of esters of retinol (as acetate, propionate or palmitate) prepared by synthesis and dispersed in a matrix of Gelatin, Acacia or any other suitable material. It may contain suitable stabilising agents such as antioxidants.

Vitamin A Concentrate Powder contains not less than 250,000 Units of Vitamin A per g, and not less than 95.0 per

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cent and not more than 115.0 per cent of the stated number of Units of Vitamin A per g.

Category. Antixerophthalmic vitamin.

Description. A yellowish powder usually in the form of pellets or particles of almost uniform size.

Identification

To a quantity containing 50,000 Units of Vitamin A, add 1.5 ml of *2 M ammonia* previously heated to 60° and heat in a water-bath at 60°, shaking occasionally. After 10 minutes add 40 ml of *ethanol*, dilute to 200 ml with *ether* and shake. Allow to stand for a few minutes and use the supernatant liquid (solution A) for the following tests.

Certain samples may not react sufficiently during the course of the above treatment. In such cases the volume of solution A used in the following tests should be increased which may be as much as 10-fold.

A. When examined in the range 230 nm to 360 nm (2.4.7), a solution in *2-propanol* containing 10 to 15 Units per ml shows an absorption maximum at about 325 nm to 327 nm.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 80 volumes of *cyclohexane* and 20 volumes of *ether*.

Test solution. Evaporate 10 ml of solution A to dryness in a current of nitrogen and dissolve the residue in 0.5 ml of *cyclohexane*.

Reference solution (a). Prepare a solution containing 5 Units per µl of *retinyl acetate IPRS* in *cyclohexane*.

Reference solution (b). Prepare 5 Units per µl of *retinyl propionate IPRS* in *cyclohexane*.

Reference solution (c). Prepare a solution containing 5 Units per µl of *retinyl palmitate IPRS* in *cyclohexane*.

Apply to the plate 2 µl of each solution. After development, dry the plate in air, spray with *antimony trichloride solution*. The principal spot or spots in the chromatogram obtained with the test solution correspond to one or more of the spots in the chromatograms obtained with reference solutions (a), (b) and (c).

C. Dilute 2 ml of solution A to 50 ml with *n-pentane* and evaporate 1 ml of the solution to dryness in a current of nitrogen. Dissolve the residue in 1 ml of *chloroform* and add 5 ml of *antimony trichloride solution*; a transient bright blue colour is produced immediately.

Tests

Related substances and degradation products. Using the relative absorbances obtained in the Assay, the ratio A_{300}/A_{325} is not more than 0.612 and the sum of the ratios A_{300}/A_{325} and A_{350}/A_{325} is not more than 1.054, where A_{300}, A_{325} and A_{350} are the absorbances measured at about 300 nm, 325 nm and 350 nm respectively.

Assay. Carry out the assay of vitamin A, Method B (2.3.41), adding 5 ml of *water* to the mixture for saponification, using *2-propanol* as the blank and taking 0.612 as the maximum value of the ratio A_{300}/A_{325}.

Storage. Store protected from light. Once the container has been opened, its contents should be used as soon as possible; any part of the contents not used at once should be protected by an atmosphere of an inert gas.

Labelling. The label states (1) the number of Units of Vitamin A per g; (2) the name of the ester or esters; (3) the name of the principal excipient or excipients used; (4) the name of any added stabilising agents.

Water-Miscible Vitamin A Concentrate

Synthetic Vitamin A Concentrate (Water-dispersible Form); Synthetic Retinol Concentrate (Water-dispersible Form).

Water-miscible Vitamin A Concentrate consists of an ester or a mixture of esters of retinol (as acetate, propionate or palmitate) prepared by synthesis to which suitable solubilisers have been added. It may contain suitable stabilising agents such as antimicrobial preservatives and antioxidants.

Water-miscible Vitamin A Concentrate contains not less than 100,000 Units of Vitamin A per g, and not less than 95.0 per cent and not more than 115.0 per cent of the stated number of Units of Vitamin A per g.

Category. Antixerophthalmic vitamin.

Description. A yellow or yellowish liquid of variable opalescence and viscosity. Highly concentrated solutions may become cloudy at low temperatures or gel at room temperature.

Identification

To a quantity containing about 10,000 Units of Vitamin A, add 5 ml of *water* and homogenise. Add 5 ml of *ethanol (95 per cent)* and 20 ml of *n-pentane* and shake vigorously for 30 seconds. Allow to stand for a few minutes and use the supernatant liquid (solution A) for the following tests.

A. Dilute solution A with sufficient *2-propanol* so that the absorbance at the wavelength of maximum absorption is 0.3 to 0.7 (2.4.7).

When examined in the range 230 nm to 360 nm (2.4.7), the solution shows an absorption maximum at 325 to 327 nm.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 80 volumes of *cyclohexane* and 20 volumes of *ether*.

Test solution. Evaporate 10 ml of solution A to dryness in a current of *nitrogen* and dissolve the residue in 0.5 ml of *cyclohexane*.

Reference solution (a). Prepare a solution containing 5 Units per µl of *retinyl acetate IPRS* in *cyclohexane*.

Reference solution (b). Prepare a solution containing 5 Units per µl of *retinyl propionate IPRS* in *cyclohexane*.

Reference solution (c). Prepare a solution containing 5 Units per µl of *retinyl palmitate IPRS* in *cyclohexane*.

Apply to the plate 2 µl of each solution. After development, dry the plate in air, spray with *antimony trichloride solution*. The principal spot or spots in the chromatogram obtained with the test solution correspond to one or more of the spots in the chromatograms obtained with reference solutions (a), (b) and (c).

C. Evaporate 0.1 ml of solution A to dryness in a current of nitrogen, dissolve the residue in 1 ml of *chloroform* and add 5 ml of *antimony trichloride solution*; a transient bright blue colour is produced immediately.

Tests

Water miscibility. Mix about 1 g with 10 ml of *water* previously heated to 50° and cool to 20°. Immediately after cooling, a uniform, slightly opalescent and slightly yellow dispersion is obtained.

Related substances and degradation products. Using the relative absorbances obtained in the Assay, the ratio A_{300}/A_{325} is not more than 0.618 and the sum of the ratios A_{300}/A_{325} and A_{350}/A_{325} is not more than 1.060, where A_{300}, A_{325} and A_{350} are the absorbances measured at about 300 nm, 325 nm and 350 nm respectively.

Assay. Carry out the assay of vitamin A, Method B (2.3.41), using *2-propanol* as the blank and taking 0.618 as the maximum value of the ratio A_{300}/A_{325}.

Storage. Store protected from light at the temperature stated on the label. Once the container has been opened, its contents should be used as soon as possible; any part of the contents not used at once should be protected by an atmosphere of an inert gas.

Labelling. The label states (1) the number of Units of Vitamin A per g; (2) the name of the ester or esters; (3) the name of the principal excipient or excipients used; (4) the temperature at which it should be stored.

Vitamin A Paediatric Oral Solution

Paediatric Retinol Oral Solution

Vitamin A Paediatric Oral Solution contains Vitamin A concentrate oil, diluted in a suitable vegetable oil. It may contain suitable antimicrobial agents and stabilizing agents such as antioxidants.

Vitamin A Paediatric Oral Solution contains not less than 90.0 per cent and not more than 120.0 per cent of the stated amount of vitamin A.

Usual strength. 100000 IU (as palmitate) per ml.

Identification

Test A may be omitted if tests B, C and D are carried out. Test C may be omitted if tests A, B and D are carried out.

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 12 volumes of *cyclohexane* and 1 volume of *ether*.

Test solution. Dissolve a quantity of the oral solution containing about 50,000 IU of vitamin A in 10 ml of *cyclohexane*.

Reference solution (a). Dissolve a quantity of *retinol acetate IPRS* in *cyclohexane* to get 5000 IU of vitamin A per ml.

Reference solution (b). Dissolve a quantity of *retinol propionate IPRS* in *cyclohexane* to get 5000 IU of vitamin A per ml.

Reference solution (c). Dissolve a quantity of *retinol palmitate IPRS* in *cyclohexane* to get 5000 IU of vitamin A per ml.

Apply 2 µl of each solution. After development dry the plate in air and examine under ultraviolet light at 254 nm. The principal spot obtained with the test solution corresponds to one or more of the spots obtained with reference solutions (a), (b) and (c).

B. Dissolve a drop of the oral solution in about 1 ml of *dichloromethane* and add 5 ml of *antimony trichloride*; a blue colour is immediately produced which turns gradually to violet-red.

C. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

D. To a quantity of the oral solution containing 50 000 IU of vitamin A, add 100 ml of *ethanol* (75 per cent). Dilute 1 ml of the solution to 50 ml with a mixture of 100 volumes of *ethanol (75 per cent)* and 1 volume of *hydrochloric acid* (42 per cent). Immediately after preparation measure the absorbance in the range 300 to 400 nm. The solution exhibits a single maximum at 326 nm. Heat the solution in a water-bath for 30 seconds and

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cool rapidly. The absorption spectrum of the resulting solution, when observed between 300 and 400 nm (2.4.7), exhibits maxima at 348, 367 and 389 nm.

Tests

Other tests. Comply with the tests stated under Oral Solution.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Transfer a quantity of the oral solution containing of about 100000 IU of vitamin A, weighed, into a 100-ml volumetric flask. Dissolve immediately in 5 ml of *n-pentane*. Add 40 ml of *0.1 M tetrabutylammonium hydroxide* in *2-propanol*. Swirl gently and let the mixture react for 10 minutes at 60° to 65° for hydrolysis, swirling occasionally. Allow to cool to room temperature, dilute to volume with *2-propanol* containing 0.1 per cent w/v solution of *butylated hydroxytoluene*, and homogenise carefully to avoid air-bubbles. Dilute 5.0 ml of the solution to 50.0 ml with *2-propanol*.

Reference solution. Transfer an amount of *retinol acetate IPRS* or *retinol palmitate IPRS* containing about 100000 IU of vitamin A, into a 100-ml volumetric flask. Prepare the solution as described under test solution.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 95 volumes of *methanol* and 5 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 325 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of vitamin A in the oral solution.

Storage. Store protected from light.

Labelling. The labelling should state the name of the retinol ester or esters present, the proportion of Vitamin A expressed in International Units (IU), and the names and proportions of any stabilizing agents added.

Vitamins A and D Capsules

Vitamins A and D Capsules contain Vitamin A Oil and a source of vitamin D such as Cholecalciferol or Ergocalciferol in an edible vegetable oil.

Vitamins A and D Capsules contain not less than 90.0 per cent of the stated number of Units of vitamin A and vitamin D.

Category. Antixerophthalmic and antirachitic vitamins.

Tests

Other tests. Comply with the tests stated under Capsules.

Assay. *For Vitamin A* — Weigh a portion of the mixed contents of 20 capsules containing about 500 Units of Vitamin A and carry out the assay of vitamin A, Method A (2.3.41).

For Vitamin D — Weigh a portion of the mixed contents of 20 capsules containing about 5000 Units of vitamin D and carry out the assay of vitamin D (2.3.42).

Storage. Store protected from light and moisture.

Labelling. The label states the number of Units of vitamin A and vitamin D per capsule.

Concentrated Vitamin D Solution

Concentrated Vitamin D Solution is a solution of Cholecalciferol or Ergocalciferol in an edible vegetable oil. It may contain suitable stabilising agents such as antioxidants.

Concentrated Vitamin D Solution contains not less than 10,000 Units of vitamin D per g and not less than 90.0 per cent and not more than 120.0 per cent of the stated number of Units of vitamin D.

Category. Antirachitic vitamin.

Description. A pale yellow to yellow, oily liquid.

Identification

Dissolve a quantity containing about 1000 Units of vitamin D in 1 ml of *chloroform* and add 10 ml of *antimony trichloride solution*; a pinkish red colour appears at once.

Tests

Acid value (2.3.23). Not more than 2.5, determined on 2.0 g.

Assay. Carry out the assay of vitamin D (2.3.42).

Storage. Store protected from light, in well-filled containers at a temperature of 6° to 15°. The contents of an opened container should be used as soon as possible.

Labelling. The label states (1) the number of Units of vitamin D per g; (2) the storage conditions; (3) the nature and concentration of any stabilising agent added.

Concentrated Vitamins A and D Solution

Concentrated Vitamins A and D Solution is a solution of Vitamin A Oil and a source of vitamin D such as Cholecalciferol

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or Ergocalciferol in an edible vegetable oil. It may contain suitable stabilising agents such as antioxidants.

Concentrated Vitamins A and D Solution contains not less than 50,000 Units of vitamin A and 5000 Units of vitamin D per g, and not less than 90.0 per cent and not more than 110.0 per cent of the stated number of Units of Vitamin A and vitamin D per g.

Category. Antixerophthalmic and antirachitic vitamins.

Tests

Acid value (2.3.23). Not more than 2.5, determined on 2.0 g.

Assay. *For Vitamin A* — Carry out the assay of vitamin A, Method A (2.3.41).

For Vitamin D — Carry out the assay of vitamin D (2.3.42).

Storage. Store protected from light and moisture.

Labelling. The label states (1) the number of Units of vitamin A and vitamin D per gram; (2) the storage conditions.

Zinc Chloride

$ZnCl_2$ Mol. Wt. 136.3

Zinc Chloride contains not less than 95.0 per cent and not more than 100.5 per cent of $ZnCl_2$.

Category. Pharmaceutical aid.

Description. A white or practically white, crystalline powder; very deliquescent.

Identification

A. To 2 g add 38 ml of *carbon dioxide-free water* prepared from *distilled water* and add *2 M hydrochloric acid* dropwise until solution is complete and dilute to 40 ml with *carbon dioxide-free water* prepared from *distilled water* (solution A). Solution A gives the reaction of zinc salts (2.3.1).

B. A 5 per cent w/v solution in *2 M nitric acid* gives the reactions of chlorides (2.3.1).

Tests

pH (2.4.24). 4.6 to 6.0, determined in a solution prepared by dissolving 1.0 g in 9 ml of freshly boiled and cooled *water*, ignoring any slight turbidity.

Aluminium, calcium, heavy metals, iron and magnesium. To 8 ml of solution A add 2 ml of *strong ammonia solution* and shake; the solution is clear (2.4.1), and colourless (2.4.1). Add 1 ml of a 9 per cent w/v solution of *disodium hydrogen phosphate*; the resulting solution remains clear for at least 5 minutes. Add 0.2 ml of *sodium sulphide solution*; a white precipitate is produced and the supernatant liquid remains colourless.

Ammonium salts. To 5 ml of a 10 per cent w/v solution add *1 M sodium hydroxide* until the precipitate first formed is redissolved and then warm the solution; no odour of ammonia is perceptible.

Oxychlorides. Dissolve 1.5 g in 1.5 ml of *carbon dioxide-free water*; the solution is not more opalescent than opalescence standard OS2 (2.4.1). Add 7.5 ml of *ethanol (95 per cent)*; the solution may become cloudy within 10 minutes but becomes clear on the addition of 0.2 ml of *2 M hydrochloric acid*.

Sulphates (2.3.17). 15 ml of solution A complies with the limit test for sulphates (200 ppm).

Assay. Dissolve 3.0 g in 125 ml of *water*, add 3 g of *ammonium chloride* and add sufficient *water* to produce 250.0 ml. To 25.0 ml of the resulting solution add 100 ml of *water* and 10 ml of *strong ammonia-ammonium chloride solution*. Titrate with *0.1 M disodium edetate*, using *eriochrome black T solution* as indicator until a deep blue colour is obtained. Carry out a blank titration.

1 ml of *0.1 M disodium edetate* is equivalent to 0.01363 g of $ZnCl_2$.

Storage. Store protected from moisture, in non-metallic containers.

Zinc Chloride Injection

Zinc Chloride Injection is a sterile solution of Zinc Chloride in Water for Injection.

Zinc Chloride Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of zinc, Zn.

Usual strength. 1 mg per ml.

Identification

In the Assay, the test solution exhibits an absorption maximum at about 213.8 nm.

Tests

pH (2.4.24). 1.5 to 2.5.

Bacterial endotoxins (2.2.3). Not more than 25.0 Endotoxin Units per mg of zinc.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. *Sodium chloride solution*. Dissolve 450 mg of *sodium chloride* in *water* and dilute to 500 ml with *water*.

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Reference solution. Dissolve 3.11 g of *zinc oxide*, in 80 ml of 0.5 *M sulphuric acid*, warm, cool and dilute to 250 ml with *water*. This stock solution contains 10 mg of zinc per ml. Dilute a volume of the solution with *water* to obtain a solution containing 125 µg of zinc per ml. Transfer 3.0, 4.0, and 5.0 ml, respectively, of the solution to three separate 500-ml volumetric flasks, each containing 5 ml of *sodium chloride solution*, dilute the contents of each flask with *water* to volume, and mix. These solutions contain, respectively, 0.75, 1.0, and 1.25 µg of zinc per ml.

Test solution. Dilute a measured volume of injection, containing about 5 mg of zinc, to 500 ml with *water*. Transfer 10.0 ml of the solution to a 100-ml volumetric flask. From the labeled amount of sodium chloride, if any, in the injection, calculate the amount, in mg, of sodium chloride in the 10.0 ml portion and add sufficient *sodium chloride solution* to bring the total sodium chloride content of the 100-ml volumetric flask to 0.9 mg and dilute to volume with *water*.

Determine the absorbances of the solutions at the zinc emission line at 213.8 nm, with a suitable atomic absorption spectrophotometry (2.4.2) equipped with a zinc hollow-cathode lamp and an air–acetylene flame, using water as the blank. Plot the absorbances of the reference solutions versus the concentration, in µg per ml, of zinc, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, in µg per ml, of zinc in the test solution. Calculate the quantity, in mg, of zinc in each ml of the injection taken by the formula:

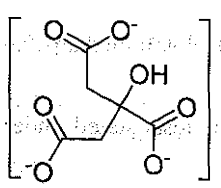
$$\frac{5C}{V}$$

in which C is the concentration, in µg per ml, of zinc in the test solution and V is the volume, in ml, of injection taken.

Storage. Store in single-dose or multiple-dose containers, preferably of Type I or Type II glass.

Labelling. The label states the injection to be diluted with Water for Injection or other suitable fluid to appropriate strength prior to administration.

Zinc Citrate



$3Zn^{++}$, $2H_2O$

$C_{12}H_{10}O_{14}Zn_3, 2H_2O$ Mol. Wt. 610.4

Zinc Citrate is 2-Hydroxy-1,2,3-propanetricarboxylic acid zinc salt.

Zinc Citrate contains not less than 31.3 per cent of zinc, calculated on the dried basis.

Category. Mineral supplement

Description. A white or almost white powder.

Identification

A. A 10 per cent w/v solution, gives reaction of Zinc salt (2.3.1).

B. A 10 per cent w/v solution, gives reaction (B) of citrate (2.3.1).

Tests

Chlorides (2.3.12). 0.5 g complies with the limit test for chlorides (500 ppm).

Sulphates (2.3.17). 0.3 g complies with the limit test for sulphates (500 ppm).

Heavy metals (2.3.13). 1.0 g complies with limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 1.0 per cent, determined on 1.0 g by drying in an oven at 105° for 2 hours.

Assay. Dissolve 0.35 g in 60 ml of *water*, add 10 ml of *ammonia-ammonium chloride buffer*, and titrate with *0.05 M disodium edetate* using 0.1 ml of *eriochrome black T solution* as indicator, until a blue colour is obtained. Carry out a blank titration.

1 ml of *0.05 M disodium edetate* is equivalent to 0.0654 g of Zn.

Storage. Store protected from moisture.

Zinc Sulphate

$ZnSO_4, 7H_2O$ Mol. Wt. 287.5

Zinc Sulphate contains not less than 99.0 per cent and not more than 104.0 per cent of $ZnSO_4, 7H_2O$.

Category. Astringent.

Description. Colourless, transparent crystals or a white, crystalline powder; efflorescent.

Identification

Dissolve 2.5 g in sufficient *carbon dioxide-free water* to produce 50 ml (solution A). Solution A gives the reactions of zinc salts and sulphates (2.3.1).

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Tests

Appearance of solution. Solution A is clear (2.4.1), and colourless (2.4.1).

pH (2.4.24). 4.4 to 5.6, determined in solution A.

Arsenic (2.3.10). Dissolve 1.0 g in 50 ml of *water* and add 10 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (10 ppm).

Iron (2.3.14). Dissolve 0.4 g in 20 ml of *water*. The resulting solution complies with the limit test for iron (100 ppm).

Chlorides (2.3.12). 20 ml of solution A complies with the limit test for chlorides (250 ppm).

Assay. Dissolve 0.5 g in 5 ml of *2 M acetic acid* and dilute to 50 ml with *water*. To the resulting solution add about 50 mg of *xylenol orange triturate* and sufficient *hexamine* to produce violet-pink colour. Further, add 2 g of *hexamine* and titrate with *0.1 M disodium edetate* until the colour changes to yellow.

1 ml of *0.1 M disodium edetate* is equivalent to 0.02875 g of $ZnSO_4, 7H_2O$.

Storage. Store protected from moisture, in non-metallic containers.

Zinc Sulphate Monohydrate

$ZnSO_4,H_2O$ Mol. Wt. 179.5

Zinc Sulphate Monohydrate contains not less than 99.0 per cent and not more than 101.0 per cent of $ZnSO_4,H_2O$.

Category. Astringent.

Description. A white or almost white, crystalline powder or colourless, transparent crystals.

Identification

A. A 5 per cent w/v solution of the substance under examination in *carbon dioxide-free water* (solution A) gives the reactions of sulphates (2.3.1).

B. Solution A gives the reaction of zinc (2.3.1).

Tests

Appearance of solution. Solution A is clear (2.4.1) and colourless (2.4.1).

pH (2.4.24). 4.4 to 5.6 for solution A.

Chlorides (2.3.12). Dissolve 0.83 g in 15.0 ml of *water*. The solution complies with the limit test for chlorides (300 ppm).

Iron (2.3.14). Dilute 8 ml of solution A to 10 ml with *water* using 0.5 ml of *thioglycollic acid*. The solution complies with the limit test for iron (100 ppm).

Assay. Dissolve 0.3 g in 5 ml of *dilute acetic acid* into a 500-ml conical flask and add 200 ml of *water*. Add about 500 mg of *xylenol orange triturate* and *hexamine* until the solution becomes violet-pink; add 2 g of *hexamine* in excess. Titrate with *0.1 M disodium edetate* until the violet-pink colour changes to yellow.

1 ml of *0.1 M disodium edetate* is equivalent to 0.01795 g of $ZnSO_4,H_2O$.

Storage. Store in a non-metallic container.

Zinc Sulphate Oral Solution

Zinc Sulphate Oral Solution contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of zinc sulphate monohydrate, $Zn\ SO_4.H_2O$. It may contain one or more suitable flavors and sweeteners.

Identification

A. Complies with the test for sulphates (2.3.1).

B. Complies with the test for zinc (2.3.1).

Tests

pH (2.4.24). 2.5 to 4.5.

Weight per ml (2.4.29). 1.18 g to 1.24 g.

Other tests. Comply with the tests stated under Oral Liquids.

Assay. To a 250-ml flask, transfer a volume of oral solution containing about 99 mg of Zinc Sulphate Monohydrate, add 50 ml of *water* and 10 ml of *ammonia- ammonium chloride buffer* and 0.3 ml of *eriochrome black T solution*. Titrate with *0.05 M disodium edetate* to a green colour.

1 ml of *0.05 M disodiuim edetate* is equivalent to 0.008973 g of $Zn\ SO_4.H_2O$.

Storage. Store protected from light and moisture.

Labelling. Label the oral solution in terms of zinc sulphate monohydrate and in terms of elemental zinc.

Zinc Sulphate Dispersible Tablets

Dispersible Zinc Sulphate Tablets

Zinc Sulphate Dispersible Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the stated

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amount of zinc sulphate monohydrate, $ZnSO_4,H_2O$. It may contain one or more suitable flavours and sweeteners.

Usual strengths. 10 mg; 20 mg of elemental zinc (as zinc sulphate monohydrate).

Identification

Solution A. Shake a quantity of powdered tablets containing about 5 g of Zinc Sulphate with 100 ml of *water* and filter. The filtrate complies with the following tests.

A. To 5 ml of solution A, add 1 ml each of 20 per cent w/w solution of *hydrochloric acid* and *barium chloride solution*. A white precipitate is formed.

B. To 5 ml of solution A, add 0.1 ml of *10 M sodium hydroxide*. A white precipitate is formed. Further add 2 ml of *10 M sodium hydroxide* to dissolve the precipitate. Add 10 ml of *ammonium chloride solution* and the solution remains clear. Add 0.1 ml of *sodium sulphide solution*. A white precipitate is formed.

Tests

Disintegration (2.5.1). Not more than 60 seconds.

Other tests. Comply with the tests stated under Tablets.

Assay. Weigh and powder 20 tablets. Dissolve a quantity of the powder containing about 90 mg of zinc in 15 ml of *dilute acetic acid* and dilute to 200 ml with *water*. Add 1 g of *xylenol orange triturate*, mix and neutralize the solution with about 2 g of *hexamine* until the solution is a violet-pink colour. Titrate with *0.1 M disodium edetate* until the solution is yellow.

1 ml of *0.1 M disodium edetate* is equivalent to 0.01795 g of $ZnSO_4, H_2O$.

Storage. Store protected from moisture, at a temperature not exceeding 30°.

Labelling. Label the tablets in terms of zinc sulphate monohydrate and in terms of elemental zinc.

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PHYTOPHARMACEUTICALS

General Requirements

Phytopharmaceuticals 4139

Monographs

Aegle marmelos PPI 4141

Andrographis paniculata PPI 4142

Curcuma longa PPI 4143

Gymnema sylvestre PPI 4145

Lawsonia inermis PPI 4146

Phyllanthus amarus PPI 4147

Silybum marianum Dry Extract PPI 4149

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Phytopharmaceuticals

The concept of science-based phytopharmaceutical drugs was first introduced through a draft amendment notified to the Drugs and Cosmetics Act 1940, and Rules 1945 there under on 24th October 2013. This notification introduced a definition for Botanical drugs for scientific evaluation for quality safety and efficacy by the office of Central Drugs Standard and Control Organisation (CDSCO) as a requirement for issue of marketing authorization. Botanical drugs were given a legal term as "Phytopharmaceutical" and the notification proposed to define it as *"Phytopharmaceutical drug includes processed or unprocessed standardized materials derived from plants or parts thereof or combination of parts of plants, extracts or fractions thereof in a dosage form for internal or external use of human beings or animals and intended to be used for diagnosis, treatment, mitigation or prevention of any disease or disorder in human beings or animals, but does not include administration by parenteral route"*. Subsequently based on stakeholder inputs the definition of phytopharmaceutical was amended in 2015 and subsequently in 2019. Currently the entire regulations related to phytopharmaceuticals as drugs has been brought under Chapter 1 of subsection (aa) of New Drugs and Clinical Trials Rules, 2018 (ref: G.S.R. 227 (E), dated 19th March 2019). The regulatory requirements for phytopharmaceutical drug are under the purview of CDSCO as it differs from *Ayurveda*, *Siddha* or *Unani* drugs which include all medicines intended for internal or external use for or in the diagnosis, treatment, mitigation or prevention of (disease or disorder in human beings or animals, and manufactured) exclusively in accordance with the formulae described in, the authoritative books of *Ayurveda*, *Siddha* and *Unani* and other systems of medicine specified in the First Schedule. However, phytopharmaceutical drug are fraction of crude extract and are distinctly differentiated by being purified and standardized. A separate category of Phytopharmaceutical Ingredient (PPI) monograph has been included in IP where, an herbal drug/extract/purified fraction has been characterized with minimum of four marker compounds. However, such PPI monographs may or may not strictly meet the requirements of definition of phytopharmaceutical drugs.

Current regulations define *"Phytopharmaceutical drugs includes a purified and standard fraction with defined minimum four bio-active or phyto-chemical compounds (qualitatively and quantitatively assessed), of an extract of a medicinal plant or its part, for internal or external use on human beings or animals, for diagnosis, treatment, mitigation or prevention of any disease or disorder but does not include drug administered through parenteral route"*.

This general chapter provides additional information and guidance to researchers, innovators, and industry on Phytopharmaceutical. As per definition:

- One step extracts of botanicals (with clear botanical identity and part /parts of the plants) involving vehicle/ solvents such as water (maceration, infusion, decoction, concentrated decoction, soft extracts, and such extracts technologically converted to powder formats), solvents such as *ethanol, ethanol: water* mixtures – of predefined ratios (liquid extract, technologically converted to powder form do not meet the requirement of phytopharmaceutical as defined above).
- Fixed oils extracted from botanicals either by cold pressing or by solvent extraction or a mix of both do not meet the requirement of phytopharmaceutical as defined above.
- Distilled oils of volatile components of botanicals do not meet the requirement of phytopharmaceutical as defined above.
- Single step oleo-resin extracts, gums, dried latexes do not meet the requirement of phytopharmaceutical as defined above.
- Extracts obtained by traditional process involving other solvents such as vegetable oil, clarified butter, milk do not meet the requirement of phytopharmaceutical as defined above.
- Steps involving more than one solvent for fractionation, purification adopting suitable technologies and such processed purified fractions subject to complying with other parts of the definition meet the requirement for the phytopharmaceutical *viz.* at least 2 or more fractionation steps involved. Fractionated fatty acids from vegetable oils, fractionated distilled oils, would meet the first requirement to be a phytopharmaceutical. The extent of fractionation and purification may vary depending on the lead being developed.
- Such fractions or purified fractions need to be tested for a defined minimum bio-active or phyto-chemical compounds quantitatively assessed.
- The term marker compound is used to refer phytochemical compounds considered for qualitative or quantitative assessment as a part of describing and maintaining similarity of the fraction to that of a fraction used in bio batch.
- Scientific approach involves selection of such markers either as analytical markers or bio active markers. Analytical marker compounds or phytochemical compounds are well characterized chemically with known knowledge, data about such marker exhibiting phytopharmaceutical drug under development. Such

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analytical markers are assessed purely for their presence and quantity by appropriate analytical methods. Phytochemical compounds well characterized chemically where knowledge or data exists of the compound exhibiting one or more biological activity *in-vitro* or *in-vivo* (in animal studies) or *in-vivo* (in human studies) are referred as bio-active markers. To meet the requirement of phytopharmaceutical, minimum four bio-active marker or analytical marker need to be assessed.

— Assessment for marker compounds is a standard practice in monographs for botanicals in various pharmacopoeias. However, it appears that there is no consensus among scientists globally on the number of marker compounds to be assessed on a botanical for quality assessment. However, innovators/ industry developing a botanical as a pharmaceutical intended to be used as a drug, need to assess minimum of four compounds. Additional information is given under 'frequently asked questions' section for phytopharmaceuticals on the website of CDSCO.

— Emerging scientific understanding reveal that in addition to well characterized single phytocompounds, other phytocomponents such as starches, fibers, inulin, phytosterols and phytostanols, provitamin components, specific minerals may also be contributing to the therapeutic activity. Where the scientific data exists, such components are to be considered as marker compounds.

— While the current definition of phytopharmaceutical does not specify the source of the 'purified fraction" to be from one botanical or more than one botanical, scientific data requirements make it that much more exhaustive data if purified fraction from more than one botanical is to be considered as "lead phytopharmaceutical". It is obvious in such cases, apart from extensive data on quality, safety and efficacy, scientifically generated data on the rationale of such leads, interaction of the fractions from each of the botanical, potential impact on bio availability of each other, and safety data would be required for assessment. Such data generation would be not only a challenge to the scientists/ innovators. Future experience basis evaluation of such cases can provide more clarity.

— The purified fraction meeting the requirements of the definition as above, would be 'phytopharmaceutical ingredient' (PPI) [on lines similar to Active Pharmaceutical Ingredient API]. The dosage form formulated containing the PPI, upon evaluation and finding suitable for marketing, will get 'marketing authorization' and will be a Phytopharmaceutical drug.

The purpose of the PPIs monograph is to provide the scientific information on the quality standards and characterization of minimum four of its bioactive / analytical markers in order to facilitate appropriate maintenance of the standards by the stakeholders. It is to be recognized that mere inclusion of a monograph for an herb or extract or fraction in IP does not give it a status of approved drug by the drug regulator and relevant regulations need to be complied with. Details of all other provision and scientific requirements for submission to CDSCO of a phytopharmaceutical are available in the relevant regulations.

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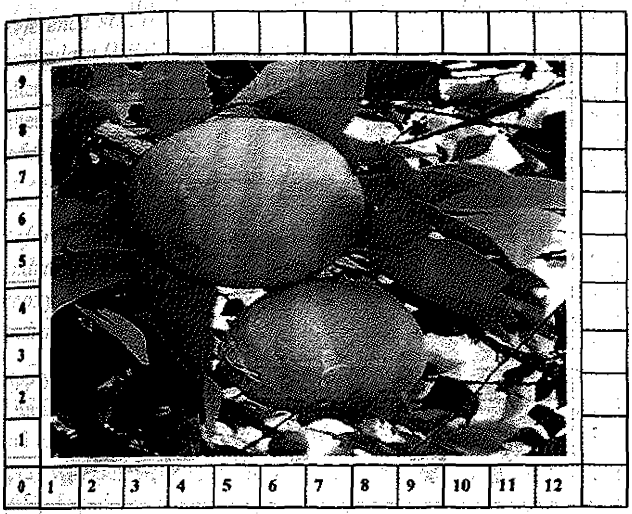
Aegle marmelos PPI

Aegle marmelos (Linn.) Correa

Marker Compounds. Marmelosin, Scopoletin, Psoralen, Umbelliferone and Marmesin.

Source. *Aegle marmelos* PPI is derived from dried pulp of ripe or half ripe fruit of *Aegle marmelos* (Linn.) Correa (Fam. Rutaceae). *Aegle marmelos* PPI contains not less than 3.0 per cent w/w of coumarins calculated on the dried basis as the sum of the marmelosin, scopoletin, psoralen, umbelliferone and marmesin.



Category. Antidiarrhoeal, antimicrobial, antiulcerative, antilipidemic, antidyslipidaemic.

Source description. The dried cut pieces of the pulp are irregularly circular or oval in outline. Outer surface is rough, shows irregular knife scars left on removal of the outer hard rind and sometime a scar left by the removal of stalk. The inner surface is hard, mucilaginous and shows a stout central axis encircled by 10 to 15 chambers separated by thick walls.

Identification

A. *Macroscopic* — Fruit, sub-globose, 5-18 cm in diameter, externally greenish when young, yellowish-brown when ripe, rind about 1.5 - 3 mm thick, hard and woody, surface smooth or slightly granular bearing a circular scar at the point of attachment with peduncle, carpels, 10-15, central, each containing several hairy seeds embedded in yellowish brown, extremely sticky mucilage. Seeds oblong, flat, woody and having white hair, fresh pulp of ripe fruit, brown, of sticky shreads, dried pulp hard and pale to dark red in colour, frequently breaks away from the rind during drying, leaving a thin layer attached to it. Odour is faintly aromatic, mucilaginous and slightly astringent.

B. *Microscopic* — Pulp constitutes mainly the mesocarp portion of the fruit devoid of seeds, scattered with vascular bundles occasionally getting anastomosing throughout the parenchymatous tissue of the ground mass, the cells of which are varying in the size and shape; the cells lying adjacent to the vascular strands and the central axis being smaller in size, vascular bundles are composed of vessels, tracheids, fibers and scalerids. Vessels are bordered pitted thickened while tracheids shows spiral thickening. Fibers are long and thick walled. Sclereids are mostly rectangular in shape with broad lumen and beaded thickened walls.

C. Thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 7 volumes of *toluene*, 3 volumes of *ethyl acetate* and 1 volume of *glacial acetic acid*.

Test solution. Reflux about 2.0 g of the coarsely powdered substance under examination with 50 ml *methanol* on a water-bath for 15 minutes, cool and filter. Reflux the residue further 2 × 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 50.0 ml.

Reference solution. Reflux about 2.0 g of the coarsely powdered *Aegle marmelos IPBRS* with 50 ml *methanol* on a water-bath for 15 minutes, cool and filter. Reflux the residue further 2 × 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 50.0 ml.

or

Reference solution. A 0.01 per cent w/v solution of *marmelosin IPRS, scopoletin IPRS, psoralen IPRS, umbelliferone IPRS* and *marmesin IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 105° for 5 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 7.0 per cent.

Water-soluble extractive (2.6.3). Not less than 45.0 per cent by Method I.

Total ash (2.3.19). Not more than 3.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

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Loss on drying (2.4.19). Not more than 7.0 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 2.0 g of the coarsely powdered substance under examination with 50 ml *methanol* on a water-bath for 15 minutes, cool and filter. Reflux the residue further 2 × 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum slightly less than 100.0 ml. Dilute with *methanol* to 100.0 ml.

Reference solution. A solution containing 0.01 per cent w/v, each of, *marmelosin IPRS, scopoletin IPRS, psoralen IPRS, umbelliferone IPRS* and *marmesin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. *water* with 0.1 per cent v/v of *glacial acetic acid*,
  B. *methanol* with 0.1 per cent v/v of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- column temperature: 35°,
- a gradient programme using the conditions given below,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	50	50
5	35	65
12	20	80
20	10	90
23	10	90
25	50	50
30	50	50

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of coumarins, calculated on the dried basis as the sum of the marmelosin, scopoletin, psoralen, umbelliferone and marmesin.

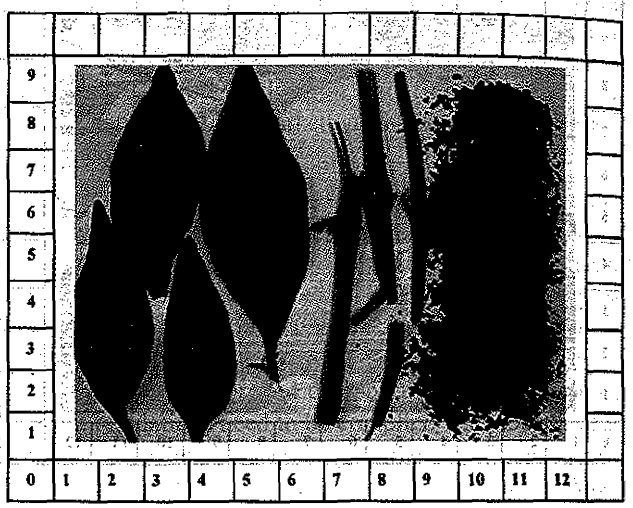
Storage. Store protected from heat and moisture.

Andrographis paniculata PPI

Andrographis paniculata (Burm.f.) Nees

Marker Compounds. Andrographolide, Neoandrographolide, 14-deoxy-11,12-didehydroandrographolide and Andrograpanin.

Source. *Andrographis paniculata* PPI is derived from dried aerial parts of the *Andrographis paniculata* (Burm.f.) Nees (Fam. Acanthaceae). *Andrographis paniculata* PPI contains not less than 1.0 per cent w/w of diterpene lactones, calculated on the dried basis as the sum of the andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide and andrograpanin.



Category. Antiinflammatory, hepatoprotectant, analgesic, antipyretic, anticancer, antimicrobial, antidiabetic, antimalarial and anthelmintic.

Source description. Stem is sharply quadrangular. Leaves are simple, opposite, glabrous, lanceolate, upper surface dark green, 5 - 8 cm long, and 1 - 2 cm wide. Fruit are glandular-hairy, longitudinally furrowed on broad faces, compressed, two celled, and acute at both ends. Seeds are small, 10-12 in numbers, round or ovoid, yellowish brown.

Identification

A. *Macroscopic* — It is a greyish-brown powder.

B. *Microscopic* — It reveals cells of the upper and lower epidermis of the leaves, some cells containing large cystoliths, up to 36 µm in diameter and 180 µm long, with a hilum-shaped scar in the large end; 1-4 celled non glandular hair; disk-shaped glandular hairs, 8 celled head and very short stalk; diacytic stomata mostly on the lower epidermis; stem epidermal cells, some cells containing cystoliths, stomata, non glandular hair and glandular hair similar to those of the leave; thin-walled

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parenchyma cells; collenchyma cells, acicular phloem fibres; tracheids; vessels; with spiral and scalariform thickening.

C. Thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 6 volumes of *chloroform*, 2.5 volumes of *toluene* and 1.5 volumes of *methanol*.

Test solution. Sonicate about 0.5 g of powdered substance under examination with 10 ml of *methanol* for 30 minutes and filter. Sonicate the residue further 2 x 10 ml of *methanol* and filter. Combine all the filtrates and concentrate under vacuum to 5.0 ml.

Reference solution. Sonicate about 0.5 g of *Andrographis paniculata IPBRS* with 10 ml of *methanol* for 30 minutes and filter. Sonicate the residue further 2 x 10 ml of *methanol* and filter. Combine all the filtrates and concentrate under vacuum to 5.0 ml.

or

Reference solution. A 0.1 per cent w/v solution of *andrographolide IPRS, neoandrographolide IPRS, 14 deoxy-11,12-didehydroandrographolide IPRS* and *andrograpanin IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands of 8 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *vanillin sulphuric acid reagent*. Heat the plate at 105° for 5 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 3.0 per cent.

Water-soluble extractive (2.6.3). Not less than 12.0 per cent by Method I.

Total ash (2.3.19). Not more than 15.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Sonicate about 0.5 g of powdered substance under examination with 10 ml of *methanol* for 30 minutes and filter. Sonicate the residue further 2 x 10 ml of *methanol* and filter. Combine all the filtrates and concentrate under vacuum to 5.0 ml.

Reference solution. A solution containing 0.1 per cent w/v, each of *andrographolide IPRS, neoandrographolide IPRS, 14-deoxy-11,12-didehydroandrographolide IPRS* and *andrograpanin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution dissolving 0.136 g of potassium *dihydrogen orthophosphate* in 500 ml of *water* and 0.5 ml of *orthophosphoric acid*, dilute to 1000 ml with *water*,

  B. *acetonitrile*,
- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 223 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of diterpene lactones, calculated on the dried basis as the sum of the andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide and andrograpanin.

Storage. Store protected from heat and moisture.

Curcuma longa PPI

Curcuma longa Linn.

Marker Compounds. Curcumin, Desmethoxycurcumin, bis-Desmethoxycurcumin and ar-turmerone.

Source. *Curcuma Longa* PPI is derived from dried root parts of the *Curcuma longa* Linn. (Fam. Zingeberaceae). It contains not less than 1.0 per cent w/w of curcuminoids calculated on

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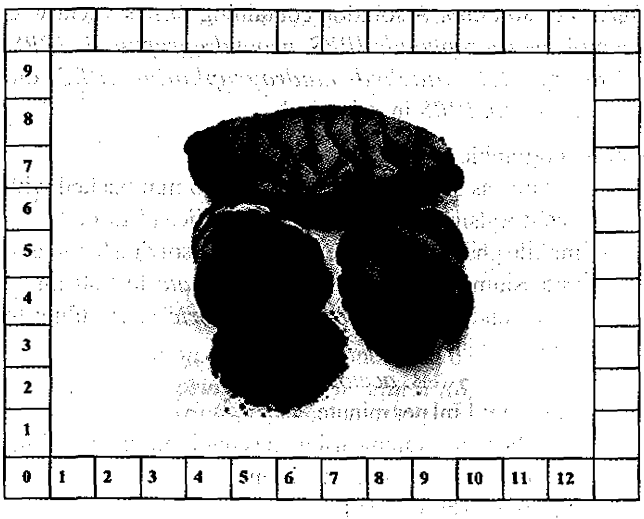
the dried basis as the sum of curcumin, desmethoxycurcumin, bis-desmethoxycurcumin and ar-turmerone.



Category. Antioxidant, antibacterial, antifungal, antiinflammatory, platelet aggregation inhibitor, cytotoxic, antiallergic, hypotriglyceridemic, enterokinase inhibitor, CNS depressant, analgesic.

Source description. *Curcuma Longa* Linn. is a stout underground rhizomatous aromatic herb. Leaves die down in late autumn and the rhizomes remain dormant during winter. The inflorescence again becomes visible in spring from the rhizome. The peduncle grows to 20-25 cm in height. Foliage appears after the emergence of the flowers. A full grown plant can reach upto height of 90 cm.

Identification

A. *Macroscopic* — Rhizomes of *Curcuma Longa* Linn. are ovate to oblong or pyriform and sometimes cylindrical and short branched. Externally the rhizomes are yellowish to yellowish-brown with root scars and signs of leaf bases.

B. *Microscopic* —Transverse section of rhizomes shows thick-walled epidermis with cubical cells of diverse dimensions. Thin-walled round shaped parenchyma cells and scattered collateral vascular bundles are present in cortex. A few layers of cork present under epidermis with scattered oleo-resin cells having brownish contents. Cork composed of 4-6 layers of brick-shaped, thin-walled and parenchymatous.

C. Thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 96 volumes of *chloroform*, 4 volumes of *methanol* and 1 volume of *formic acid*.

Test solution. Sonicate 0.2 g of the powdered substance under examination in 3 ml of *acetone* for 5 minutes and filter. Supernatant is used for spotting.

Reference solution. Sonicate 0.2 g of the *Curcuma Longa IPBRS* in 3 ml of *acetone* for 5 minutes and filter. Supernatant is used for spotting.

or

Reference solution. A 0.02 per cent w/v solution of *curcumin IPRS*, *desmethoxycurcumin IPRS*, *bis-desmethoxycurcumin IPRS* and *ar-turmerone IPRS* in *acetone*.

Apply to the plate 10 µl of each solution as bands of 8 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm and in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 6.0 per cent.

Water-soluble extractive (2.6.3). Not less than 12.0 per cent by Method I.

Total ash (2.3.19). Not more than 10.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not more than 12.0 per cent, determined on 0.2 g.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Sonicate about 0.1 g of powdered substance under examination with 5 ml *acetone* for 30 minutes, filter and make up the volume to 5.0 ml.

Reference solution. A solution containing 0.1 per cent w/v, each of, *curcumin IPRS*, *desmethoxycurcumin IPRS*, *bis-desmethoxycurcumin IPRS* and *ar-turmerone IPRS* in *acetone*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. *water*,
  B. *acetonitrile*,
- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 240 nm,
- injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
40	20	80
45	85	15

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Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of curcuminoids calculated on the dried basis as the sum of curcumin, desmethoxycurcumin and bis-desmethoxycurcumin and ar-turmerone.

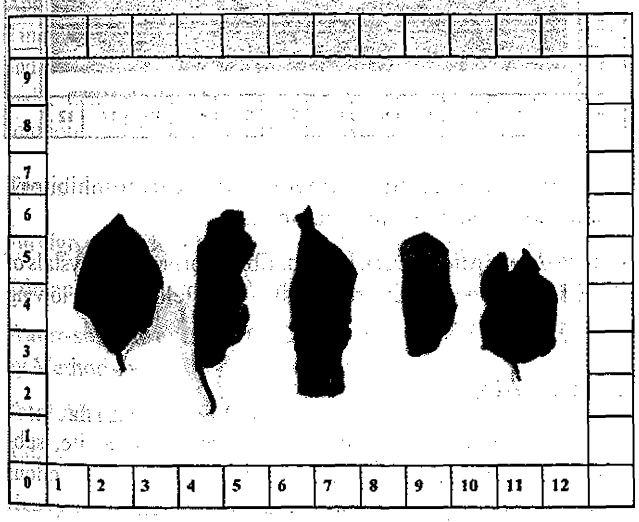
Storage. Store protected from heat and moisture.

Gymnema sylvestre PPI

Gymnema sylvestre (Retz.) R.Br. ex Sm.

Marker Compounds. Gymnemagenin, Deacylgymnemic acid, Gymnemic acid IV and Gymnemic acid XVII.

Source. *Gymnema sylvestre* PPI is derived from the dried leaves of *Gymnema sylvestre* (Retz.) R.Br. ex Sm. (Fam. *Apocynaceae*). *Gymnema sylvestre* PPI contains not less than 10.0 per cent w/w of gymnemic acid calculated on the dried basis as the sum of the gymnemagenin, deacylgymnemic acid, gymnemic acid IV and gymnemic acid XVII.



Category. Antidiabetic, hepatoprotectant, antimicrobial and antiinflammatory.

Source description. It is a slow growing, perennial large woody, branched and more or less pubescent liana. Leaves are elliptical and opposite, and flowers are small and yellow in colour.

Identification

A. *Macroscopic* — Leaves are opposite, petiolate and elliptical to ovate in shape. Usually the size of the petiole ranges from 0.6-1.2 cm and lamina is 0.3-0.6 cm long and 0.1-0.3 cm broad with reticulate venation and unpleasant odour.

B. *Microscopic* — Midrib is a layer of epidermis followed by 2-3 collenchyma layers, center placed, crescent shaped, conjoint and bicollateral vascular bundle. The tissue between vascular bundles and collenchymas composed of thin walled parenchyma cells. Petiole is semicircular outline with profound furrow having single layered epidermis below thick cuticle. Uniseriate multicellular trichomes, cortex consists of 3-4 layers of collenchyma and parenchyma cells. Vascular bundles are 3 in number, conjoint and bicollateral. Leaf powder is light green in colour, microscopic view reveals epidermal cells with straight wall and surface view shows stomata with one or more pairs of parallel oriented lateral subsidiary cells along with calcium oxalate crystals and broken trichomes.

C. Thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 80 volumes of *chloroform*, 18 volumes of *methanol* and 2 volumes of *formic acid*.

Test solution. Sonicate about 0.2 g of powdered substance under examination with 10 ml of 70 per cent v/v *methanol* in *water* for 10 minutes and filter. Sonicate the residue further 2 × 10 ml of 70 per cent v/v *methanol* in *water* and filter. Combine all the filtrates and concentrate under vaccum up to the volume 10.0 ml.

Reference solution. Sonicate about 0.2 g of *Gymnema sylvestre IPBRS* with 10 ml of 70 per cent v/v *methanol* in *water* for 10 minutes and filter. Sonicate the residue further 2 × 10 ml of 70 per cent v/v *methanol* in *water* and filter. Combine all the filtrates and concentrate under vaccum up to the volume 10.0 ml.

or

Reference solution. A 0.1 per cent w/v solution of *gymnemagenin IPRS, deacylgymnemic acid IPRS, gymnemic acid IV IPRS* and *gymnemic acid XVII IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands of 8 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *dilute sulphuric acid reagent*. Heat the plate at 120° for 5 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 5.0 per cent.

Water-soluble extractive (2.6.3). Not less than 20.0 per cent by Method I.

Total ash (2.3.19). Not more than 15.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 6.0 per cent.

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Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 14.0 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Sonicate about 0.5 g of powdered substance under examination with 25 ml *methanol* for 5 minutes and filter. Sonicate the residue further 2 x 10 ml of *methanol* and filter. Combine all the filtrates and make up the volume to 10.0 ml.

Reference solution. A solution containing 0.01 per cent w/v, each of, *gymnemagenin IPRS, deacylgymnemic acid IPRS, gymnemic acid IV IPRS* and *gymnemic acid XVII IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution dissolving 0.136 g of *anhydrous potassium dihydrogen phosphate* in 900 ml of *water* and 0.5 ml of *orthophosphoric acid* and dilute to 1000 ml with *water*,
  B. *acetonitrile*,
- flow rate: 1.5 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 205 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
5	85	15
10	75	25
15	65	35
20	60	40
25	50	50
30	35	65
35	20	80
40	95	5

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of gymnemic acid calculated on the dried basis as the sum of the gymnemagenin, deacylgymnemic acid, gymnemic acid IV and gymnemic acid XVII.

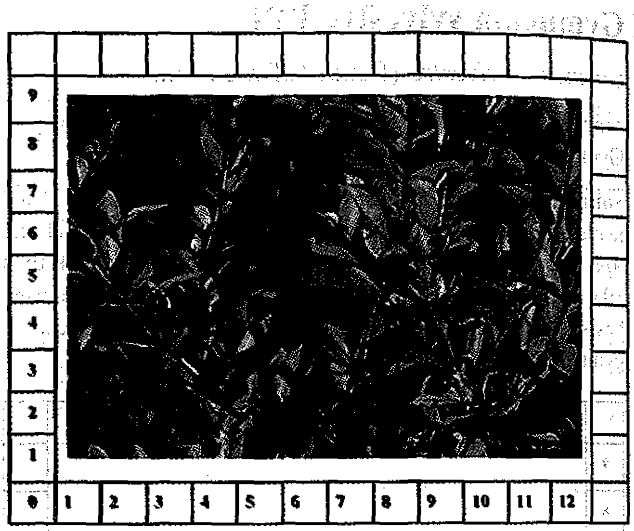
Storage. Store protected from heat and moisture.

Lawsonia inermis PPI

Lawsonia inermis Linn.

Marker Compounds. Lawsone, Quercetin, Luteolin-7-O-glucoside and Apigenin.

Source. *Lawsonia inermis* PPI is derived from dried leaves of *Lawsonia inermis* Linn. (Fam. Lythreaceae). *Lawsonia inermis* PPI contains not less than 0.03 percent w/w of lawsone and not less than 0.3 percent w/w of flavanoids calculated on the dried basis as the sum of the quercetin, luteolin-7-O-glucoside and apigenin.



Category. Astringent, antineoplastic, cardioinhibitory, hypotensive, sedative, antimicrobial.

Source description. Dark brown, fibres, prismatic crystals of calcium oxalate, druses (rosettes), vessel elements showing annular thickenings.

Identification

A. *Macroscopic* — The leaves are simple, opposite, sub-sessile, elliptic, entire, acute or obtuse, petiole short, odour aromatic; mucilagionous and slightly astringent.

B. *Microscopic* — Upper epidermis possessing elliptical mucilagionous sacs. The dorsal side of the midrib is marked by a narrow V-shaped groove while the ventral side is convex. Groove is lined by epidermis and a few layers of collenchymas. 2 or 3 layers of collenchyma are also present sub epidermally on the ventral convex side. Vascular bundle crescent shaped with a central xylem surrounded by a phloem elements, fibres and parenchyma. Lamina mesophyll differentiated into an upper palisade and lower spongy parenchyma. Cells of palisade parenchyma are columnar and compactly arranged in 2 rows. Spongy parenchyma composed of rounded cells with intercellular spaces. Lamina transverse by a few small vascular

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bundles comprising a xylem core surrounded by phloem. Some of the parenchyma cells of midrib and lamina region contain rosettes and prismatic crystals.

C. Thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 55 volumes of *ethyl acetate*, 40 volumes of *toluene* and 5 volumes of *formic acid*.

Test solution. Reflux about 5.0 gm of coarsely powder substance under examination with 100 ml *methanol* for 30 minutes, cool and filter. Reflux the residue further 2 x 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10.0 ml.

Reference solution. Reflux about 5.0 gm of *Lawsonia inermis IPBRS* with 100 ml *methanol* for 30 minutes, cool and filter. Reflux the residue further 2 x 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10.0 ml.

or

Reference solution. A 0.1 per cent w/v solution of *lawsone IPRS*, *quercetin IPRS*, *luteolin-7-O-glucoside IPRS* and *apigenin IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands of 8 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm and under white light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 10.0 per cent.

Water-soluble extractive (2.6.3). Not less than 20.0 per cent by Method I.

Total ash (2.3.19). Not more than 14.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 4.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 5.0 gm of coarsely powder substance under examination with 100 ml *methanol* for 30 minutes, cool and filter. Reflux the residue further 2 x 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10.0 ml.

Reference solution. A solution containing 0.1 per cent w/v, each of *lawsone IPRS*, *quercetin IPRS*, *luteolin-7-O-glucoside IPRS* and *apigenin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. *water* with 0.1 per cent v/v of *glacial acetic acid*,
  B. *methanol* with 0.1 per cent v/v *glacial acetic acid*,
- flow rate: 1 ml per minute,
- column temperature: 35°
- a gradient programme using the conditions given below,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	50	50
5	35	65
12	20	80
20	10	90
23	10	90
25	50	50
30	50	50

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of lawsone and flavanoids calculated on the dried basis as the sum of the quercetin, luteolin-7-O-glucoside and apigenin.

Storage. Store protected from heat and moisture.

Phyllanthus amarus PPI

Phyllanthus amarus Schumach. and Thonn.

Marker Compounds. Phyllanthin, Hypophyllanthin, Nirtetralin and Niranthin.

Source. *Phyllanthus amarus* PPI is derived from dried aerial part collected at fruiting stage of the *Phyllanthus amarus* Schumach. and Thonn. (Fam. Phyllanthaceae). *Phyllanthus amarus* PPI contains not less than 0.25 per cent w/w of lignans, calculated on the dried basis as the sum of the phyllanthin, hypophyllanthin, nirtetralin and niranthin.

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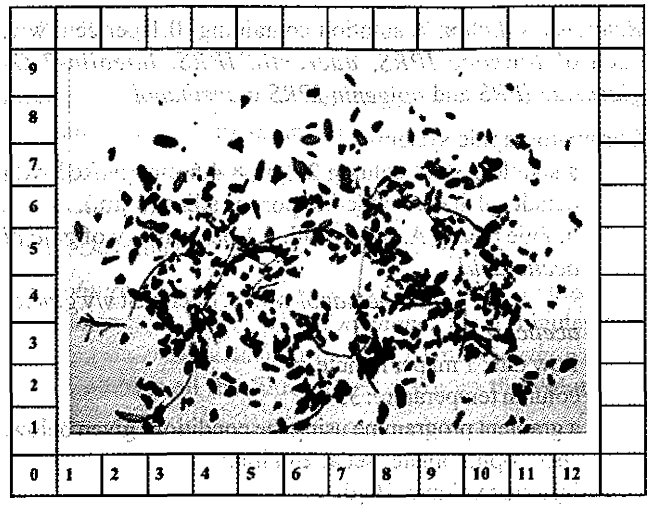

Category. Hepatoprotective, astringent, stomachic, diuretic, febrifugal, antiseptic.

Source description. *Phyllanthus amarus* Schumach. and Thonn. is an annual herbs, with erect, simple and branched stem. The plant is about 10-60 cm tall. Leaf blades are petiolate, oblong to obovate ranging from 3-11 mm long, and 1.5-6 mm wide. Flowers are minutes in unisexual cymules. Seeds are about 1 mm long, triangular, light brown, with 5-6 ribs on the back.

Identification

A. *Macroscopic* — Organoleptic assessment revealed the deep green colour and non-specific odour and tastes of the leaves. *Phyllanthus amarus* Schumach. and Thonn. has pinnate and compound leaves with alternate arrangement on the stem. The leaf blades are oblong to elliptical with entire margins, glabrous surface and reticulate venation. Small, ovoid fruitlets were arranged on the lower side of each rachis.

B. *Microscopic* — Leaves showed the presence of wavy epidermal cells, paracytic stomata and prismatic calcium oxalate crystals that were lined up along the length of the veins. Stomatal cells are 2-8, epidermal cells are 33-37 and vein islets are 8-12 in numbers.

C. Thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 2 volumes of *hexane* and 1 volume of *ethyl acetate*.

Test solution. Reflux about 0.5 g of powdered substance under examination with 10 ml *methanol* for 30-45 minutes, cool and filter. Reflux the residue further 2 x 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 5.0 ml.

Reference solution. Reflux about 0.5 g of powdered *Phyllanthus amarus IPBRS* with 10 ml *methanol* for 30-45 minutes, cool and filter. Reflux the residue further 2 x 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 5.0 ml.

or

Reference solution. A 0.01 per cent w/v solution of *phyllanthin IPRS, hypophyllanthin IPRS, nirtetralin IPRS* and *niranthin IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands of 8 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *vanillin sulphuric acid reagent*. Heat the plate at 120° for 5 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 6.0 per cent.

Water-soluble extractive (2.6.3). Not less than 15.0 per cent by Method I.

Total ash (2.3.19). Not more than 8.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 5.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 0.5 g of powdered substance under examination with 10 ml *methanol* for 30-45 minutes, cool and filter. Reflux the residue further 2 × 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 5.0 ml.

Reference solution. A solution containing 0.01 per cent w/v, each of, *phyllanthin IPRS, hypophyllanthin IPRS, nirtetralin IPRS* and *niranthin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 10 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (2 µm),
- mobile phase: 0.5 per cent v/v of *1,4 dioxan* in a mixture of 40 volumes of *water* and 60 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 10 µl.

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Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of lignans, calculated on the dried basis as the sum of the phyllanthin, hypophyllanthin, nirtetralin and niranthin.

Storage. Store protected from heat and moisture.

Silybum marianum Dry Extract PPI

Silybum marianum (Linn.) Gaertn.

Marker Compounds. *Silychristin*, *Silybin A*, *Silybin B*, *Isosilybin A* and *Isosilybin B*.

Source. *Silybum marianum* Dry Extract PPI is derived by extracting *Silybum marianum* (Linn.) Gaertn. (Fam. Asteraceae) fruit or seed with *methanol* or any other suitable solvent. *Silybum marianum* Dry Extract PPI contains not less than 40.0 per cent w/w flavanolignans, calculated on the dried basis as sum of *silychristin*, *silybin A*, *silybin B*, *isosilybin A* and *isosilybin B*.

Category. Hepatoprotective, chemopreventive.

Source description. Yellowish, amorphous powder.

Identification

A. Thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 9 volumes of *chloroform*, 2.5 volumes of *acetone* and 1 volume of *formic acid*.

Test solution. Dissolve about 0.1 g of the extract to be examined in 10 ml of *methanol* then sonicate for 30 minutes.

Reference solution. Reflux 2.0 g of the *Silybum marianum extract IPBRS* with 10.0 ml *methanol* for 15 minutes, cool and filter.

or

Reference solution. A 0.1 per cent w/v solution of *silybin A IPRS*, *silybin B IPRS*, *isosilybin A IPRS* and *isosilybin B IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands of 8 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *vanillin sulphuric acid reagent*. Heat the plate at 105° for 5 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Total ash (2.3.19). Not more than 8.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 0.5 g of the extract to be examined in 10 ml of *methanol* then sonicate for 30 minutes. Concentrate under vacuum to 5.0 ml.

Reference solution. A solution containing 0.1 per cent w/v, each of, *silychristin IPRS*, *silybin A IPRS*, *silybin B IPRS*, *isosilybin A IPRS* and *isosilybin B IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 45 volumes of *methanol* and 55 volumes of 0.1 per cent v/v *formic acid* in *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 288 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of flavanolignans, calculated on the dried basis as the sum of silychristin, silybin A, silybin B, isosilybin A and isosilybin B.

Storage. Store protected from heat and moisture.

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HERBS AND HERBAL PRODUCTS

General Requirements

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Monographs

Acacia (*Vachellia nilotica* (Linn.) P.J.H. Hurter and Mabb.) 4162

Ajwain (*Trachyspermum ammi* (Linn.) Sprague) 4162

Amalaki (*Phyllanthus emblica* Linn.) 4164

Amaltas (*Cassia fistula* Linn.) 4165

Amarbel (*Cuscuta reflexa* Decne.) 4166

Amla Juice Powder (*Phyllanthus emblica* Linn. Powder) 4167

Amra (*Mangifera indica* Linn.) 4168

Anantmula (*Hemidesmus indicus* Linn.) 4169

Anise Oil (*Pimpinella anisum* Linn. Oil) 4170

Arachis Oil (*Arachis hypogea* Linn. Oil) 4171

Arjuna (*Terminalia arjuna* (Roxb. ex DC.) Wight and Arn.) 4172

Arjuna Dry Extract (*Terminalia arjuna* (Roxb. ex DC.) Wight and Arn. Dry Extract) 4173

Artemisia (*Artemisia annua* Linn.) 4174

Ashwagandha (*Withania somnifera* (Linn.) Dunal) 4175

Ashwagandha Dry Extract (*Withania somnifera* (Linn.) Dunal Dry Extract) 4176

Asoka (*Saraca asoca* (Roxb.) J.J.de Wilde) 4177

Asthisamhrta (*Cissus quadrangularis* Linn.) 4178

Bakuci (*Cullen corylifolium* (Linn.) Medik) 4179

Bala (*Sida acuta* Burm.f.) 4180

Basil Oil {(Methyl Chavicol Type) (*Ocimum basilicum* Linn. Oil)} 4181

Bassant (*Hypericum perforatum* Linn.) 4182

Bassant Dry Extract (*Hypericum perforatum* Linn. Dry Extract) 4183

Belladona Leaf (*Atropa bella-donna* Linn. Leaf) 4184

Belladona Dry Extract (*Atropa bella-donna* Linn. Dry Extract) 4185

Belladona Dry Extract Tablets (*Atropa bella-donna* Linn. Dry Extract Tablets) 4186

Belladona Soft Extract (*Atropa bella-donna* Linn. Soft Extract) 4187

Belladona Tincture (*Atropa bella-donna* Linn. Tincture) 4188

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Berberine Chloride 4189
Bhibhitaki (*Terminalia bellirica* Gaertn.) 4190
Bhibhitaki Aqueous Extract (*Terminalia bellirica* Gaertn. Extract) 4191
Bhringraj (*Eclipta prostrata* Linn.) 4192
Bhuiamla Dry Extract (*Phyllanthus amarus* Schum. and Thonn. Dry Extract) 4193
Birmi (*Taxus wallichiana* Zucc.) 4194
Black Pepper Oil (*Piper nigrum* Linn. Oil) 4195
Brahmi (*Bacopa monnieri* (Linn.) Wettst.) 4196
Brahmi Extract (*Bacopa monnieri* (Linn.) Wettst. Extract) 4197
Caraway Oil (*Carum carvi* Linn. Oil) 4198
Cardamom Oil (*Elletaria cardamomum* Oil) 4198
Cassia Oil (*Cinnamomum cassia* Blume Oil) 4199
Castor Oil (Refined grade) (*Ricinus communis* Linn.) 4199
Castor Oil (Virgin grade) (*Ricinus communis* Linn.) 4200
Chaulai (*Amaranthus tricolor* L.) 4200
Chitrak (*Plumbago zeylanica* Linn.) 4201
Cinnamon Bark Oil (*Cinnamomum zeylanicum* Nees. Bark Oil) 4202
Cinnamon Leaf Oil (*Cinnamomum verum* J.S.Presl. leaf Oil) 4203
Citronella Oil {(Geraniol Type) (*Cymbopogon nardus* Oil)} 4204
Citronella Oil {(Java Type) (*Cymbopogon winterians* Jowitt Oil)} 4204
Clove Bud Oil (*Syzygium aromaticum* Linn. bud Oil) 4205
Clove Leaf Oil (*Syzygium aromaticum* Linn. leaf Oil) 4205
Clove Stem Oil (*Syzygium aromaticum* Linn. stem Oil) 4206
Coconut Oil (*Cocos nucifera* Linn. Oil) 4207
Coleus (*Coleus forskohlii* Briq.) 4208
Coleus Dry Extract (*Coleus forskohlii* Willd. Briq. Dry Extract) 4209
Coriander Oil (*Coriandrum sativum* Oil) 4209
Cumin Oil (*Cuminum cyminium* Linn. Oil) 4210
Daruharidra Roots (*Berberis aristata* DC roots) 4211
Daruharidra Stems (*Berberis aristata* DC stems) 4212
Dill Seed Oil (*Anethum sowa* Oil) 4214
Draksha (*Vitis vinifera* Linn.) 4214
Ergot (*Claviceps purpurea* Fries) 4215

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Prepared Ergot (*Claviceps purpurea* Fries) 4216
Eucalyptus Oil (*Eucalyptus globulus* Oil) 4217
Garcinia (*Garcinia cambogia* Desr.) 4218
Garcinia Aqueous Extract (*Garcinia cambogia* Desr. Extract) 4219
Ginkgo Leaf (*Gingko biloba* Linn. Leaf) 4220
Ginkgo Dry Extract (*Gingko biloba* Linn. Dry Extract) 4221
Ginkgo Tablets (*Gingko biloba* Linn. Tablets) 4222
Ginseng (*Panax ginseng* C.A Myer) 4223
Ginseng Dry Extract (*Panax ginseng* Dry Extract) 4224
Gokhru (*Tribulus terrestris* Linn.) 4225
Green Coffee Bean Extract (*Coffea arabica/Coffea robusta* Linn. Bean Extract) 4226
Guar Gum (*Cyamopsis tetragonolobus* Linn.) 4227
Gudmar Dry Extract (*Gymnema sylvestre* R.Br. Dry Extract) 4228
Guduchi (*Tinospora cordifolia* Willd.) 4229
Guggul Resin (*Commiphora wightii* Arnott) 4230
Gugulipid (*Commiphora wightii* Arnott) 4231
Gugulipid Tablets (*Commiphora wightii* Arnott Tablets) 4232
Haridra Dry Extract (*Curcuma longa* Linn. Dry Extract) 4232
Haritaki (*Terminalia chebula* Retz.) 4233
Haritaki Extract (*Terminalia chebula* Retz. Extract) 4235
Haritaki Aqueous Extract (*Terminalia chebula* Retz. Aqueous Extract) 4235
Hingu (*Ferula asafoetida* Regel.) 4236
Horse Chestnut Dry Extract (*Aesculus hippocastanum* Dry Extract) 4237
Hydrogenated Castor Oil (*Ricinus communis* Oil) 4238
Ipecac Tincture {Cephaelis ipecacuanha (Brot.) A. Rich.} 4239
Ispaghula Husk (*Plantago ovate* Forssk.) 4240
Ivy Leaf (*Hedera helix* Linn. leaf) 4240
Ivy Leaf Dry Extract (*Hedera helix* Linn. Dry Extract) 4241
Jangali Haldi (*Curcuma aromatica*) 4242
Jatamansi (*Valeriana jatamansi*) 4243
Juniper Oil (*Juniperus communis* Linn. Oil) 4244
Kalmegh Dry Extract (*Andrographis paniculata* Nees. Dry Extract) 4245
Karipatta (*Murraya koenigii* Linn.) 4246

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Kasni (*Cichorium intybus* Linn.) 4247
Kaunch (*Mucuna pruriens* Linn.) 4248
Kunduru (*Boswellia serrata* Roxb.) 4249
Kunduru Dry Extract (*Boswellia serrata* Roxb. Dry Extract) 4250
Kutki (*Picrorhiza kurroa* Royle) 4251
Lasuna (*Allium sativum* Linn.) 4252
Lavang (*Syzygium aromaticum* Linn) 4253
Lavender Oil (*Lavendula angustifolia* P. Miller Oil) 4254
Lemon Grass Oil (*Cymbopogon flexuosus* Stapf Oil) 4254
Lemon Oil (*Citrus limon* Linn. Oil) 4255
Lime Oil (*Citrus aurantifolia* Oil) 4256
Lodhra (*Symplocos racemosa* Roxb.) 4256
Malt Extract (*Hordeum vulgare*) 4257
Mandarin Oil (*Citrus reticulata* Blanco Oil) 4258
Mandukaparni (*Centella asiatica* Linn.) 4259
Mandukaparni Dry Extract (*Centella asiatica* Linn. Dry Extract) 4260
Manjistha (*Rubia cordifolia* Linn.) 4261
Maricha (*Piper nigrum* Linn.) 4262
Mentha Oil (*Mentha species* Oil) 4263
Mentha Arvensis Oil (*Mentha arvensis* Linn. Oil) 4263
Methi (*Trigonella foenumgraecum* Linn.) 4264
Milk Thistle (*Silybum marianum* Linn.) 4265
Mirch (*Capsicum annum* Linn.) 4267
Nagakesar (*Mesua ferrea* Linn.) 4268
Neem (*Azadirachta indica* A. Juss.) 4269
Nirgundi (*Vitex negundo* Linn.) 4270
Noni (*Morinda citrifolia* Linn.) 4271
Nutmeg Oil (*Myristica fragrans* Houttuyn Oil) 4272
Opium (*Papaver somniferum* Linn.) 4273
Opium Powder (*Papaver somniferum* Linn. powder) 4275
Papain (*Carica papaya* Linn.) 4276
Patha (*Cissampelos pareira* Linn.) 4277
Peppermint Oil (*Mentha piperita* Linn. Oil) 4278

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Pippali Large (*Piper retrofractum* Vahl.) 4279
Pippali Small (*Piper longum* Linn.) 4280
Punarnava (*Boerhaavia diffusa* Linn.) 4281
Puskara (*Inula racemosa* Hook. F.) 4282
Rosemary Oil (*Rosemarinus officinalis* Linn. Oil) 4283
Sahajana Leaf (*Moringa oleifera* Lamk. Leaf) 4284
Sahajana Stick (*Moringa oleifera* Lamk. Stem) 4285
Sarpagandha (*Rauwolfia serpentina* Kurz) 4285
Sarpagandha Powder (*Rauwolfia serpentina* Kurz Powder) 4286
Sarpagandha Tablets (*Rauwolfia serpentina* Kurz Tablets) 4286
Saunf (*Foeniculum vulgare* Mill.) 4288
Schisandra Fruit (*Schisandra chinensis* Turcz. Baill Fruit) 4289
Schisandra Dry Extract (*Schisandra chinensis* Turcz. Baill Extract) 4290
Senna Leaf (*Cassia angustifolia* Vahal. Leaf) 4291
Powdered Senna Leaf (*Cassia angustifolia* L.Vahal) 4292
Senna Pods (*Cassia angustifolia* Vahal. Pods) 4292
Senna Dry Extract (*Cassia angustifolia* Vahal. Dry Extract) 4294
Senna Tablets (*Cassia angustifolia* Vahal. Tablets) 4294
Shankhpushpi (*Convolvulus pluricaulis* Choisy) 4295
Shatavari (*Asparagus racemosus* Willd.) 4296
Shati (*Hedychium spicatum* Buch.-Ham. Ex Smith) 4300
Shellac (*Laccifer lacca* Kerr) 4301
Siris (*Albizia lebbeck* (Linn.) Benth.) 4302
Starch (*Zea mays* Linn.) 4303
Sunthi (*Zingiber officinale* Roscoe.) 4304
Sunthi Extract (*Zingiber officinale* Roscoe. Extract) 4305
Sweet Orange Oil (*Citrus sinensis* Linn. Oil) 4306
Tea Tree Oil (*Melaleuca alternifolia* Cheel Oil) 4306
Thyme Oil (*Thymus zygis* Loefl. Linn. Oil) 4307
Tolu Balsam (*Myroxylon balsamum* Linn.) 4308
Tragacanth (*Astragalus gummifer* Labill.) 4309
Tulasi (*Ocimum sanctum* Linn.) 4310
Tulasi Dry Extract (*Ocimum sanctum* Linn. Dry Extract) 4311

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Tvak (*Cinnamomum zeylanicum* Blume.) 4311
Upakunchika (*Nigella sativa* Linn.) 4313
Valerian Root (*Valeriana officinalis* Linn. Root) 4314
Valerian Dry Extract (*Valeriana officinalis* Linn. Dry Extract) 4315
Vasaka (*Adhatoda vasica* Nees.) 4316
Vasaka Extract (*Adhatoda vasica* Nees. Extract) 4317
Vidanga (*Embelia ribes* Burm.) 4318
Vijayasara (*Pterocarpus marsupium* Roxb.) 4319
Yasti (*Glycyrrhiza glabra* Linn.) 4320
Yasti Dry Extract (*Glycyrrhiza glabra* Linn. Dry Extract) 4321

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Herbs and Herbal Products

Herbs and products containing herb(s) have been in trade and commerce and are currently used for a variety of purposes. As a country, India has a rich history of use of herbs, processed herbs and formulations containing herbs both from traditional wisdom as well as cultural usage. Herbs and herbal products are also regulated by various laws. For the purposes of pharmacopoeial standards various considerations have been given. This monograph provides a general outline and policies towards the same.

Crude Herbs

This term means, unless specified otherwise, mainly whole, fragmented or cut, plants, parts of plants, algae, fungi, and lichen in a form which is not processed. Herbs are usually in dried form, but sometimes, when specified, may also be in a fresh form. In specific cases exudates which have not been processed further also are covered under the term herbs. Processing, does not include, normally expected value addition steps like grading, sizing, removal of weeds or parts of plants other than those specified herbs and removal of adulterants. The term herbs, though botanically generally refer to plants of specified height and nature, for the purposes of pharmacopoeial reference, shall mean and include plants and parts of plants not necessarily from herbs and shrubs, but cover the entire range namely creepers, climbers, trees etc. Each monograph of a herb in the pharmacopoeia shall specify the botanical scientific name according the binomial system specifying the genus, species, variety and author. In cases where there are controversial botanical identity, as is seen with mainly herbs known in the Indian traditional system, the monograph shall specify the official name of the herb along with its botanical scientific name and guidance is taken from Ayurvedic Pharmacopoeia of India* to decide the same. In cases where, the same herb is available in different grades or sizes, if found appropriate and necessary, separate monographs may be introduced in the pharmacopoeia to cover each of them with appropriate standards. For example- Pippali (large) and Pippali (small).

In order to improve authentication of botanical identity, especially in those cases where there are controversial botanical identity or chances of substitution/adulteration is expected, IP has provided general test method adopting DNA-based identification test. This method has been provided in appendix 2.2.17 under General Tests. A detailed test method adopting this has been introduced in the monograph of Shatavari to begin with. Testing by this method has been made mandatory only in case of a dispute.

While deciding to introduce a monograph for a herb in the pharmacopoeia, the criteria that would be kept in mind, but not limited to are – herbs with specific name and a definitive botanical identity up to species, availability and usage in trade and commerce, regulatory interest, knowledge of and availability of a specific chemical compound of well characterised structure [either responsible for the biological activity of the herb (bio-marker) or a chemical compound known to be present in the herb even if not responsible for biological activity (chemical/analytical marker)], availability of a quantitative method for estimation of such a compound, knowledge of safety of the herb, and its sustainability. Herbs which may figure in a regulated list under appropriate forest and other laws, may still be taken up for a monograph for inclusion in pharmacopoeia, if there is knowledge of efforts to cultivate or take care of sustainability issues and /or specific permission is available under law for use of the herb. As already specified under "General Notices" in the pharmacopoeia, appearance of a monograph does not mean its approval as a drug under the law. Monograph of a herb in the pharmacopoeia is to provide qualitative and quantitative standards of quality for the herb for its use either as a food item or food ingredient or food supplement/ nutraceuticals, as a drug, and/or as an ingredient in cosmetics. Each such use would need to comply with applicable regulations. Each herb is regarded as one active substance, irrespective of the knowledge about the active constituents of the herb is available or not.

* Ayurvedic Pharmacopoeia of India, Vol. 1-6, Ministry of Health and Family Welfare, Govt. of India.

Herbs may be exposed to low dose gamma radiation for purposes of reducing their microbial contamination. Herbs treated with low dose gamma radiation shall meet national laws related to such treatment and shall be labeled as per law.

Processed Herbs

Processed herbs means preparations obtained by subjecting herbs to treatment such as extraction, distillation, expression, fractionation, purification, concentration and partial or full fermentation. Processing may also be done by way of powdering herbs, preparing tincture, preparing extract, distilling to get essential oils, fatty oils (either expressed or solvent extracted or a blend of both) expressed juices, extracted exudates, gums and oleo resins, liquid extract where the solvent is evaporated to yield concentrated semi solid mass or dried mass. Extraction may be performed by means of appropriate technology such as infusion, maceration, soxhleting, boiling under ambient or higher pressure, with or without specified enzymes, with or without agitation and combination thereof. Drying of liquid extracts for removal of the solvent may be done by using various appropriate technologies like air drying, sun drying, drying under vacuum or with forced air circulation, drying at low temperature with air circulation, by way of lyophilization or freeze drying.

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Extracts of herbs may also be prepared by using carbon dioxide as a solvent-super critical fluid extraction.

Usage of distillates of herbs especially adopting steam distillation is increasing – for use to provide health benefits/ pharmacological actions when used as products for external use as well as in some cases internally used, and also for flavour and fragrance purposes. Such distillates may be referred as distilled oils. Distilled oils may be used with or without partial fractionation, purification, selective removal of identified components (De-mentholized Mentha Oil etc.) and blending of natural distilled oil with fractionated oils. Distilled oils are normally packed in tightly closable glass containers or glass-lined aluminium container or aluminium containers and are to be stored and transported at temperature below 25°. Exposure to heat and light are to be prevented recognizing their high flammability, and potential oxidation.

Extracts may be liquid extracts and tinctures, semi solid (soft extracts) or solid dry extracts of known consistency obtained from herbs. Standardized extract, a term commonly employed, would for pharmacopoeial purposes, mean an extract adjusted with in an acceptable tolerance to a given content of bio-marker or chemical/ analytical marker. Standardization may be achieved by adjusting the extracts with approved inert material or by blending one or more batches of extracts. Wherever possible, extracts shall specify the defined range of the constituents (bio-marker or chemical/ analytical marker). Extracts not covered in the above description would be defined by the process of production of the herb to the extract, solvent used and technology applied. The difference between extracts and tinctures would be, in the type of solvent used for extracting a herb, and tincture would normally mean an extract where aqueous-ethanol is used as a solvent for extraction. Dry extracts usually have a loss on drying or water content not greater than 5 per cent w/w, unless specified otherwise in any monograph. It is normal to extrapolate safety aspects and history of use information for extracts as long as the process, solvents, extraction ratios are comparable to the processes used in documented traditional knowledge. Additionally in cases of standardized extracts the inert excipients(s), if any used for standardization or adjustment of the content of constituents should also be declared on the label of such extracts. Extracts shall be free from solvent used for extraction and shall comply with the respective limits as given in Appendix 5.4. Harmful and carcinogenic solvents shall not be used for extraction purposes. Solvents and solvent systems may include use of propylene glycol, glycerin, sorbitol and such other polyhydroxy alcohols, as long as the content of such polyhydroxy alcohol are within safe limits in the final product.

In cases where extraction and fractionation process leads to preparation of an extract, which consists of a single chemical compound of more than at least 70 per cent purity, such extracts shall be treated as an active pharmaceutical ingredient or a food additive or a cosmetic ingredient and would be required to meet relevant laws.

Extracts may also be offered as purified or enriched extracts. Such extract of a herb is processed in such a way to provide higher than normal proportion of the active constituent (s) of the herb as long as the active constituent (s) is/are known. Such purified or enriched extracts may contain additional valuable components which may provide specific properties like enhanced efficacy or stability or solubility and availability of the active constituent (s). Purified and enriched extracts may also be prepared to reduce or remove other specific compound or group of compounds that is scientifically considered undesirable in the herb extracts. Pharmacological, toxicological, pharmaceutical considerations need to be applied while preparing such purified or enriched extracts. Mixed extracts may also be offered which would cover combination of more than one herb extract for purposes of providing simplification or economical way to manufacture herbal formulations.

Herbs may also be extracted using vegetable oils (approved by Food Law) for extraction purposes and such extracts shall specify the oil used for processing.

Approved preservatives or preservatives system may be used during preparation of extracts. The names of such preservatives used which would remain in the final extract shall be listed on the label of such extract, and the proportion of preservatives used shall not exceed normally accepted safe limits of their usage as per relevant laws or pharmacopoeial standards. No artificial colours may be used in extracts of herbs unless and otherwise specified in the specific monograph.Only approved colours shall be used.

Extracts may be exposed to ethylene oxide fumigation or low dose gamma radiation for purposes of reducing their microbial contamination. In cases where they are fumigated, the final extracts exposed shall meet residual levels of ethylene oxide limits as applicable. Herbs treated with low dose gamma radiation shall meet national laws related to such treatment and shall be labelled as per law.

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Herbal Formulations

Herbal formulation shall mean a dosage form consisting of one or more herbs or processed herb(s) in specified quantities to provide specific nutritional, cosmetic benefits, and/or other benefits meant for use to diagnose treat, mitigate diseases of human beings or animals and/or to alter the structure or physiology of human beings or animals. Dosage forms commonly employed for food or cosmetic or pharmaceuticals may be employed to formulate one or more herb or processed herbs. Dosage forms known in traditional medicines may also be adopted for preparing herbal formulations, either for external use or for internal administration. Adequate consideration for uniform distribution of herb or processed herbs as well as stability of the same in the dosage form shall be provided during formulation development.

Herbal formulation shall be labelled to comply with relevant labelling requirements under food or drug or cosmetics laws as applicable. Additionally, adequate information shall be provided on label of such formulations to include the name of the herb, parts used, nature and type of extract or processed herb used, extraction ratios, quantity per unit dose or per serving, name (s) of inert excipients used and any preservatives added shall be provided on the label.

Appearance of a monograph of a herbal formulation in the pharmacopoeia does not mean its approval as a drug under the law. Monograph of a herbal formulation in the pharmacopoeia is to provide qualitative and quantitative standards of quality for the formulation for its use either as a food item or food ingredient or food supplement/ nutraceuticals, as a drug and / or as a cosmetic. Each such use would need to comply with applicable regulations.

Botanical Extracts

In the extraction practice for articles of botanical origin, the constituents of interest are completely or partially separated from other components with the aid of *water, alcohol, alcohol-water* mixtures, or other suitable solvents. This extraction process involves the removal of the desired constituents from the plant matter with suitable solvent, the evaporation of all or nearly all of the solvent, and the adjustment of the residual fluids, masses, or powders to the prescribed standards. Suitable inert substances may be added as carriers or diluents to improve physical characteristics. Suitable antimicrobials and other preservatives may be added to preserve the integrity. Extracts may be subjected to processes that increase the content of characterized constituents, decrease the content of unwanted constituents, or both. Extracts with no added inert substances and no processing beyond the extraction are called native extracts. In some preparations, the plant matter may be pre-treated by inactivation of enzymes and microbial contaminants, grinding, defatting, or a similar procedure. Extracts may be defined as preparations with liquid, solid, or semisolid consistency. The products obtained by extraction are fluid extracts, powdered extracts, semisolid extracts, and tinctures.

Methods of Extraction

Percolation

In the manufacture of extracts, percolation is a commonly used method. The crude material being extracted is reduced to pieces of suitable size, if necessary, then mixed thoroughly with a portion of the specified solvent, and allowed to stand for about 15 minutes. The mixture is transferred to a percolator, sufficient amount of the specified solvent is added to cover the entire solid mass, and the mixture is allowed to percolate slowly (at a rate of not more than 1 ml per minute for 1000 g of material), the matter to be extracted being always covered with a layer of solvent. The residue may be pressed, and the obtained fluid is combined with the percolate. The entire percolates are concentrated, generally by distillation under reduced pressure, so as to subject the constituents of interest in the article under extraction to as little heat as possible.

Maceration

Unless otherwise specified, the crude material being extracted is reduced to pieces of suitable size, mixed thoroughly with the specified extracting solvent, and allowed to stand at room temperature in a closed container for an appropriate time, with frequent agitation until soluble matter is dissolved. The mixture is filtered, the insoluble material is washed with the same solvent used for maceration, and the filtrates are combined and concentrated, usually under reduced pressure, to the desired consistency.

Preparations

Fluid Extracts

Fluid extracts, also known as liquid extracts, are preparations of plant matter, containing alcohol as a solvent or as a preservative, or both, and are so made that each ml contains the extracted constituents of 1 g of the crude material that it represents, unless otherwise specified in the individual monograph. They may be prepared from suitable extracts and may contain suitable antimicrobial or other preservatives.

Pharmacopoeial fluid extracts are made by percolation, often following a period of maceration. The required solvent is specified in the individual monograph. The common manufacturing procedure includes concentration of the more diluted portion of percolate by evaporation or distillation under vacuum at temperatures below 60°. The time of maceration and the rate of flow during percolation may be varied to adjust

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for the quantity and nature of the crude material under extraction, provided that the composition of the extracted constituents of interest is not adversely affected. The rate of flow of the percolate can be slow, moderate, or rapid. With reference to the extraction of 1000 g of the starting material, at a slow rate, not more than 1 ml of percolate is produced per minute; at a moderate rate, between 1 and 3 ml per minute is produced; and at a rapid rate, between 3 and 5 ml per minute is produced. A fluid extract that tends to deposit sediment may be aged and filtered, or the clear portion may be decanted, provided that the resulting clarified liquid conforms to the Pharmacopoeial standards.

Powdered Extracts

Powdered extracts are solid preparations having a powdery consistency obtained by evaporation of the solvent used for extraction. They may contain suitable added substances such as excipients, stabilizers, and preservatives. Standardized powdered extracts are adjusted to the defined content of constituents, using suitable inert materials or a powdered extract of the plant matter used for preparation. Where applicable, a limit for the solvent used for extraction is specified in the individual monograph.

Semisolid Extracts

Semisolid extracts, also known as soft extracts or pillular extracts, are preparations having consistencies between those of fluid extracts and those of powdered extracts, and are obtained by partial evaporation of the solvent, water, alcohol, or hydroalcoholic mixtures being used as extracting solvents. They may contain suitable antimicrobial or other preservatives. A semisolid extract and a powdered extract obtained from the same material are interchangeable as drugs or as supplements, but each has its own advantages.

General Pharmacopoeial Requirements

Unless otherwise specified in the individual monographs, Pharmacopoeial requirements for the fluid extracts, powdered extracts, and semisolid extracts are as follows.

Packaging and Storage. Store in tight, light-resistant containers.

Labelling. Label it to indicate the name of the plant part used; the names of solvents, other than the hydroalcoholic solvents, used in preparation; the content, in percentage, of active principles or marker compounds identified in the individual monograph; and the name and concentration of any added antimicrobial or other preservative. Where active principles are unknown, the ratio of starting material to final product is stated. For semisolid extracts and powdered extracts, the identity and quantity of any added excipient is also indicated. In such cases the percentage of native extract may also be stated.

Residue on Evaporation. Transfer promptly about 2 ml, accurately measured, of fluid extract, about 0.5 g of powdered extract, or about 2 g of semisolid extract to a suitable tared, round-bottom flask. Evaporate to dryness on a water bath, and dry the residue at 100° to 105° for 3 hours. Allow to cool in a desiccator over *phosphorus pentaoxide*, and determine the weight of the residue obtained is not less than 95 per cent of powdered extract specimen remains as residue; or not less than 70 per cent of semisolid extract specimen remains as residue.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm)

Alcohol Content Method (2.6.6). Between 90 per cent and 110 per cent of the labelled amount of *ethanol* is found in fluid extract and semisolid extract.

Tinctures

Tinctures are liquid preparations usually prepared by extracting plant materials with alcohol or hydroalcoholic mixtures. Traditionally, tinctures of potent articles of botanical origin represent the activity of 10 g of the drug in each 100 ml of tincture, the strength being adjusted following the test for content of active principles or marker compounds. Most other plant tinctures represent 20 g of the respective plant material in each 100 ml of tincture. Different tinctures are not always diluted to obtain the same ratio of starting plant material to final tincture. This ratio will depend on the requirements prescribed in the specific tests for content of active principles or marker compounds included in the individual monographs. As tinctures are being prepared, they are assayed in accordance with these content tests. Using the values obtained from such assays, the final concentration of a tincture is adjusted by adding more solvent or by evaporating part of the solvent.

Unless otherwise specified, tinctures are usually prepared from coarse powder or fine cuttings of plant materials either by a percolation process or a maceration process.

Percolation process

Carefully mix the ground mixture of ingredients with a sufficient quantity of the prescribed extracting solvent to render it evenly and distinctly damp, allow it to stand for 15 minutes, transfer it to a suitable percolator, and pack the mass firmly. Pour on enough of the specified extracting solvent to saturate the drug, and cover the top of the percolator. When the liquid is about to drip from the percolator, close the lower orifice, and allow the drug to macerate for 24 hours or for the time specified in the monograph. If the test for content of active principles or marker compounds is not required in the individual monograph, allow the percolation to proceed slowly or at the

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specified rate (for definitions of flow rates, see under Fluid extracts), gradually adding sufficient quantity of extracting solvent to produce 1000 ml of tincture, and mix. If a test for content of active principles or marker compounds is required, collect only 950 ml of percolate, mix, and test a portion of it as directed in the individual monograph. Dilute the remainder of the percolate with as much of the prescribed extracting solvent as calculation from the content test indicates is necessary to produce a tincture that conforms to the requirements, and mix.

Maceration process

Macerate the drug with 750 ml of the prescribed extracting solvent in a closed container, and put in a warm place. Agitate it frequently for 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter. When most of the liquid has drained, wash the residue on the filter with a sufficient quantity of the prescribed extracting solvent, combining the filtrates, to produce 1000 ml of tincture, and mix.

Packaging and Storage. Store in tight, light-resistant containers, and avoid exposure to direct sunlight and excessive heat.

Labelling. Label it to indicate the name of the plant part used for preparation; the name of the solvent or solvent mixture used for extraction; and the content of the constituents of interest and the ratio of starting material to final product.

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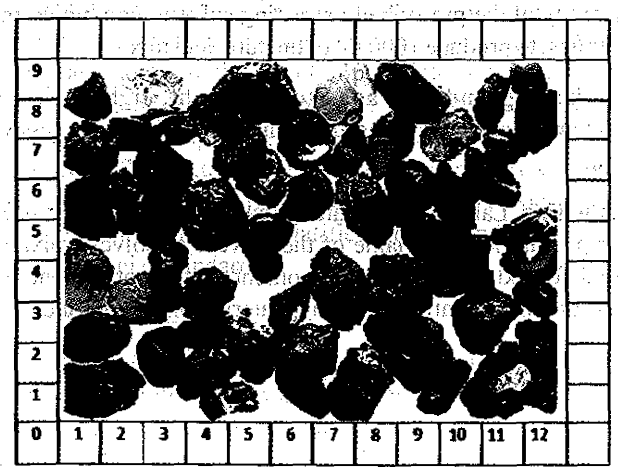
Acacia

Gum Acacia; Indian Gum



Acacia is the air-hardened, gummy exudation from the stem and branches of *Vachellia nilotica* (Linn.) P.J.H. Hurter and Mabb. (Fam. Leguminosae), or other species of *Acacia*. It is available as pieces (tears) or in the form of a powder.

Category. Pharmaceutical aid, antidiabetic.

Description

Tears — Irregular and broken pieces of varying size, yellowish-white, yellow or amber in colour, with numerous minute fissures; brittle fractured surface, glassy and occasionally iridescent; odourless.

Powder — A white or yellowish-white powder; odourless; on treatment with *water* it dissolves to give a mucilaginous liquid which is colourless or yellowish, dense, viscous, adhesive and translucent.

Identification

A. An aqueous solution is gelatinised by the addition of *lead subacetate solution*.

B. To 5 ml of a 10 per cent w/v solution add gradually, while shaking, 10 ml of *ethanol (95 per cent)*. The cloudy liquid, on addition of 0.5 ml of *acetic acid*, gives a white precipitate. Filter and add to the clear filtrate 50 ml of *ammonium oxalate solution*; the filtrate becomes cloudy.

C. A 10 per cent w/v solution is either dextro-rotatory or slightly laevo-rotatory.

Tests

Sterculia gum and agar. To 50 mg of the powdered substance under examination add 0.2 ml of freshly prepared *ruthenium red solution* and examine microscopically; the particles do not acquire a red colour after irrigation with *water*.

Agar and tragacanth. To 10 ml of a 10 per cent w/v solution add 0.2 ml of *lead acetate solution*; no precipitate is produced.

Starch and dextrin. Boil 10 ml of a 10 per cent w/v solution and cool, add 0.1 ml of *0.05 M iodine*; no blue or brown colour is produced.

Tannins. To 10 ml of a 10 per cent w/v solution add 0.1 ml of *ferric chloride test solution*; a gelatinous precipitate is formed, but neither the precipitate nor the liquid shows a dark blue colour.

Sucrose and fructose. To 1 ml of a 10 per cent w/v solution add 4 ml of *water*, 0.1 g of *resorcinol* and 2 ml of *hydrochloric acid* and heat on a water-bath; no yellow or pink colour develops.

Water-insoluble matter. Dissolve 5 g of fine powder in 100 ml of *water* in a 250-ml flask, add 10 ml of *dilute hydrochloric acid* and boil gently for 15 minutes. Filter by suction while hot through a sintered-glass crucible, previously tared, wash thoroughly with hot *water*, dry at 105° and weigh; the residue does not exceed 50 mg.

Microbial contamination (2.2.9). 1.0 g is free from *Escherichia coli*.

Sulphated ash (2.3.18). Not more than 5.0 per cent.

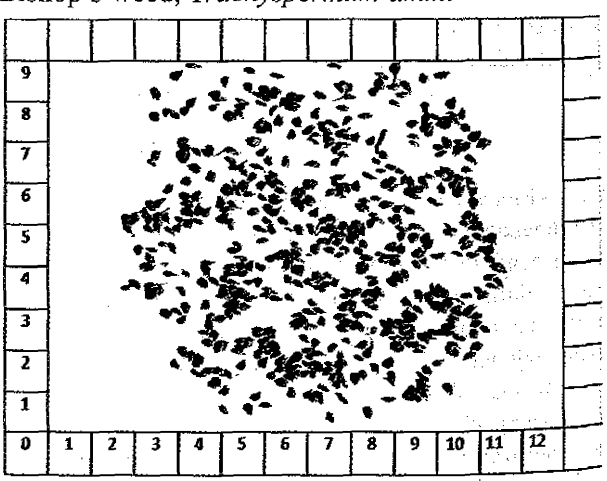
Acid-insoluble ash (2.3.19). Not more than 1.0 per cent, determined on 1.0 g by Method C.

Loss on drying (2.4.19). Not more than 15.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Ajwain

Bishop's weed; *Trachyspermum ammi*



Ajwain consists of the dried fruits of *Trachyspermum ammi* (Linn.) Sprague (Fam. Apiaceae).

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Ajwain contains not less than 1.0 per cent w/w of thymol, calculated on the dried basis.

Category. Antiasthmatic, carminative, germicidal, antioxidant, dipana.

Description. The fruits are oval-oblong, greenish brown to yellowish brown in colour. They have an aromatic characteristic odour and the taste is sweet aromatic.

Identification

A. *Macroscopic* — The fruit consists of two portions each called mericarp and connected by central stalk (carpophore). A single seed is seen in each mericarp. Fruit surface is glabrous and the five primary ridges of each mericarp are prominent, straight and pale straw coloured.

B. *Microscopic* — Epicarp is composed of polygonal cells. In the mesocarpic region, reticulate and lignified parenchymas are seen at vascular strands. Tracheids show helical thickening. Endocarp consists of narrow elongated cells having a parquetry arrangement. Polyhedral, thick walled endosperm contains aleurone grains and oil globules. Vittae are six in number, four on the dorsal surface at the mesocarpic region below the secondary ridges and two on the commissural surface of the mericarp. Vittae long, slender composed of thin walled polygonal cells and is lined by an epithelium of small polygonal tubular cells; 10-15 separate, septum transverse or curved.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 93 volumes of *toluene* and 7 volumes of *ethyl acetate*.

Test solution. To 1 g of the coarsely powdered substance under examination, add 25 ml of *dichloromethane,* reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 25 ml of *dichloromethane*, cool and filter. Combine all the filtrates and concentrate under vacuum to 25 ml.

Reference solution. To 1 g of the *ajwain IPBRS*, add 25 ml of *dichloromethane,* reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 25 ml of *dichloromethane*, cool and filter. Combine all the filtrates and concentrate under vacuum to 25 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent.* Heat at 110° for 10 minutes and examine the plate in daylight. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 2.0 per cent.

Water-soluble extractive (2.6.3). Not less than 15.0 per cent by Method I.

Total Ash (2.3.19). Not more than 15.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 7.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 10 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by gas chromatography (2.4.13).

Test solution. Weigh 2.0 g of the coarsely powdered substance under examination, add 50 ml of *methanol,* and reflux on a water-bath for 15 minutes, cool and filter. Reflux the residue further with *methanol* till the extract turns colourless, cool and filter. Combine all the filtrates and concentrate to a volume slightly less than 100 ml. Dilute with *methanol* to 100.0 ml.

Reference solution. A 0.01 per cent w/v solution of *thymol IPRS* in *methanol*.

Chromatographic system

- a capillary column 30 m x 0.25 mm x 0.25 µm, packed with methyl polysiloxane,
- temperature:
  oven 90° to 260° at a rate of 10° per minute and maintain at this temperature for 5 minutes,
  injector 240° and detector 280°,
- flow rate: 0.8 ml per minute, using nitrogen as the carrier gas,
- split flow: 20 ml per minute.

Inject the reference solution and the test solution.

Calculate the content of thymol.

Storage. Store protected from light in well-filled containers, at a temperature not exceeding 30°.

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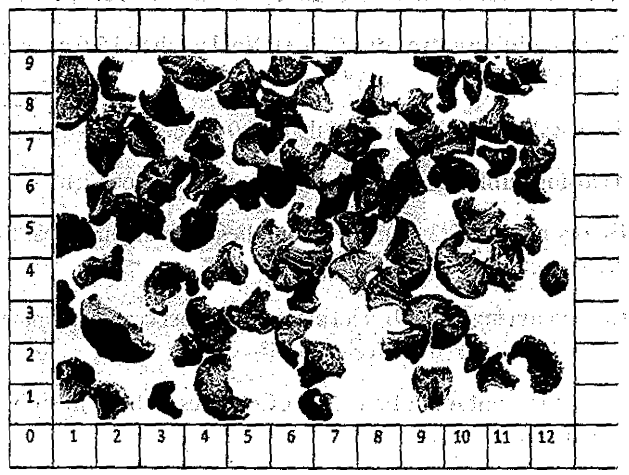
Amalaki

Emblic Myrobalan; Indian Gooseberry; *Phyllanthus emblica*



Amalaki consists of the dried fruit pericarp of *Phyllanthus emblica* Linn. (Fam. Euphorbiaceae).

Amalaki contains not less than 1.0 per cent w/w gallic acid calculated on the dried basis.

Category. Antiscorbutic, antacid, carminative, hepato-protective, raktapitta

Description. The dried fruit has a highly shriveled and wrinkled external surface. The taste is sour and astringent followed by delicately sweet tinge.

Identification

A. *Macroscopic* — The dried fruit shows a broad, highly shriveled and wrinkled external convex surface, lateral surface transversely wrinkled, external surface exhibits few whitish specks, occasionally some pieces show a portion of stony testa.

B. *Microscopic* — The epicarpic cells are rectangular in shape and their walls are highly cuticularized. Anomocytic type of stomata is found rarely. Collateral fibrovascular bundles are scattered throughout the inner mesocarp. Pitted and helical tracheids with tapering ends are seen. At places in the phloem, large cavities filled with crystal mass are present.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 20 volumes of *toluene*, 45 volumes of *ethyl acetate*, 20 volumes of *glacial acetic acid* and 5 volumes of *formic acid*.

Test solution. Reflux 2 g of the coarsely powdered substance under examination with 50-75 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further for two times with 75 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 50 ml.

Reference solution. Reflux 0.4 g of the coarsely powdered *amalaki IPBRS* with 50-75 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further for two times with 75 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 100° for 5-10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 3 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 30 per cent.

Water-soluble extractive (2.6.3). Not less than 40 per cent by Method 1.

Total ash (2.3.19). Not more than 5.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh about 0.5 g of coarsely powdered substance under examination, add 50 ml of *water*, sonicate for 3 minutes and heat on a boiling water-bath for 15 minutes, cool and dilute to 100.0 ml with *water* and filter.

Reference solution. A 0.01 per cent w/v solution of *gallic acid IPRS* in *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a solution prepared by dissolving 0.136 g of *potassium di-hydrogen orthophosphate* in 500 ml of *water*, add 0.5 ml of *orthophosphoric acid* and dilute to 1000 ml with *water*,
  B. *acetonitrile*
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

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Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
18	55	45
25	20	80
30	100	0

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

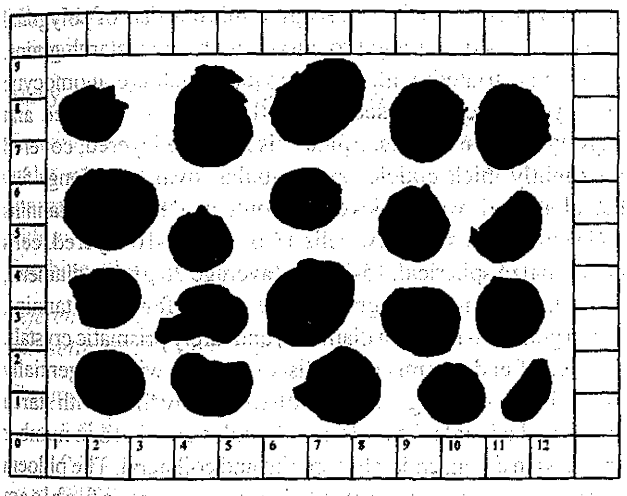
Inject the reference solution and the test solution.

Calculate the content of gallic acid.

Storage. Store protected from light, heat, moisture and against attack by insects and rodents.

Amaltas

Sonhali; *Cassia fistula*



Amaltas consists of dried pulp of fruits of *Cassia fistula* Linn. (Fam. Fabaceae).

Amaltas contains not less than 0.07 per cent w/w of rhein, calculated on the dried basis.

Category. Purgative, diuretic, antipruritic, febrifuge, vibandha.

Description. Pulp is dark brown, sticky, sweet and mucilaginous; odour characteristic, somewhat disagreeable.

Identification

A. *Macroscopic* — The fruits are 40-70 cm long and 20-27 mm in diameter, straight or slightly curved, smooth but finely striated transversely, the striations appearing as fine fissures. The seed contains a whitish endosperm in which the yellowish embryo is embedded. The fresh fruit is filled with black-brown pulp which contracts on drying.

B. Microscopic —The pericarp about 1.5 mm thick, shows the presence of epicarp, mesocarp and endocarp. Epicarp is made up of single layer of brownish coloured rectangular cells. Mesocarp is composed of polygonal thin walled parenchymatous cells and endocarp consists of many layers of stone cells of varying shapes and sizes. Vascular elements present in the mesocarp are composed of small tracheids and long tracheids. The characteristically smelling sweetish pulp is formed of parenchymatous cells which are thin walled, filled with a brownish black content.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 3 volumes of *toluene*, 3 volumes of *ethyl acetate*, 0.8 volumes of *formic acid* and 0.2 volume of *methanol*.

Test solution. Reflux 1 g of coarsely powdered substance under examination with 25 ml of *0.01 M methanolic potassium hydroxide* for 1 hour on water-bath. Cool and filter. Extract three times with 25 ml of *diethyl ether*. Combine all the extracts and concentrate under vacuum to dryness. Dissolve the residue in 25 ml of *methanol*.

Reference solution. Reflux 1 g of *amaltas IPBRS* with 25 ml of *0.01M methanolic potassium hydroxide* for 1 hour on water-bath. Cool and filter. Extract three times with 25 ml of *diethyl ether*. Combine all the extracts and concentrate under vacuum to dryness. Dissolve the residue in 25 ml of *methanol*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air, examine in the ultraviolet light at 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 8.0 per cent.

Water-soluble extractive (2.6.3). Not less than 41.0 per cent by Method I.

Total ash (2.3.19). Not more than 18.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.3 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 6.2 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 1 g of coarsely powdered substance under examination three times with 50 ml of *methanol* for 3 hours,

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cool and filter. Combine the filtrates and evaporate to dryness. Dissolve the residue in *methanol* and dilute to 15 ml with *methanol*.

Reference solution. A 0.01 per cent w/v solution of *rhein IPRS* in *methanol*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with phenyl (5 µm),
- mobile phase: A. a mixture of 90 volumes of *methanol* and 10 volumes *acetonitrile*,
  B. 0.1 per cent of *orthophosphoric acid*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0 - 3	57	43
3 - 15	90	10

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

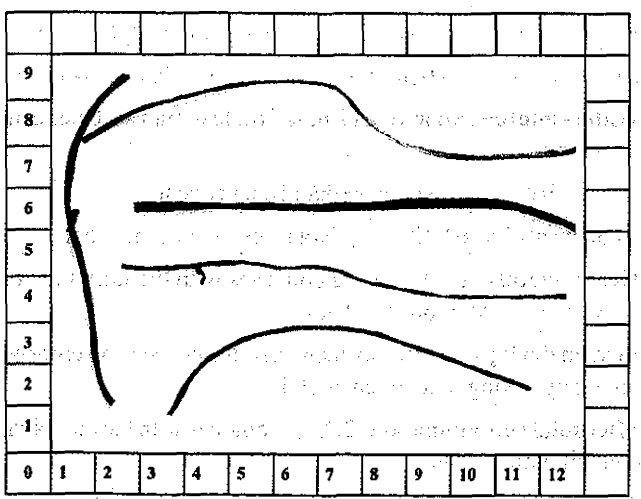
Inject the reference solution and the test solution.

Calculate the content of rhein.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Amarbel

Akashbel; Nirmali; *Cuscuta reflexa*



Amarbel consists of dried whole plant of *Cuscuta reflexa* Decne. (Fam. Convolvulaceae).

Amarbel contains not less than 0.1 per cent w/w of quercetin calculated on the dried basis.

Category. Antidiabetic, aphrodisiac, antiaging, hepatoprotective, purgative.

Description. The dried whole plant is yellowish brown to dark brown in colour and 5-15 cm in length and 1-2 mm in thickness and has characteristic odour.

Identification

A. *Macroscopic* — Amarbel is an unusual parasitic vine related to the morning glory family. It grows in a prolific manner over host plants (or other support) with inter-twined stems, The plant is leafless and rootless. Initially the starter plant would have had some roots. Within a few days of germination, the plant, which is touch sensitive, finds a host or dies. After establishing itself on a host body, it draws nutrition from the host as a stem parasite and the roots wither away. The twining stem develops haustoria, which are root like and penetrate the host stem to draw water and nourishment.

B. *Microscopic* — In surface, epidermis consists of polygonal linear cells; sides straight to curved with a few starch grains. Surface is striated; with few stomata, which are anomocytic and cyclocytic. (Transection of the stem is rounded and slightly wavy in outline. Epidermis is single layered, covered by slightly thick cuticle; cells tubular, oval to oblong, few barrel shaped; walls thickened; contents dense with tannins and starch grains in a few cells.) Cortex is 7-10 layered, cells polygonal to spherical, 15-38 µm (average 28 µm) in diameter, walls thin, contents slightly dense and a few with tannins, starch grains (8-15 µm in diameter) and rarely prismatic crystals. A layer of endodermis often discontinuous with tangentially long, oblong to polygonal cells, often densely filled with starch grains and crystals. The vascular bundles are 12-16 in number arranged in a ring, endarch, conjoint and collateral. The phloem is present towards outside and consists of phloem parenchyma, sieve cells and companion cells. Internal phloem is present in patches.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 7.5 volumes of *formic acid*, 7.5 volumes of *glacial acetic acid* , 17.5 volumes of *water* and 67.5 volumes of *ethyl acetate*.

Test solution. Reflux 2 g of coarsely powdered substance under examination with 25 ml of *methanol* on a water bath. Cool and filter and further reflux the residue with 3 x 25 ml *methanol*. Combine the extracts and concentrate under vacuum to 25 ml with *methanol*.

Reference solution. Reflux 2 g of *amarbel IPBRS* with 25 ml of *methanol* on a water bath. Cool and filter. Reflux the residue

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with 3 x 25 ml *methanol*. Combine the extracts and concentrate under vacuum to 25 ml with *methanol*.

Apply to the plate 10 µl of each solution as band length 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in ultraviolet light at 254 nm and 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 15.0 per cent.

Water-soluble extractive (2.6.3). Not less than 12.0 per cent.

Total ash (2.3.19). Not more than 6.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.2 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 17.0 per cent.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 2 g coarsely powdered substance under examination with 100 ml of *methanol* by soxhlet apparatus for 3 hours, cool and filter. Reflux the residue further with *methanol* till last extract turn colourless, cool & filter. Combine all the filtrates and concentrates to 10 ml.

Reference solution. A solution containing 0.04 per cent w/v *quercetin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. *water* and adjusted to pH 2.0 with *orthophosphoric acid*,
  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 370 nm,
- injection volume: 10 µl.

Time (in min.)	mobile phase A (per cent v/v)	mobile phase B (per cent v/v)
0	60	40
1	60	40
20	45	55
21	0	100
25	60	40
27	60	40

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of quercetin.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Amla Juice Powder

Amla Juice Powder is obtained by spray drying cold pressed juice of fresh fruits of *Phyllanthus emblica* Linn. (Fam. Euphorbiaceae)

Amla Juice Powder contains not less than 90.0 per cent w/w and not more than 110 per cent w/w of the stated amount of polyphenols, calculated on the anhydrous basis. It may contain suitable added substances.

Category. Antiscorbutic, antacid, carminative, hepato-protective, raktapitta.

Usual strength. 40 per cent w/w.

Description. A beige to off-white powder.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 15 volumes of *ethyl acetate*, 1.5 volumes of *methanol* and 1 volume of *water*.

Test solution. Dissolve 0.5 g of the extract under examination with 10 ml of *water* and filter.

Reference solution. A 0.01 per cent w/v solution of *gallic acid IPRS* in *water*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. Spray the plate with *anisaldehyde-sulphuric acid reagent*. Heat the plate at 110° for 10 minutes and examine the plate under 366 nm and in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Water-soluble extractive (2.6.3). Not less than 85.0 per cent by Method I.

Total ash (2.3.19). Not more than 15.0 per cent.

Water (2.3.43). Not more than 5.0 per cent.

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Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Gallic acid. Not less than 0.1 per cent and not more than 2.0 per cent.

Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 50 mg of the extract under examination or quantity equivalent to 10 mg of *gallic acid* in *water*, by frequent shaking and dilute to 50.0 ml with *water* and filter.

Reference solution. A 0.01 per cent w/v solution of *gallic acid IPRS* in *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded porous silica (5 µm),
- mobile phase: A. 0.1 per cent *formic acid* in *water*,
  B. *methanol*,
- a gradient programme using the conditions given below,
- flow rate: 0.7 ml per minute,
- spectrophotometer set at 272 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
25	95	5
30	0	100
40	0	100
42	95	5

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of the gallic acid in extract.

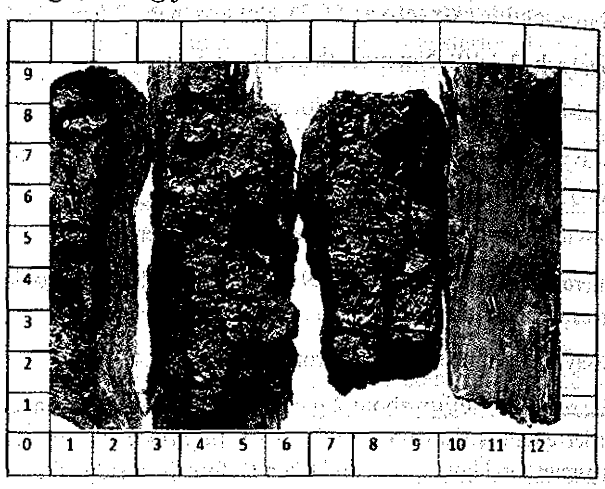
Assay. Weigh about 2 g of the extract, add 50 ml boiling *water* and heat it on a water-bath for 30 minutes with frequent stirring. Allow the solution to settle and carefully decant it through a piece of cotton wool to a 500 ml volumetric flask. Repeat the extraction for 5 times with 50 ml of boiling *water*. To confirm that all tannins have been extracted, add 3-4 drops of *ferric ammonium sulphate solution* to 5 ml of the extract. Blue colour will not be produced, if all tannins have been extracted. If blue colour develops extract again 2 times with 50 ml of boiling *water* and check with *ferric ammonium sulphate solution*. Cool the extracts and make up to the mark with *water*. Take 50 ml of this into a 250.0 ml conical flask and add 50 ml of *indigo sulphonic acid solution*, prepared by dissolving 1 g of *indigo carmin* in 50 ml of *sulphuric acid*, add 500 ml of *water* and dilute this solution to 1000 ml. Titrate, with *0.1 M potassium permanganate* using *indigo sulphonic acid* as indicator until a golden yellow colour is produced. Carry out a blank titration.

1 ml of *0.1 M potassium permanganate* is equivalent to 0.004157 g of polyphenols calculated as tannic acid.

Storage. Store protected from heat and moisture.

Amra

Mango; *Mangifera indica*



Amra consists of dried stem bark of *Mangifera indica* Linn. (Fam. Anacardiaceae).

Amra contains not less than 1.5 per cent of mangiferin calculated on the dried basis.

Category. Cardiotonic, astringent, nourishing tonic in haemoptysis, atisara.

Description. The dried stem bark occurs in pieces of variable size and thickness, surface rough. Odour pleasant and taste astringent.

Identification

A. *Macroscopic* — The surface is rough due to longitudinal cracks, fissures and scattered, raised lenticels, greyish to dark brown externally and yellowish-white to reddish internally.

B. *Microscopic* — The mature bark shows a wide cork which has tangentially elongated cells a few outer layers are brown and inner lighter in colour, resin canals and yellow coloured tannin sacs are found in the phloem region, stone cells are thick walled and lignified, prismatic calcium oxalate crystals are present.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

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Mobile phase. A mixture of 100 volumes of *ethyl acetate*, 11 volumes of *formic acid*, 11 volumes of *acetic acid* and 25 volumes of *water*.

Test solution. To 5 g of the coarsely powdered substance under examination, add 50 ml of *methanol* and reflux for 15 minutes, cool and filter. Reflux the residue further for three times with 50 ml of *methanol*, cool and filter. Combine all the filtrates and evaporate under vacuum to 10 ml.

Reference solution. Weigh about 2 g of *amra IPBRS*, add 50 ml of *methanol* and reflux for 15 minutes, cool and filter. Reflux the residue further three times with 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 4 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *vanillin glacial acetic acid reagent*. Heat the plate at 100° for 5-10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 10.0 per cent.

Water-soluble extractive (2.6.3). Not less than 10.0 per cent by Method I.

Total ash (2.3.19). Not more than 16.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 5.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh 2 g of coarsely powdered substance under examination, add 10 ml of *dimethylformamide*, sonicate for 5 minutes and add 50 ml of *methanol* and boil on a water-bath for 10 minutes, cool and dilute to 100.0 ml with *methanol* and filter. Dilute 5.0 ml of this solution to 50.0 ml with *methanol*.

Reference solution. Dissolve 10 mg of *mangiferin IPRS* in 10 ml of *dimethylformamide* and dilute to 100.0 ml with *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: filtered and degassed mixture of 15 volumes of *acetonitrile* and 85 volumes of buffer pH 2.8 prepared by dissolving 1.36 g of *potassium dihydrogen orthophosphate* in 950 ml of *water*, adjusted to pH 2.8 with *orthophosphoric acid* and make upto 1000 ml,
- flow rate: 1 ml per minute,
- spectrophotometer set at 365 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

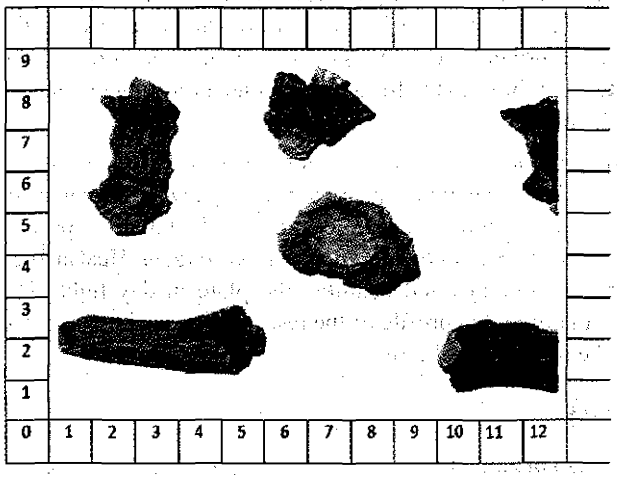
Inject the reference solution and the test solution.

Calculate the content of mangiferin.

Storage. Store protected from moisture and against attack by insects and rodents.

Anantmula

Sariva; Indian Sarsaparilla; *Hemidesmus indicus*



Anantmula consists of the dried roots of *Hemidesmus indicus* (Linn) R.Br. (Fam. Apocynaceae).

Anantmula contains not less than 0.02 per cent of iso-vanillin, calculated on the dried basis.

Category. Antioxidant, antileprotic, in skin diseases, arnci.

Description. The roots are cylindrical, yellowish brown in colour. They have vanillin like odour and the taste is acrid.

Identification

A. *Macroscopic* — Root is 30 cm or more long and from 3 to 6 mm thick, rigid, tortuous, cylindrical, and little branched, consisting of a ligneous center, and a brownish, corky bark, marked with longitudinal furrows and transverse fissures. The odour is aromatic, recalling that of tonqua bean, the taste is aromatic and sweetish. On one side of the root the cork is frequently separated from and raised above the cortex and is transversely fissured.

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B. *Microscopic* —Transverse section of root shows 2-3 layered cork cambium having compressed cells filled with brown contents. Xylem traversed by narrow medullary rays. Pith absent and central region occupied by woody tissues. Vessel shows pitted thickening. The transverse section exhibits numerous laticiferous cells in the secondary cortex. Powder in chloral hydrate solution shows unicellular and branched latex ducts; lignified elements and vessels with pitted thickenings.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 75 volumes of *toluene,* 15 volumes of *ethyl acetate,* 5 volumes of *glacial acetic acid* and 5 volumes of *methanol.*

Test solution. To 2 g of the coarsely powdered substance under examination, add 40 ml of *methanol,* reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 25 ml of *methanol,* cool and filter. Combine all the filtrates and concentrate under vacuum to 25 ml.

Reference solution. To 2 g of the *anantmula IPBRS*, add 40 ml of *methanol,* reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 25 ml.

Apply to the plate 20 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat at 105° for 5-10 minutes and examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 8.0 per cent.

Water-soluble extractive (2.6.3). Not less than 12.0 per cent by Method I.

Total ash (2.3.19). Not more than 15.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh 2 g of the coarsely powdered substance under examination, add 30 ml of *water,* and reflux on a water-bath for 15 minutes, cool and filter. Reflux the residue further with *water* till the extract turns colourless, cool and filter. Combine all the filtrates and concentrate to a volume slightly less than 50 ml. Dilute to 50.0 ml with *methanol.*

Reference solution. A 0.002 per cent w/v solution of *iso-vanillin IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. 0.1 per cent w/v solution of *formic acid* in *water*

B. *acetonitrile*,

- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
10	70	30
35	10	90
36	90	10
40	90	10

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of isovanillin.

Storage. Store protected from heat, moisture and against attack of insects and rodents.

Anise Oil

Saunf ka tail, *Pimpinella anisum*

Anise oil is the essential oil obtained by steam distillation from the dry ripe fruits of *Pimpinella anisum* Linn. (Fam. Apiaceae).

Anise oil contains not more than 1.5 per cent of linalol, not more than 1.2 per cent of α-terpineol not less than 87.0 per cent and not more than 94.0 per cent of trans-anethole.

Category. Antispasmodic, expectorant, carminative, antibacterial, anticancer.

Description. A clear, colourless to pale yellow liquid.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

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Reference solution. A 2.0 per cent w/v solution of *anise oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described under Assay.

The chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 83.3° to 90.0°.

Tests

Relative density (2.4.29). 0.980 to 0.990.

Refractive index (2.4.27). 1.552 to 1.561.

Freezing point (2.4.11). 15° to 19°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *linalol IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *α-terpineol IPRS* in *ethanol (95 per cent)*.

Reference solution (c). A 2.0 per cent w/v solution of *trans-anethole IPRS* in *ethanol (95 per cent)*.

Chromatographic system
- a capillary column 30 m x 0.25 mm, coated with 5 per cent methyl phenylpolysiloxane,
- temperature:
  column. 60° for 1 minute, increase from 60° to 210° at a rate of 10° per minute and maintain at this temperature for 20 minutes,
- inlet port at 200° and detector at 220°,
- detection by flame ionization detector,
- flow rate: 1 ml per minute using helium as carrier gas,
- injection volume: 0.2 µl,
- split ratio: 1:100.

Inject the test solution and reference solution.

Inject reference solutions (a), (b), (c) and the test solution.

Calculate the content of linalol, α-terpineol and trans-anethole in the oil using area normalization procedure.

Storage. Store protected from light and moisture.

Arachis Oil

Moongphali ka tail; Groundnut Oil; Peanut Oil

Arachis Oil is the refined fixed oil obtained from the seed kernels of one or more of the cultivated varieties of *Arachis hypogaea* Linn. (Fam. Fabaceae) and may contain suitable antioxidants as stabilisers.

Category. Pharmaceutical aid.

Description. A clear, colourless or pale yellow oily liquid; odour, faint and nutlike.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *kieselguhr G*.

Mobile phase. Glacial acetic acid.

Test solution. Dissolve 20 mg (one drop) of the substance under examination in 4 ml of *chloroform*.

Reference solution (a). Dissolve 20 mg (one drop) of *arachis oil IPRS* in 4 ml of *chloroform*.

Reference solution (b). Dissolve 20 mg (one drop) of cottonseed oil in 4 ml of *chloroform*.

Reference solution (c). Dissolve 20 mg (one drop) of sesame oil in 4 ml of *chloroform*.

Impregnate the plate by placing it to a depth of about 5 minutes in a tank containing a shallow layer of a mixture of 95 volumes of *light petroleum (60° to 80°)* and 5 volumes of *liquid paraffin*, allowing the solvent to rise at least 14 cm, removing the plate and allowing it to dry in air for 5 minutes; use with the flow of the mobile phase in the direction in which impregnation was carried out.

Apply to the plate 2 µl of each solution. Allow the mobile phase to rise 12 cm. Dry the plate at 110° for 10 minutes, allow to cool and expose it to iodine vapour in a saturated chamber. Remove the plate and allow it to stand for a few minutes until the brown background colour has disappeared. Spray the plate with *starch solution*; blue spots appear which become brown on drying and become blue again on spraying with *water*. The spots in the chromatogram obtained with the test solution correspond to those in the chromatogram obtained with reference solution (a).

Tests

Weight per ml (2.4.29). 0.908 g to 0.920 g.

Refractive index (2.4.27). 1.467 to 1.470.

Alkaline impurities. Mix 10 ml of recently distilled *acetone* and 0.3 ml of *water* in a test-tube, add 0.05 ml of a 0.04 per cent w/v solution of *bromophenol blue in ethanol (95 per cent)* and neutralise the solution, if necessary, with *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide*. Add 10 ml of the substance under examination, shake and allow to stand. Not more than 0.1 ml of *0.01 M hydrochloric acid* is required to change the colour of the upper layer to yellow.

Semi-drying oils. Boil 1 g in a flask under a reflux condenser for 5 minutes with 5 ml of a mixture of 3 volumes of *2 M ethanolic potassium hydroxide* and 1 volume of *ethanol (95 per cent)*, add 1.5 ml of *6 M acetic acid* and 50 ml of *ethanol (70 per cent)*, warm until the solution is clear. Cool

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slowly with a thermometer in the liquid; the temperature at which the solution becomes turbid is not lower than 36°.

Peroxide value (2.3.35). Not more than 5.0.

Acid value (2.3.23). Not more than 0.5.

Iodine value (2.3.28). 85 to 105.

Saponification value (2.3.37). 185 to 196.

Rancidity. Shake 1 ml of 10 per cent v/v solution in *ether* with 1 ml of *hydrochloric acid*, add 1 ml of 0.1 per cent w/v solution of *phloroglucinol* in *ether*; no red or pink colour is produced.

Unsaponifiable matter (2.3.39). Not more than 1.0 per cent.

Cottonseed oil. In the Identification test, the chromatogram obtained with the test solution does not correspond to that obtained with reference solution (b)

Sesame oil. In the Identification test, the chromatogram obtained with the test solution does not correspond to that obtained with reference solution (c).

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (10 ppm).

Arachis Oil intended for use in the manufacture of parenteral preparations complies with the following additional requirement.

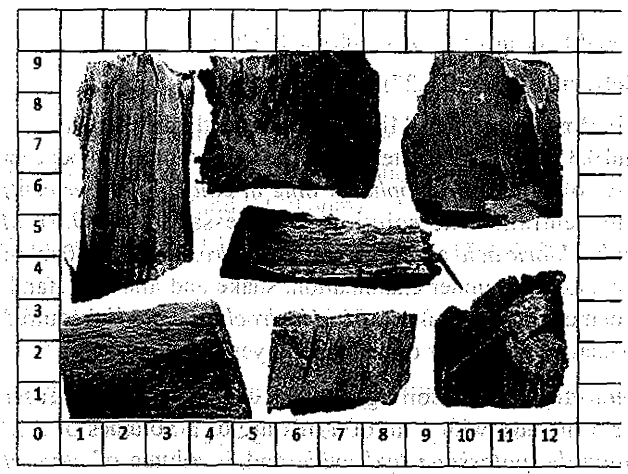
Water (2.3.43). Not more than 0.3 per cent, determined on 3.0 g.

Storage. Store protected from light in a well-filled container.

Labelling. The label states name and quantity of the added antioxidants.

Arjuna

Terminalia arjuna



Arjuna consists of dried stem bark of *Terminalia arjuna* (Roxb ex DC.) Wight and Arn. (Fam. Combretaceae)

Arjuna contains not less than 0.02 per cent of arjungenin calculated on the dried basis.

Category. Antihyperlipidaemic, antihypertensive, astringent, cardioprotective, hridroga.

Description. A flat or minutely curved thick pieces of bark with reddish gray colour and astringent taste.

Identification

A. *Macroscopic* — Stem bark pieces, flat or minutely curved, with reddish gray external surface and darker inner surface. Internal surface has longitudinal minute ridges. Fractures longitudinal.

B. *Microscopic* — Cork consisting of 6-10 layers of elongated cells, phloem broad, medullary rays uniseriate. Calcium oxalate clusters abundant. Few of the parenchyma cells contain colouring matter.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 5 volumes of *toluene*, 5 volumes of *ethyl acetate* and 0.5 volume of *acetic acid*.

Test solution. Reflux 2 g of coarsely powdered substance under examination with 50 ml of *chloroform* for 15 minutes, cool and filter. Reflux the residue further with 50 ml of *chloroform*. Combine the filtrate and concentrate under vacuum to dryness. Dissolve the residue in 10 ml of *ethanol* at 50° for 10 minutes and filter.

Reference solution. Reflux 1 g of *arjuna IPBRS* with 50 ml of *chloroform* for 15 minutes, cool and filter. Reflux the residue further with 50 ml of *chloroform*. Combine the filtrate and concentrate under vacuum to dryness. Dissolve the residue in 5 ml of *ethanol* at 50° for 10 minutes and filter.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with 10 per cent v/v solution of *sulphuric acid* in *methanol*. Heat the plate at 110° for 5 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 20.0 per cent.

Water-soluble extractive (2.6.3). Not less than 20 per cent by Method I.

Total ash (2.3.19). Not more than 30.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

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Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 5 g of coarsely powdered substance under examination with 50 ml of *chloroform* for 15 minutes, cool and filter. Reflux the residue further with 50 ml of *chloroform*, cool and filter. Combine the filtrates and concentrate under vacuum to dryness, then extract dried residue with 10 ml of *ethanol* at 50° for 10 minutes and filter.

Reference solution. A 0.1 per cent w/v solution of *arjungenin IPRS* in *ethanol*.

Chromatographic system

– a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
– mobile phase: A. *acetonitrile (70 per cent)* in *water*,
B. *acetonitrile (30 per cent)* in *water*,
– a gradient programme using the condition given below,
– flow rate: 1.2 ml per minute,
– spectrophotometer set at 210 nm,
– injection volume: 20 µl

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	30	70
10	50	50
30	70	30
50	30	70

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections for the analyte peak corresponding to arjungenin is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the contents of arjungenin.

Storage. Store protected from light, heat, moisture and against attack by insects and rodents.

Arjuna Dry Extract

Arjuna Dry Extract is obtained by extracting Arjuna (*Terminalia arjuna* (Roxb ex DC.) Wight and Arn., Fam. Combretaceae) bark with *methanol* or any other suitable solvent and evaporation of solvent.

Arjuna Dry Extract contains not less than 90.0 per cent w/w and not more than 110.0 per cent w/w of stated amount of the arjunolic acid on the dried basis. It may contain suitable added substances.

Category. Antihyperlipidaemic, antihypertensive, astringent, cardioprotective, hridroga.

Usual strength. 60 per cent w/w.

Description. A light brown to beige powder with or without red tinge.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 92 volumes of *chloroform* and 8 volumes of *methanol*.

Test solution. Dissolve 50 mg of the extract under examination with 50.0 ml of *methanol* and filter.

Reference solution. A 0.1 per cent w/w solution of *arjunolic acid IPRS* in the *methanol*.

Apply to the plate 5 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 110° for 10 minutes and examine the plate under ultraviolet light at 366 nm and day light. The chromatographic profile of the test solution is similar to that of the reference solution.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 35 volumes of *chloroform*, 10 volumes of *methanol* and 2 volumes of *water*.

Test solution. Dissolve 50 mg of the extract under examination with 10.0 ml of *methanol* and filter.

Reference solution. A 0.05 per cent w/w solution of *arjungenin IPRS* in the *methanol*.

Apply to the plate 5 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and spray with *vanilline sulphuric acid reagent*. Heat the plate at 110° for 10 minutes and examine the plate under ultraviolet light at 366 nm and day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Ethanol-soluble extractive (2.6.2). Not less than 80.0 per cent.

Total ash (2.3.19). Not more than 5.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

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Test solution. Shake a quantity of the extract under examination containing about 50 mg of *arjunolic acid* in 50.0 ml of the *methanol*, filter.

Reference solution. A 0.1 per cent w/v solution of *arjunolic acid IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of *5 mM α-cyclodextrin* and 65 volumes of *methanol,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

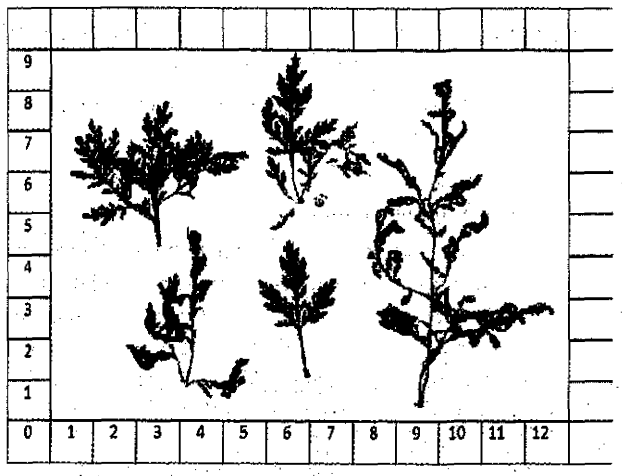
Inject the reference solution and the test solution.

Calculate the content of the arjunolic acid in the extract.

Storage. Store protected from heat and moisture.

Artemisia

Artemisia annua



Artemisia consists of dried leaves or the dried leaves and flowering tops of *Artemisia annua* Linn. (Fam. Asteraceae), known as *Qinghao.*

Artemisia contains not less than 0.8 per cent of artemisinin, calculated on the dried basis.

Category. Antimalarial, antibacterial, cytotoxic, antihelmintic.

Description. Slightly camphoraceous odour and bitter in taste.

Identification

A. The leaves grayish green, slightly darker upper surface, glabrous to sparsely hairy, break easily into small fragments, 3-pinnatipartite, petiolate and much variable in size (2.5-10.0 cm long). Leaf lobes narrow, oblong to elliptical with acuminate tip, about 1.0 mm wide. Petioles up to 1.0 cm long, base amplexicaul. Inflorescence panicled raceme. Dry capitula yellowish brown, pedicellate, heterogamous globose to sub-globose or disc shaped, 2.0 mm in diameter, flower heads arranged in lax or drooping, involucral bracts 3- seriate, greenish yellow, glabrous and oblong in shape, measure 1.0-1.2 x 0.5 mm in size. Inner involucral bracts elliptic having a median greenish streak on its outer surface. Ray florets pistillate, 6-8 in number per capitulum and 1.0-1.2 mm long. Disc florets hermaphrodite, 20-36 florets per capitulum and 0.8-1.0 mm long. All florets possess capitate oil glands on the middle of the outer surface that are 54-83 µm in diameter. Stamens 0.7 mm long attached to the corolla base, anther appendages lanceolate to triangular with acuminate tip. Anthers oblong and introrse. Pollen grains tricolpate, rounded 21-33 µm in diameter. In dry condition, capitula become empty because florets/achenes come out of it. Receptacle globular to oblong. Achenes minute; possess striated surface, yellowish brown in colour, 0.7-1.0 mm long, and oval to elliptic in shape. Stomatal index: 8-10-12.

B. The powder of the herb is grayish green to greenish yellow. Examined under a microscope using chloral hydrate solution. The powder shows the following diagnostic characteristics: T-shaped and globular trichomes, epidermal cells with wavy walls, anomocytic to anisocytic stomata, minute druse crystals, tricolpate rounded pollen grains, stone cells, annular vessels, rod shaped palisade cells and stigma with small and club shaped papillae.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 1 volume of *hexane* and 1 volume of *diethyl ether.*

Test solution. Boil 0.1 g of the coarsely powdered substance under examination with 10 ml of *hexane* for 10 minutes and filter. Evaporate the filterate to 1 ml.

Reference solution. Boil 0.1 g of *artemisia IPBRS* with 10 ml of *hexane* for 10 minutes and filter. Evaporate the filtrate to 1 ml.

Apply to the plate 5 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent.* Heat the plate at 100° for 15 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Total ash (2.3.19). Not more than 11.0 per cent.

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Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Assay. Determine by high performance thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 1 volume of *hexane* and 1 volume of *diethyl ether*.

Test solution. To 0.1 g of the coarsely powdered substance under examination, add 10 ml of *hexane* and keep for 12 hours, filter. Repeat the process of extraction 3 times. Combine the extracts, evaporate and dissolve in 1.0 ml of *hexane*.

Reference solution. A 0.1 per cent w/v solution of *artemisinin IPRS* in *hexane*.

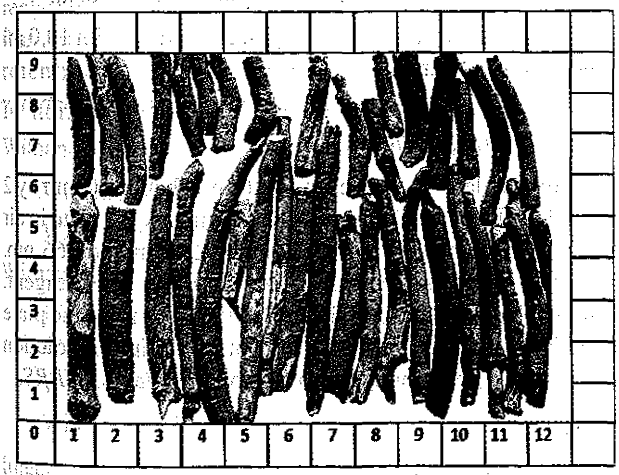
Apply to the plate 5 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and spray with a mixture of 50 volumes of *glacial acetic acid*, 1 volume of *sulphuric acid* and 0.5 volume of *anisaldehyde*. Heat the plate at 100° for 15 minutes, scan the plate in absorbance mode at 540 nm. Record the chromatograms and measure the responses for the analyte peak.

Calculate the content of artemisinin.

Storage. Store protected from light and moisture and against attack by insects and rodents.

Ashwagandha

Indian Ginseng; *Withania somnifera*



Ashwagandha consists of the dried mature roots of *Withania somnifera* (Linn.) Dunal (Fam. Solanaceae).

Ashwagandha contains not less than 0.3 per cent w/w of total withanolide (sum of withanolide glycosides and withanolide aglycones), calculated on the dried basis.

Category. Adaptogenic, alterative, antidepressant, ksaya, rasayana.

Description. Buff to greyish-yellow roots. Taste, slightly mucilaginous, bitter and acrid.

Identification

A. *Macroscopic* — Primary roots are straight, conical or finger like in shape, variable in thickness with the age. Secondary roots are thin and fibrous. Surface buff to greyish-yellow with longitudinal wrinkles.

B. *Microscopic* — Vessels with bordered pits and horizontal perforations. Fibres aseptate with pointed ends. Wood elements lignified. Starch grains abundant, simple, mostly spherical, reniform oval with central hilum. Microcrystals in parenchyma cells.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 9 volumes of *chloroform* and 1 volume of *methanol*.

Test solution. Reflux 3 g of coarsely powdered substance under examination with 50 ml *methanol* for 15 minutes, cool and filter.

Reference solution. Reflux 0.6 g of coarsely powdered *ashwagandha IPBRS* with 10 ml *methanol* for 15 minutes, cool and filter.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 100° for 5-10 minutes and examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 10.0 per cent.

Water-soluble extractive (2.6.3). Not less than 15.0 per cent by Method I.

Total ash (2.3.19). Not more than 7.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.2 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 5 g of the coarsely powdered substance under examination with 50 ml of *acetonitrile* on a

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water-bath for 15 minutes, cool and filter. Reflux the residue further with *acetonitrile* till the last extract turns colourless, cool and filter. Combine all the filtrates and concentrate to 50.0 ml.

Reference solution. A solution containing 0.01 per cent w/v each of *withanolide A IPRS* and *withanoside IV IPRS* in *acetonitrile*, prepared by heating gently on a water-bath.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in 900 ml *water*, adjusted to pH 2.8 with *dilute orthophosphoric acid* and make up it to 1000 ml with *water*,

B. *acetonitrile*,

- a gradient programme using the conditons given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 227nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
18	55	45
25	20	80
27	95	5
37	95	5

Inject the reference solution.The relative retention time of *withanolide A* is 1.0 and *withanoside IV* is about 0.7.The test is not valid unless the relative standard deviation for the replicate injections for both the analyte peaks is not more than 2.0 per cent and resolution is not less than 2.0. The relative retention time of *withanolide glycosides* and *withanolide aglycones* are as follows.

Withanolide glycoside

Withanoside IV	0.7
Withanoside V and VI	0.89

Withanolide aglycones

12, deoxywithastramonolide	0.96
Withanolide A	1.0
Withanolide B	1.14
Withanone	1.01

Inject the reference solution and the test solution.

Calculate the contents of withanolide glycosides in the sample as withanoside IV by summing the peak areas of withanoside IV,V and VI. Calulate the content of withanolide aglycones in the sample as withanolide A by summing the peak areas of 12, deoxywithastramonolide, withanolide A, withanolide B and withanone. Sum the content of withanolide glycosides and withanolide aglycones to get total withanolides.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Ashwagandha Dry Extract

Ashwagandha Dry Extract is obtained by extracting ashwagandha (*Withania somnifera* (Linn.) Dunal, Fam.Solanaceae) roots with *methanol*, *alcohol*, *water* or mixture of these solvents and evaporation of solvent.

Ashwagandha Dry Extract contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of withanolides, calculated on the dried basis as sum of withanolideaglycones calculated as withanolide A and withanolide glycosides calculated as withanoside IV. The extract may contain suitable added substances.

Category. Adaptogenic, alterative, antidepressant, ksaya, rasayana.

Description. A light brown to brown powder.

Identification

Determine by thin layer chromatography (2.4.17), coating the plate with silica gel *GF254*.

Mobile phase. A mixture of 55 volumes of *toluene*, 45 volumes of *ethyl acetate* and 3 volumes of *glacial acetic acid*.

Test solution. Dissolve about 0.2 g of the extract with 10.0 ml of *methanol* and filter.

Reference solution. A 0.05 per cent w/v solution of *withaferin A IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8-9cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. Spray the plate with *anisaldehyde-sulphuric acid* reagent. Heat the plate at 100° for 5-10 minutes and examine the plate under day light. The chromatogram obtained with test solution shows a band corresponding to that of *withaferin A IPRS*.

Tests

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals Method B (20 ppm).

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

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Test solution. Transfer extract equivalent to 0.1 mg of *Withanoside IV* and add 50.0 ml of *methanol* with gentle heating and make up to 100.0 ml.

Reference solution. A solution containing 0.01 per cent w/v each of *withanolide A IPRS* and *withanoside IV IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in 900.0 ml *water*, adjusted to pH 2.8 with *dilute orthophosphoric acid* and make up it to 1000.0 ml with *water*,

B. *acetonitrile*,

- a gradient program using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 227 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
18	55	45
25	20	80
27	95	5
37	95	5

Inject the reference solution. The relative retention time of *withanolide A* is 1.0 and *withanolide IV* is about 0.7. The test is not valid unless the relative standard deviation for the replicate injections for both the analyte peaks is not more than 2.0 per cent and resolution is not less than 2.0. The relative retention time of *withanolide glycosides* and *withanolideaglycones* are as follow.

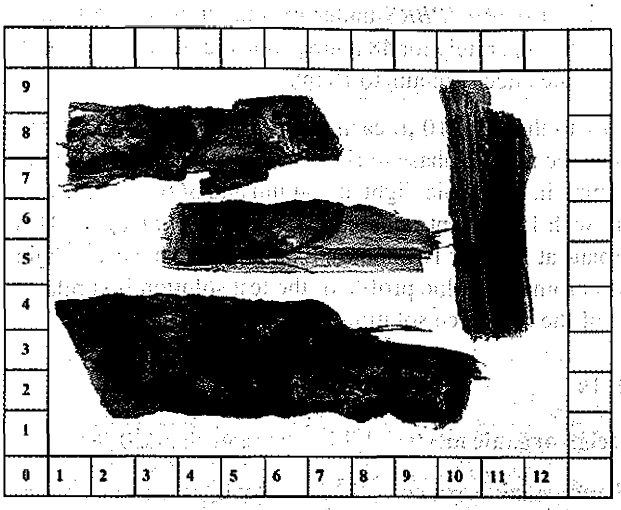
Withanolide glycoside	
Withanoside IV	0.70
Withanoside V and VI	0.89
Withanolideaglycones	
12, deoxywithastramonolide	0.96
Withanolide A	1.0
Withanone	1.01
Withanolide B	1.14

Inject the reference solution and the test solution.

Calculate the contents of withanolide glycosides in the sample as withanoside IV by summing the peak areas of withanoside IV, V and VI. Calculate the content of withanolideaglycones in the sample as withanolide A by summing the peak areas of 12, deoxywithastramonolide, withanolide A, withanolide B, and withanone. Sum the content of withanolide glycosides and withanolideaglycones to get total withanolides.

Asoka

Ashoka, *Saraca asoca*



Asoka consists of dried stem bark of *Saraca asoca* (Roxb.) J.J.de Wilde (Fam. Fabaceae).

Asoka contains not less than 0.4 per cent w/w of β-sitosterol.

Category. Tonic, oxytoxic.

Description. The dried stem bark occurs in pieces of variable size and thickness, surface rough. It is externally brown in colour with no characteristic odour.

Identification

A. *Macroscopic* — Stem bark externally brown and elongated; with circular lenticels and transversely ridged, cracked; internally reddish-brown with fine longitudinal strands and fibres. Texture rough; fracture splinting exposing striated surface, a thin whitish and continuous layer is seen beneath the cork layer.

B. *Microscopic* — Wide layer of cork, secondary cortex wide with one or two continuous layers of stone cells with many patches of sclereids, parenchymatous tissue containing yellow masses. Secondary phloem consists of phloem parenchyma, sieve tubes, fibres, with medullary rays, with companion cells and phloem fibres occurring in groups.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *toluene*, 15 volumes of *ethyl acetate* and 1 volume of *glacial acetic acid*.

Test solution. Macerate about 1 g of the coarsely powdered substance under examination with 50 ml of *methanol* for

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48 hours, cool and filter. Concentrate the filtrate under vacuum to 10 ml.

Reference solution. Macerate about 1 g of the coarsely powdered *asoka IPBRS* under examination with 50 ml of *methanol* separately for 48 hours, cool and filter. Concentrate the filtrate under vacuum to 10 ml.

Apply to the plate 10 µl each solution as band length 8 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in ultraviolet light at 254 nm and 366 nm. Spray the plate with 10 per cent *methanolic sulphuric acid reagent*. Heat the plate at 100° for 10 minutes. Examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent

Ethanol-soluble extractive (2.6.2). Not less than 15.0 per cent

Water-soluble extractive (2.6.3). Not less than 11.0 per cent.

Total Ash (2.3.19). Not more than 11.0 per cent

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on Drying (2.4.19). Not more than 5.0 per cent.

Microbial contamination (2.2.9) Complies with the microbial contamination tests.

Assay. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *toluene*, 15 volumes of *ethyl acetate* and 1 volume of *glacial acetic acid*.

Test solution. Macerate about 1 g of the coarsely powdered substance under examination with 50 ml of *methanol* and *methanol* separately for 48 hours, cool and filter.

Reference solution. A 0.0025 w/v solution of *β-sitosterol IPRS* in *methanol*.

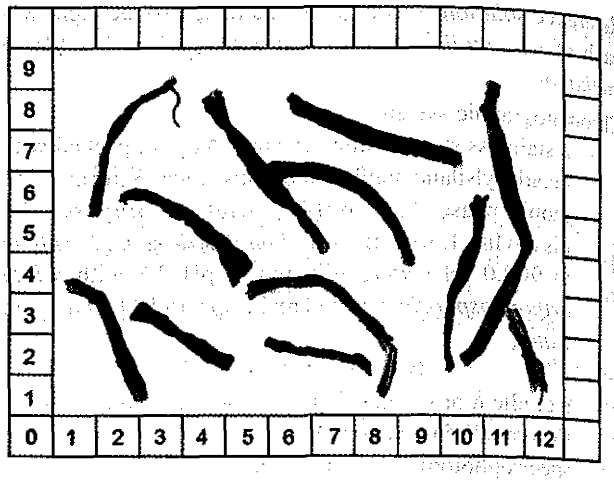
Apply to the plate, 10 µl of test and reference solution as band length 8 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and scan the plate in absorbance mode at 366 nm. Record the chromatograms and measure the responses for the analyte peak.

Calculate the content of β-sitosterol.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Asthisamhrta

Hadjod, Harjora, *Cissus quadrangularis*



Asthisamhrta consists of dried stem of *Cissus quadrangularis* Linn. (Fam. Vitaceae).

Asthisamhrta contains not less than 0.3 per cent of quercetin, calculated on the dried basis.

Category. Anti-osteoporotic, anti-haemorrhoidal, analgesic, anti-oxidant, anti-inflammatory.

Description. Stems are longitudinally striated, transversely cracked and pale green in colour. Fracture short and fibrous, odour characteristic and taste bitter.

Identification

A. *Macroscopic* — Fresh stems are fleshy, glabrous, succulent, quadrangular, internodes measuring 7 to 21 cm in length, 1 to 2 cm in breadth, nodes constricted with attached leaf and tendril, stem leafless, woody and fibrous, with four winged projections. Dried stems highly shrivelled; longitudinally striated, ridged and transversely cracked, fracture short and fibrous, pale greenish in colour.

B. *Microscopic* —Transverse section of the young stem shows a rectangular outline with discontinuous rings of vascular bundles, parallel to dermis, 3 to 4 vascular bundles under the wings are more developed than that on the flat sides, collateral with the cap of bast fibres and encircled by idioblasts containing cluster crystals of calcium oxalate with numerous air cavities in the section.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 80 volumes of *toluene*, 20 volumes of *ethyl acetate* and 2 volumes of *glacial acetic acid*.

Test solution. Reflux 2 g of coarsely powdered substance under examination with 100 ml of *n-hexane* for 30 minutes. Reflux the

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residue again two times with 50 ml of *methanol*. Cool and filter. Combine the extracts and concentrate under vacuum to 5 ml.

Reference solution. Reflux 2 g of *asthisamhrta IPBRS* with 100 ml of *n-hexane* for 30 minutes. Reflux the residue again two times with 50 ml of *methanol*, 30 minutes for each extraction. Cool and filter. Combine the extracts and concentrate under vacuum to dryness. Dissolve the residue in 5 ml of *methanol*.

Apply to the plate 10 µl of each solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. Spray the plate with *anisaldehyde-sulphuric acid reagent* and heat the plate at 105°. Examine the plate in visible light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol- soluble extractives (2.6.2). Not less than 5.0 per cent.

Water soluble extractive (2.6.3). Not less than 21.0 per cent.

Total ash (2.3.19). Not more than 20.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.5 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 9.0 per cent determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 5 g of coarsely powdered substance under examination two times with 50 ml of *methanol* for 2 hours, cool and filter. Combine the filtrates and evaporate to dryness. Dissolve the residue in *methanol* and dilute to 25 ml with *methanol*.

Reference solution. A 0.01 per cent w/v solution of *quercetin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 40 volumes of *acetonitrile* and 60 volumes of a buffer solution prepared by dissolving 3.01g of *potassium di-hydrogen orthophosphate* in 1000 ml of *water* and adjust to pH 3.4 with *acetic acid*,
- flow rate: 1ml per minute,
- spectrophotometer set at 370 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injection is not more than 2.0 per cent.

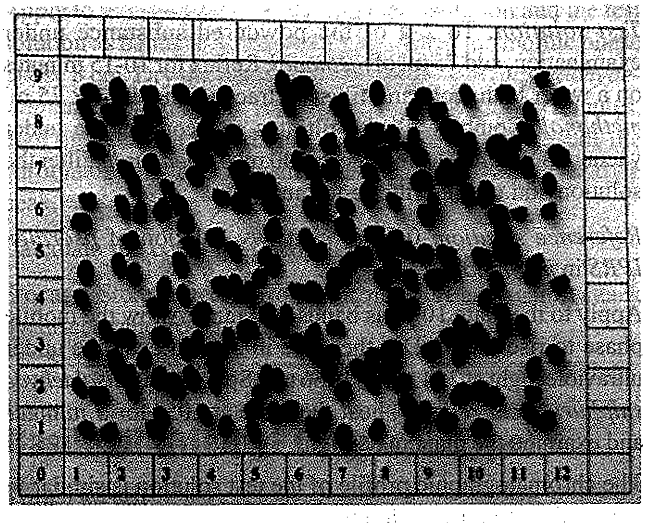
Inject the reference solution and the test solution.

Calculate the content of quercetin.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Bakuci

Bakuchi; Babchi; *Cullen corylifolium*



Bakuci consists of dried ripe fruits of *Cullen corylifolium* (Linn.) Medik. (Fam. Fabaceae).

Bakuci contains not less than 0.50 per cent w/w of Psoralen, calculated on the dried basis.

Description. Fruit is externally brown in colour and internally pale brown to whitish in colour. Odourless, but when chewed, smell of a pungent essential oil felt; taste bitter, unpleasant and acrid.

Identification

A. *Macroscopic*—Fruits dark brown or yellowish brown in colour, one seeded pod with pericarp adhering to the seed coat. Fruit is 3-5 mm in length and 2.5-3.5 mm in breadth, and 2-3 mm in thickness ovoid-oblong or kidney-shaped, somewhat compressed rounded or mucronate, closely pitted; seeds campylotropous, non-endospermous, oily and free from starch.

B. *Microscopic*—Transverse section of the fruit shows an outer layer of epicarp composed of rectangular to tangentially elongated dark brown coloured cells covered with thin cuticle, 5-6 rows of irregularly arranged parenchymatous cells of hypodermis filled with dark brown contents. The inner zone

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of testa is composed of outer tangentially running parenchyma and inner collapsed parenchymatous tissue traversed by vascular strands, and a layer of endosperm lying underneath. The epidermal cells of the cotyledon are squarish to somewhat rectangular; cells of the mesophyll are radially arranged, with aleurone grains and oil globules.

Powder. Epidermal cells with dark brown pigment, large secretory canals surrounded by dark brown parenchymatous cells of sub-epidermis.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 7 volumes of *toluene* and 3 volumes of *ethyl acetate*.

Test solution. To 2 g of the powdered substance under examination, add 75 ml of *methanol*. Reflux for 10-15 minutes on a water bath, cool and filter. Extract again with 2 x 75 ml *methanol*. Combine all the filtrates and concentrate under vacuum. Dissolve the residue in 100 ml of *methanol*. Filter the solution and use the filtrate for analysis.

Reference solution. 0.2 per cent w/v solution of *psoralen IPRS* in *methanol*.

Apply to the plate 10 µl as bands of 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. Spray with *anisaldehyde sulphuric acid reagent*, heat the plate at 105° for 5 minutes and examine the plate in daylight.

The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 11.0 per cent.

Water-soluble extractive (2.6.3). Not less than 9.0 per cent.

Total ash (2.3.19). Not more than 9.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.8 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 7 volumes of *toluene* and 3 volumes of *ethyl acetate*.

Test solution. To 2 g of the powdered substance under examination, add 75 ml of *methanol*. Reflux for 10-15 minutes on a water bath, cool and filter. Extract again with 3 x 75 ml *methanol*. Combine all the filtrates and concentrate under vacuum. Dissolve the residue in 100 ml of *methanol*.

Reference solution. A 0.02 per cent w/v solution of *psoralen IPRS* in *methanol*.

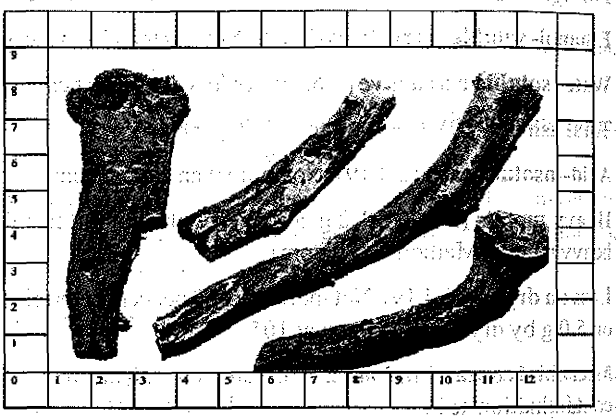
Apply to the plate, 10 µl of test and reference solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. Record the chromatograms and measure the responses for the analyte peak.

Calculate the content of psoralen.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Bala

Mahabala; *Sida acuta*



Bala consists of dried roots of *Sida acuta* Burm.f.

Bala contains not less than 0.016 per cent w/w of ecdysterone, calculated on the dried basis.

Category. Astringent, diuretic, urinary infections, snigdha, rasayana.

Description. The roots have a pale yellow colour; outer surface is fairly smooth

Identification

A. *Macroscopic* — Root consists of a short stout taproot system attaining a diameter of about 1 cm and often long and flexuous with fairly thick lateral roots. Outer surface of the root is fairly smooth except for the small filmy strips of exfoliating cork. The bark has a thickness of about 1 to 1.5 mm and can be easily peeled off in fresh condition or by wetting.

B. *Microscopic* — Transverse section of root shows thin cork consisting of 4 to 9 rows of rectangular or tangentially

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elongated, thin-walled cells followed by 2 to 3 layers of cork cambium towards inner side. Secondary cortex inner to cork cambium consists of 2 to 3 rows of tangentially elongated cells. Inner to the cortex, phloem elements (composed mainly of thin-walled cells) alternate with groups of thick-walled cells. This region is interrupted by several, 1 to 3 seriate, medullary rays. Cambium consists of 1 to 2 rows of narrow, almost rectangular, thin walled cells. Inner to cambium, the wood is composed of many vessel elements mostly having simple pits, xylem parenchyma, xylem fibres and 1 to 2 seriate medullary rays.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 4 volumes of *chloroform* and 1 volume of *methanol*.

Test solution. Reflux 2 g of powdered substance under examination with 150 ml of *n-hexane* in a soxhlet apparatus to defat the material. Dry the residue and reflux it further with three times with 25 ml of *methanol*, cool and filter. Combine the filtrates and evaporate to dryness. Dissolve the residue in *methanol* and dilute to 5 ml with *methanol*.

Reference solution. Reflux 2 g of *bala IPBRS* with 150 ml of *n-hexane* in a soxhlet apparatus to defat the material. Dry the residue and reflux it further with three times with 25 ml of *methanol*, cool and filter. Combine the filtrates and evaporate to dryness. Dissolve the residue in 5 ml of *methanol*.

Apply to the plate 10 µl of the reference solution and the test solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. Spray with *anisaldehyde-sulphuric acid reagent*, heat at 110° for 30 minutes and examine in daylight.

The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 3.0 per cent.

Water-soluble extractive (2.6.3). Not less than 8.0 per cent.

Total ash (2.3.19). Not more than 5.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 7.8 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 4 volumes of *chloroform* and 1 volume of *methanol*.

Test solution. Extract 20 g of powdered substance under examination with 150 ml of *n-hexane* in a soxhlet apparatus to defat the material. Dry the residue and reflux it further three times with 25 ml of *methanol*, cool and filter. Combine the filtrates and evaporate to dryness. Dissolve the residue in *methanol* and dilute to 5 ml with water.

Reference solution. A 0.1 per cent w/v solution of *ecdysterone IPRS* in *methanol*.

Apply to the plate 10 µl of the reference solution and the test solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and spray with *anisaldehyde-sulphuric acid reagent*. Heat the plate at 110° for 30 minutes, cool and scan in absorbance mode at 560 nm. Record the chromatograms and measure the responses for the analyte peak.

Calculate the content of ecdysterone.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Basil Oil (Methyl Chavicol Type)

Tulasi ka tail

Basil Oil is the essential oil obtained by steam distillation of part of plant (leaves and flowering tops) *Ocimum basilicum* Linn. (Fam. Lamiaceae).

Basil Oil contains not less than 50.0 per cent and not more than 75.0 per cent of methyl chavicol, and not less than 15.0 per cent and not more than 25.0 per cent of limonene.

Description. A clear, pale yellow to amber liquid, free from sediment and suspended matter.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *basil oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). Not less than 100.0°.

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Tests

Relative density (2.4.29). 0.930 to 0.960.

Refractive index (2.4.27). 1.488 to 1.500.

Optical rotation (2.4.22). – 6.0° to +7.5°.

Acid value (2.3.23). Not more than 1.0.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

Reference solution(a). A 2.0 per cent w/v solution of *methyl chavicol IPRS* in *ethanol (95 per cent).*

Reference solution (b). A 2.0 per cent w/v solution of *limonene IPRS* in *ethanol (95 per cent).*

Chromatographic system

- a capillary column 30 m x 0.25 mm x 0.25 µm, coated with methyl 15 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio : 1 : 25.

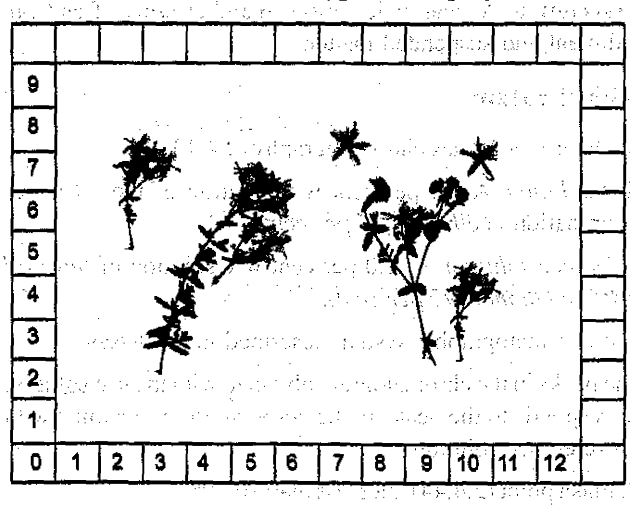
Inject reference solutions (a), (b) and the test solution.

Calculate the content of methyl chavicol and limonene in the oil using area normalisation procedure.

Storage. Store protect from light and moisture.

Bassant

Dendhu; *St. Johns wort; Hypericum perforatum*



Bassant consists of whole or cut, dried flowering tops or aerial parts of *Hypericum perforatum* Linn. (Fam. Hypericaceae).

Bassant contains not less than 0.08 per cent of total hypericins, calculated on the dried basis.

Category. Antidepressant, anxiolytic, sedative, anti-oxidant and anti-ulcerogenic.

Description. The flower is yellow with conspicuous black dots. The sepals are pointed, with glandular dots. It has a characteristic balsamic odour, and a bitter resinous, astringent taste.

Identification

A. *Macroscopic* — Stem shows two more or less prominent longitudinal ridges. Leaves are opposite, sessile, exstipulate, oblong-oval and 15-30 mm long; present on the leaf margins are glands which appear as black dots and over all the surface of the leaves. Many small, strongly translucent excretory glands which are visible in transmitted light. The flowers are regular and form corymbose clusters at the apex of the stem. They have five green, acute sepals, with black secretory glands on the margins; five orange-yellow petals, also with black secretory glands on the margins; three staminal blades, each divided into many orange-yellow stamens and three carpels surmounted by red styles. Immature fruits are green or yellowish, seeds are whitish. Occasional ripe fruits may be present; these are dry trilocular capsules containing numerous seeds, brown, broad or small-ovate, 5-10 mm long, with broad linear or punctiform glands, irregularly striated ducts, conducting secretions. Ripe seeds are 1-1.3 mm long, cylindrical or trigonous, shortly pointed at both ends, brown or almost black, minutely pitted longitudinally.

B. *Microscopic*—The powder is greenish-yellow. When examined under a microscope it shows the following diagnostic characters; fragments of the leaf epidermis or stems with paracytic, anisocytic or anomocytic stomata fragments of the leaf epidermis often accompanied by palisade parenchyma; polygonal cells of the upper epidermis with thickened and beaded walls; more or less sinuous, thin-walled cells of the lower epidermis; fragments of the leaf and sepal with large, red-pigmented oil glands associated with palisade parenchyma and small vessels elongated cells of fragments of the petal epidermis with straight or wavy anticlinal walls; vessels with reticulate or pitted walls and groups of thick-walled fibres; fragments of the central parenchyma of the stems with lignified and pitted rectangular cells sometimes associated with vessels; fragments of the anthers showing the central part consisting of small cells containing cluster crystals of calcium oxalate and cells from the fibrous layer; fragments of the staminal filament with elongated, thin-walled cells with a striated cuticle; numerous pollen grains with three germinal pores and a smooth exine, occurring singly or in dense groups.

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C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 6 volumes of *formic acid*, 9 volumes of *water* and 90 volumes of *ethyl acetate*.

Test solution. Reflux 0.5 g of coarsely powdered substance under examination with 10 ml of *methanol* in water bath for 10 minutes, cool and filter.

Reference solution. Reflux 0.5 g of coarsely powdered *bassant IPBRS* with 10 ml of *methanol* in water bath for 10 minutes, cool and filter.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. Spray the plate with 10.0 per cent v/v solution of *diphenylboric acid aminoethyl ester* in *methanol* and heat the plate at 105°. Examine the plate in visible light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 3.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 13.0 per cent.

Water-soluble extractive (2.6.3). Not less than 24.0 per cent.

Total ash (2.3.19). Not more than 7.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.8 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 10.0 per cent determined on 1.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9).Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 1 g of the coarsely powdered substances under examination with 50 ml of *methanol* on water bath for 2 hours and cool and filter. Evaporate to dryness and dissolve in 10 ml of *methanol*.

Reference solution. A 0.01 per cent w/v solution of *hypericin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 39 volumes of *ethyl acetate*, 160 volume of *methanol* and 41 volumes of buffer solution prepared by dissolving 15.6 g of *sodium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 2.0 with *orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 590 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injection is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of hypericins.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Bassant Dry Extract

Bassant Dry Extract is obtained from dried flowering tops or aerial parts of *Hypericum perforatum* Linn.(Fam. Hypericaceae).

Bassant Dry Extract contains not less than 0.1 per cent of hypericin, calculated on the dried basis.

Category. Antidepressant, anxiolytic, sedative, anti-inflammatory and anti-ulcerogenic.

Description. A greenish brown colored powder.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 6 volumes of *formic acid*, 9 volumes of *water* and 90 volumes of *ethyl acetate*.

Test solution. Dissolve about 0.25 g of extract under examination in 5 ml of *methanol*.

Reference solution. Reflux 0.25 g of *bassant dry extract IPBRS* with 5 ml of *methanol*.

Apply to the plate 10 µl of each solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in ultraviolet light at 254 nm and 366 nm. Spray with 10.0 per cent v/v solution of *diphenylboric acid aminoethyl ester* in *methanol* and heat the plate at 105°. Examine the plate in visible light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent determined on 1.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

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Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 70 mg of the extract under examination in 25.0 ml of *methanol* and filter.

Reference solution. A 0.006 per cent w/v solution of *hypericin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 39 volumes of *ethyl acetate*, 160 volumes of *methanol* and 41 volumes of buffer solution prepared by dissolving 15.6 g of *sodium dihydrogen orthophosphate* in 1000 ml of *water*, adjusted to pH 2.0 with *orthophosphoric acid*,
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 590 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

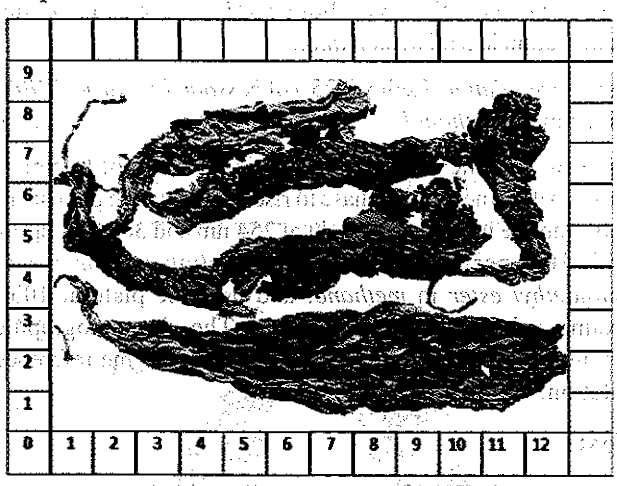
Inject the reference solution and the test solution.

Calculate the content of hypericin.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Belladonna Leaf

Atropa bella-donna



Belladonna Leaf consists of the dried leaf and flowering tops of *Atropa bella-donna* Linn. or of *A. acuminata* Royle ex Lindley (Fam. Solanaceae) or a mixture of both species.

Belladonna Herb contains not less than 0.30 per cent of total alkaloids, calculated as hyoscyamine with reference to the material dried at 100° to 105°.

Category. Anticholinergic.

Description. Green to greenish-brown leaves, slightly darker on the upper surface, often crumpled and rolled and partly matted together in the drug. When whole, the lamina is 5 to 25 cm long and 3 to 12 cm wide, elliptical to ovate; acuminate at the apex, narrowing at the base; margin entire. Petiole 0.5 to 4 cm in length. The young leaves are highly pubescent, the older leaves are slightly pubescent along the veins. In the flowering tops, the stems are hollow and flattened, with leaves in pairs of unequal size, in the axils of which are single flowers with campanulate corolla, about 2 cm long and 1.5 cm wide, purple or yellow-brown in colour, with five short, reflexed lobes, five epipetalous stamens and one bilocular ovary with numerous ovules.

Identification

A. When examined under a microscope it shows epidermal cells with sinuate anticlinal walls and cuticle which is often striated and furrowed. Covering and glandular hairs infrequent, though more frequent in the young leaves and around the veins; covering hairs, multicellular, uniseriate, with thin smooth walls; glandular hairs; short clavate glands with multicellular heads and glands with a long uniseriate stalk and ovoid unicelluar head. Stomata, anisocytic, more frequent on the lower epidermis. The midrib is characterised by an open arc of vascular bundles with isolated groups of perimedullary phloem. Mesophyll dorsiventral with a single palisade layer. Throughout the parenchyma and particularly just below the palisade layer are cells containing microsphenoidal crystals of calcium oxalate or, very rarely, cluster crystals. The stems show pericyclic fibres and perimedullary bundles of phloem, few trichomes; the cortical parenchymatous cells and the pith cells contain microsphenoidal crystals of calcium oxalate.

B. Powder 1 g and shake for 2 minutes with 10 ml of *0.05 M sulphuric acid*. Filter and add to the filtrate 1 ml of *strong ammonia solution* and 5 ml of *water*. Extract with 15 ml of *ether*, taking care to prevent the formation of an emulsion. Dry the ether extract over *anhydrous sodium sulphate* and filter. Evaporate the filtrate to dryness, add 0.5 ml of *fuming nitric acid* and evaporate to dryness. Add 10 ml of *acetone* and, dropwise, a 3 per cent w/v solution of *potassium hydroxide* in *ethanol (95 per cent)*; a deep violet colour develops.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 90 volumes of *acetone*, 7 volumes of *water* and 3 volumes of *strong ammonia solution*.

Test solution. Add 15 ml of *0.05 M sulphuric acid* to 0.6 g of the material under examination, in fine powder, shake for 15 minutes, filter and wash the filter with *0.05 M sulphuric acid* until 20 ml of filtrate is obtained; add 1 ml of *strong*

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ammonia solution to the filtrate, extract with two quantities, each of 10 ml, of *peroxide-free ether*, separate the ether layer, by centrifugation if necessary, dry the combined ether extracts over *anhydrous sodium sulphate*, filter, evaporate to dryness on a water-bath and dissolve the residue in 0.5 ml of *methanol*.

Reference solution. Add 15 ml of *0.05 M sulphuric acid* to 0.6 g of the *belladonna leaf IPBRS*, shake for 15 minutes, filter and wash the filter with *0.05 M sulphuric acid* until 20 ml of filtrate is obtained, add 1 ml of *strong ammonia solution* to the filtrate, extract with two quantities, each of 10 ml, of *peroxide-free ether*, separate the ether layer, by centrifuging if necessary, dry the combined ether extracts over *anhydrous sodium sulphate*, filter, evaporate to dryness on a water-bath and dissolve the residue in 0.5 ml of *methanol*.

or

Dissolve 50 mg of *hyoscyamine sulphate IPRS* in 9 ml of *methanol* (solution A) and dissolve 15 mg of *hyoscine hydrobromide IPRS* in 10 ml of *methanol* (solution B), mix 8 ml of solution A with 1.8 ml of solution B.

Apply to the plate 10 µl and 20 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate at 105° for 15 minutes, allow to cool and examine under ultraviolet light at 254 nm and 366 nm, spray with 10 ml of *modified potassium iodobismuthate solution* until the bands become visible as orange or brown on a yellow background. The bands in the chromatogram obtained with test solution have similar Rf values to those in the chromatogram obtained with reference solution (hyoscyamine in the lower third of the chromatogram; hyoscine in the upper third) and are similar in colour and atleast equal in size. Faint secondary bands may appear, particularly in the middle of the chromatogram obtained with 20 µl of the test solution or near the line of application in the chromatogram obtained with 10 µl of test solution. Spray the plate with a freshly prepared 10 per cent w/v solution of *sodium nitrite* until transparent and examine after 15 minutes. The colours due to *hyoscyamine* in the chromatogram change from brown to reddish-brown but not to greyish-blue (atropine); any secondary bands are no longer visible.

Tests

Foreign organic matter (2.6.1). Not more than 3.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Assay. Powder 50 g and determine the loss on drying (2.4.19), by drying 2 g, accurately weighed, in an oven at 105°. From the remaining sample, weigh accurately about 10 g, moisten with a mixture of 5 ml of *dilute ammonia solution*, 10 ml of *ethanol (95 per cent)* and 30 ml of *ether* and mix thoroughly. Transfer the mixture to a percolator with the aid of an extracting solvent mixture consisting of 3 volumes of *ether* and 1 volume of *chloroform*. Allow to macerate for 4 hours and percolate with the solvent mixture until complete extraction of the alkaloids is effected (2.6.4).

Concentrate the percolate to about 50 ml by distilling off the solvent mixture on a water-bath, and transfer to a separator, previously rinsed with *ether*. Add a quantity of *ether* at least equal to 2.1 times the volume of the percolate and extract with three quantities, each of 20 ml, of *0.5 M sulphuric acid*. Transfer each acid extract to another separating funnel. Combine the acid extracts, make the solution alkaline with *dilute ammonia solution* and extract with *chloroform* until complete extraction of the alkaloids has been effected. Wash the combined chloroform extracts with 10 ml *water*, discard the *water*, evaporate the chloroform layer to dryness and heat the residue for 15 minutes on a water-bath. Redissolve the residue in successive small quantities of *chloroform*, evaporating to dryness on a water-bath each time before adding the solvent. Heat for 15 minutes on a water-bath and dissolve the residue in 5 ml of *chloroform*. Add 20.0 ml of *0.01 M sulphuric acid*, remove the chloroform by evaporation on a water-bath and titrate the excess of acid with *0.02 M sodium hydroxide* using *methyl red solution* as indicator.

1 ml of *0.01 M sulphuric acid* is equivalent to 0.005788 g of total alkaloids calculated as hyoscyamine. Calculate the content of total alkaloids with reference to the dried material.

Storage. Store protected from light and moisture.

Belladonna Dry Extract

Belladonna Dry Extract is obtained from dried Belladonna leaf and root of one or more of the cultivated varieties of *Atropa bella-donna* Linn. or *A. acuminata* Royle ex Lindley (Fam Solanaceae) or a mixture of both species by extraction with a suitable solvent using an appropriate procedure.

Belladonna Dry Extract contains not less than 90.0 per cent and not more than 110.0 per cent of stated amount of alkaloids of belladonna calculated as sum of atropine and hyosine calculated on dried basis.

Category. Anticholinergic.

Usual strength. 1.0 per cent.

Description. Greenish brown colored powder.

Identification

Determine by thin-layer Chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 90 volumes of *acetone*, 7 volumes of *water* and 3 volumes of strong *ammonia solution*.

Test solution. Disslove 0.6 g of the substance under examination in 20 ml of *0.05 M sulphuric acid*, shake for

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15 minutes, filter and wash the filter with *0.05 M sulphuric acid* until 20 ml filtrate is obtained; add 1 ml of strong *ammonia* solution to the filtrate, extract with two quantities, each of 20 ml of *peroxide-free-ether*, separate the ether layer by centrifugation, if necessary, dry the combined ether extract over *anhydrous sodium sulphate*, filter evaporate to dryness on a water-bath and dissolve the residue in 0.5 ml of *methanol*.

Reference solution. Dissolve 50 mg of *atropine sulphate IPRS* in 9 ml of *methanol* (solution A). Dissolve 15 mg of *hyoscine hydrobromide IPRS* in 10 ml of *methanol* (solution B). Mix 1.8 ml of *hyoscine hydrobromide* solution (solution B) and 8 ml of the *atropine sulphate* solution (solution A).

Apply to the plate 20 µl of each solution as bands 10 mm. Allow the mobile phase to rise 12 cm. Dry the plate at 105° for 15 minutes, allow to cool and examine under ultraviolet light at 254 nm and 366 nm, spray with 10 ml of modified *potassium iodobismuthate solution* until the bands become visible as orange or brown on a yellow background. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 0.5 g of the dry extract dried at 105°.

Assay. Determine by Liquid Chromatography (2.4.14).

Test solution. Transfer an accurately weighed quantity of the substance under examination, equivalent to about 5 mg of total alkaloids, into a 50 ml volumetric flask. Add about 40 ml *0.05M sulphuric acid* and sonicate for about 30 minutes. Make volume with *0.05M sulphuric acid*, mix and filter.

Reference solution (a). A solution containing 0.012 per cent w/v of *atropine sulphate IPRS* and 0.0014 per cent w/v of *hyoscine hydrobromide IPRS* in *0.05M sulphuric acid*.

Reference solution (b). A 0.0014 per cent w/v solution of *hyoscine hydrobromide IPRS* in *0.05M sulphuric acid*, for peak identification.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm).
- mobile phase: a mixture of 13 volumes of *acetonitrile* and 87 volumes of buffer (pH 3.5), prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 900 ml water, add 10 ml *triethylamine*, adjusted to pH 3.5 with *dilute orthophosphoric acid* and diluting it to 1000 ml with *water*.
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent and resolution factor should not be less than 3.0.

Inject reference solutions (a), (b) and the test solution.

Calculate the contents of atropine and hyoscine.

1mg *atropine sulphate* contains 0.8551 mg of *atropine* and 1 mg *hyoscine hydrobromide* contains 0.7894 mg of *hyoscine*.

Storage. Store protected from light and moisture.

Belladonna Dry Extract Tablets

Belladona Dry Extract Tablets contain not less than 90.0 per cent w/w and not more than 110.0 per cent w/w stated amount of belladonna dry extract equivalent to alkaloids of belladonna calculated as sum of atropine and hyoscine, on the dried basis.

Category. Anticholinergic.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 90 volumes of *acetone*, 7 volumes of *water* and 3 volumes of *strong ammonia solution*.

Test solution. Disslove 0.6 g of the substance under examination in 20 ml of *0.05 M sulphuric acid*, shake for 15 minutes, filter and wash the filter with *0.05 M sulphuric acid* until 20 ml filtrate is obtained; add 1 ml of *strong ammonia solution* to the filtrate, extract with two quantities, each of 20 ml of *peroxide-free-ether*, separate the ether layer by centrifugation, if necessary, dry the combined ether extract over *anhydrous sodium sulphate*, filter evaporate to dryness on a water-bath and dissolve the residue in 0.5 ml of *methanol*.

Reference solution. Dissolve 50 mg of *atropine sulphate IPRS* in 9 ml of *methanol* (solution A). Dissolve 15 mg of *hyoscine hydrobromide IPRS* in 10 ml of *methanol* (solution B). Mix 1.8 ml of *hyoscine hydrobromide solution* (solution B) and 8 ml of the *atropine sulphate solution* (solution A).

Apply to the plate 20 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 12 cm. Dry the plate at 105° for 15 minutes, allow to cool and examine under ultraviolet light at 254 nm and 366 nm, spray with 10 ml of modified *potassium iodobismuthate solution* until the bands become visible as orange or brown on a yellow background. The chromatographic profile of the test solution is similar to that of the reference solution.

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Tests

Loss on drying (2.4.19). Not more than 5.0 per cent determined on 0.5 g of the dry extract dried at 105° to constant weight.

Other tests. Comply with tests stated under tablets.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh accurately a quantity of the powder containing 1.0 mg of total alkaloids, into a 100.0 ml volumetric flask, dissolve in 70 ml of *0.05 M sulphuric acid*, ultrasonicate for 30 minutes. Dilute to 100.0 ml with *0.05 M sulphuric* acid and filter.

Reference solution (a). A solution containing 0.012 per cent w/v of *atropine sulphate IPRS* and 0.0014 per cent w/v of *hyoscine hydrobromide IPRS in 0.05 M sulphuric acid.*

Reference solution (b). A solution containing 0.0014 per cent w/v of *hyoscine hydrobromide IPRS* in *0.05 M sulphuric acid.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 13 volumes of *acetonitrile* and 87 volumes of a buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 900 ml of *water*, add 10 ml *triethylamine* adjusted to pH 3.5 with *dilute orthophosphoric acid* and dilute to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent and resolution factor should not be less than 3.0.

Inject reference solutions (a), (b) and the test solution.

Calculate the content of alkaloids of belladonna as sum of atropine and hyoscine.

1.0 mg atropine sulphate contains 0.8551 mg of atropine and 1.0 mg hyoscine hydrobromide contains 0.7894 mg of hyoscine.

Storage. Store protected from light, at temperature not exceeding 30°.

Labeling. The quantity of active ingredient is stated in terms of belladonna dry extract equivalent to alkaloids of belladonna.

Belladonna Soft Extract

Belladonna Soft Extract is obtained from dried Belladonna leaf and root of one or more of the cultivated varieties of *Atropa bella-donna* Linn. or *A. acuminata* Royle ex Lindley (Fam. Solanaceae) or a mixture of both species by extraction with a suitable solvent using an appropriate procedure.

Belladonna soft extract contains not less than 90.0 per cent and not more than 110.0 per cent of stated amount of total alkaloids of Belladonna calculated as sum of *atropine* and *hyoscine.*

Category. Anticholinergic.

Usual strength. 1.0 per cent.

Description. Greenish brown soft extract.

Identification

A. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 3 volumes of strong *ammonia* solution, 7 volumes of *water* and 90 volumes of *acetone.*

Test solution. To 0.5 g of the soft extract under examination, add 15 ml of *0.05 M sulphuric acid* and add 1 ml of *strong ammonia* solution. Extract with two quantities each of 10 ml of *peroxide* free *ether.* Separate the ether layer by centrifugation if necessary dry the combined ether extracts over *anhydrous sodium sulphate*, filter and evaporate to dryness on a water bath. Dissolve the residue in 0.5 ml of *methanol.*

Reference solution. Dissolve 50 mg of *atropine sulphate IPRS* in 9 ml of *methanol* (solution A). Dissolve 15 mg of *hyoscine hydrobromide IPRS* in 10 ml of *methanol* (solution B). Mix 1.8 ml of *hyoscine hydrobromide* solution (solution B) and 8 ml of the *atropine sulphate* solution (solution A).

Apply to the plate 20 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 12 cm. Dry the plate at 105° for 15 minutes, allow to cool and examine under ultraviolet light at 254 nm and 366 nm, spray with 10 ml of *modified potassium iodobismuthate* solution until the bands become visible as orange or brown on a yellow background. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Transfer an accurately weighed quantity of the substance under examination, equivalent to 5 mg of total alkaloids, into a 50 ml volumetric flask. Add about 40 ml *0.05 M sulphuric acid* and sonicate for about 30 minutes. Make volume with *0.05 M sulphuric acid*, mix and filter.

Reference solution (a). A solution containing a 0.012 per cent w/v of *atropine sulphate IPRS* and 0.0014 per cent w/v of *hyoscine hydrobromide IPRS* in *0.05 M sulphuric acid.*

Reference solution (b). A solution containing 0.0014 per cent w/v of *hyoscine hydrobromide IPRS* in *0.05 M sulphuric acid*, for peak identification.

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Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous *silica* (5 µm),
- mobile phase: a mixture of 13 volumes of *acetonitrile* and 87 volumes of *buffer* (pH 3.5), prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 900 ml water, add 10 ml *triethylamine*, adjusted to pH 3.5 with dilute *orthophosphoric acid* and diluting it to 1000 ml with *water*.
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent and resolution factor should not be less than 3.0.

Inject reference solutions (a), (b) and the test solution.

Calculate the contents of atropine and hyoscine.

1mg *atropine sulphate* contains 0.8551 mg of *atropine* and 1mg *hyoscine hydrobromide* contains 0.7894 mg of *hyoscine*.

Storage. Store in wide mouthed tightly closed containers, protected from light and moisture.

Belladonna Tincture

Belladonna Tincture obtained from *Belladonna leaf* or roots of one or more of the cultivated varieties of *Atropa belladonna* Linn. or *A. acuminata* Royle ex Lindley (Fam. Solanaceae) or a mixture of both species.

Belladonna Tincture contains not less than 90 per cent w/w and not more than 110 per cent w/w of total alkaloids, calculated as hyoscyamine, $C_{17}H_{23}NO_3$.

Category. Anticholinergic.

Usual strength. 0.03 per cent w/w.

Description. A clear green or brownish green liquid.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 10 volumes of *anhydrous formic acid*, 10 volumes of *water*, 30 volumes of *methyl ethyl ketone* and 50 volumes of *ethyl acetate*.

Test solution. Evaporate 10 ml of the tincture under examination in a water-bath at 40° under reduced pressure. Dissolve the residue in 1.0 ml of the *methanol*.

Reference solution. Dissolve 1 mg of *chlorogenic acid IPRS* and 2.5 mg of *rutin IPRS* in 10 ml of the *methanol*.

Apply to the plate 40 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 15 cm. Dry the plate in air, heat the plate at 110° for 10 minutes. Spray the warm plate with 1 per cent v/v solution of *diphenylboric acid aminoethyl ester* in *methanol*. Allow to cool and spray with 5 per cent v/v solution of *macrogol 400* in *methanol*, allow the plate to dry in air for 30 minutes and examine under ultraviolet light at 254 nm and 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

B. **Atropine**. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 3 volumes of *strong ammonia solution*, 7 volumes of *water* and 90 volumes of *acetone*.

Test solution. To 15.0 ml of the tincture under examination, add 15 ml of *0.05 M sulphuric acid* and filter. Add 1 ml of *strong ammonia solution* to the filtrate, with two quantities, each of 10 ml, of *peroxide-free ether*. Separate the ether layer by centrifugation if necessary, dry the combined ether extracts over *anhydrous sodium sulphate*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 ml of *methanol*.

Reference solution. Dissolve 50 mg of *hyoscyamine sulphate IPRS* in 9 ml of *methanol* and 15 mg of *hyoscine hydrobromide IPRS* in 10 ml of *methanol*, separately. Mix 1.8 ml of the *hyoscine hydrobromide solution* and 8 ml of the *hyoscyamine sulphate solution*.

Apply to the plate 20 µl and 40 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 10 cm. Dry the plate at 105° for 15 minutes, spray with *potassium iodobismuthate solution*, dry the plate and spray with *sodium nitrite solution* until the plate is transparent. Examine the plate after 15 minutes in day light. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution.

Tests

Ethanol (2.3.45). 64 to 69 per cent v/v by Method III.

Assay. Evaporate about 50 g of the tincture under examination to a volume of about 10 ml. Transfer quantitatively to a separating funnel, with the minimum volume of *ethanol (70 per cent v/v)*. Add 5 ml of strong *ammonia solution* and 15 ml of *water*. Extract with three quantities, each of 40 ml of a mixture of 1 volume of *dichloromethane* and 3 volumes of *peroxide-free ether*, carefully to avoid emulsion, until the alkaloids are completely extracted. Combine the *dichloromethane* and *ether* extracts, concentrate the solution to a volume of about 50 ml by heating on a water-bath. Transfer the resulting solution quantitatively to a separating funnel, rinsing with *peroxide-free ether*. Add a quantity of *peroxide-free ether* equal to at least 2.1 times the volume of the solution

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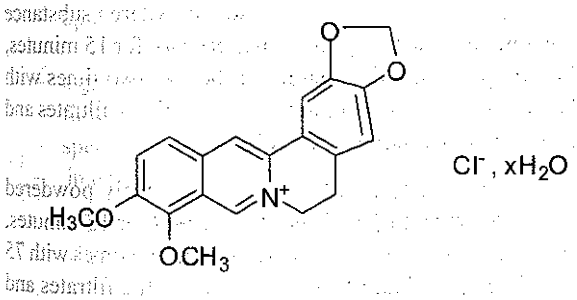
to produce a layer having a density well below that of water. Extract the resulting solution with minimum of three quantities, each of 20 ml of *0.25 M sulphuric acid* until the alkaloids are completely extracted. Separate the layers by centrifugation if necessary and transfer the layers to a separating funnel. Make the combined layers alkaline with *strong ammonia solution* and extract with minimum of three quantities, each of 30 ml of *dichloromethane* until the alkaloids are completely extracted. Combine the *dichloromethane* and *ether* extracts, add 4 g of *anhydrous sodium sulphate* and allow to stand for 30 minutes with occasional shaking. Decant the *dichloromethane* and filter. Wash the *sodium sulphate* with three quantities, each of 10 ml of *dichloromethane*. Combine the *dichloromethane* and *ether* extracts, evaporate to dryness on a water-bath. Heat the residue in an oven at 105° for 15 minutes. Dissolve the residue in a few ml of *dichloromethane*, evaporate to dryness on a water-bath and heat the residue in an oven at 105° for 15 minutes again. Dissolve the residue in a few ml of *dichloromethane*. Add 20.0 ml of *0.01 M sulphuric acid* and remove the *dichloromethane* by evaporation on a water-bath. Titrate the excess of acid with *0.02 M sodium hydroxide* using *methyl red mixed solution* as indicator.

1 ml of *0.01 M sulphuric acid* is equivalent to 0.005788 g of total alkaloids calculated as hyoscyamine.

Calculate the content of total alkaloids with reference to the dried material.

Berberine Chloride



$C_{20}H_{18}ClNO_4, xH_2O$ Mol. Wt. 392.47

Berberine Chloride is 5,6-dihydro-9,10 dimethoxy-2,3-methylene-dioxydibenzo [a,g]-quindizimium chloride hydrate.

Berberine Chloride contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of $C_{20}H_{18}ClNO_4$, calculated with reference to the anhydrous substance.

Category. Bitter stomachic, anti-bacterial.

Dose. 0.1 g as a single dose.

Description. A yellow crystals or crystalline powder, odourless or almost odourless bitter in taste.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

B. Determine by infrared absorption spectrophotometry (2.4.6). Compare the spectrum with that obtained with *berberine chloride IPRS* or with reference spectrum of *berberine chloride*.

C. Dissolve 0.1 g of the sample in 20.0 ml of *water* by warming; add 0.5 ml of *nitric acid*. Cool, set aside for ten minutes and filter. To 3.0 ml of the filtrate add 1 ml of *silver nitrate solution* and filter. The precipitate dissolves in an excess of *dilute ammonia solution*.

Tests

Clarity of solution. Dissolve 0.1 g in 50.0 ml of *water* by warming on a water-bath. The resulting solution is clear.

Acidity. Shake about 0.1 g with 30.0 ml of *water* and filter. To the filtrate add two drops of *phenolphthalein solution* and titrate with *0.1 M sodium hydroxide* upto red colour is obtained, not more than 0.1 ml required.

Sulphate (2.3.17). Not more than 0.048 per cent.

Test solution. Weigh accurately 1.0 g of *berberine chloride* add 48 ml *water* and 2 ml *dilute hydrochloric acid* and shake for 1 minute then filter. Discard the first 5 ml of the filtrate, take the subsequent 25 ml of the filtrate and dilute to 50.0 ml with *water*.

Reference solution. To 0.50 ml of *0.005 M sulphuric acid*, add 1 ml of *dilute hydrochloric acid*, 5 to 10 drops of *bromophenol blue*, dilute to 50.0 ml with *water*.

Add 2 ml solution of *barium chloride* to each of the test and standard solution, mix well and allow to stand for 10 minutes. Compare the turbidity produced in both solutions against a black background by viewing downward or transversely. The turbidity produced in the test solution is not than that of the reference solution.

Heavy metals (2.3.13). 0.67 g complies with the limit test for heavy metals, Method B (30 ppm).

Sulphated ash (2.3.18). Not more than 0.1 per cent.

Water (2.3.43). Not more than 16.5 per cent.

Related substances. Determine by liquid chromatography (2.4.14)

Test solution. Dissolve 10 mg of sample under examination in mobile phase and dilute to 100.0 ml with mobile phase.

Reference solution. Dilute 2.0 ml of the test solution to 50.0 ml with mobile phase.

Use chromatographic system as described under Assay.

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Inject the reference solution and the test solution. In the chromatographic obtained with the test solution, the sum of the area of all the secondary peaks is not more than the area of the peak in the chromatogram obtained with reference solution. (4.0 per cent)

Assay. Determine by liquid chromatography (2.4.14)

Test solution. Dissolve 10 mg of sample under examination in mobile phase and dilute to 100.0 ml with mobile phase.

Reference solution. Dissolve 10 mg *berberine chloride IPRS* in 100.0 ml mobile phase.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5µm),
- mobile phase: dissolve 3.4 g of *potassium dihydrogen orthophosphate* in 500 ml of *water*. Add 1.7 g of *sodium dodecyl sulphate* in 500 ml *acetonitrile* and mix,
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 345 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injection is not more than 2.0 per cent.

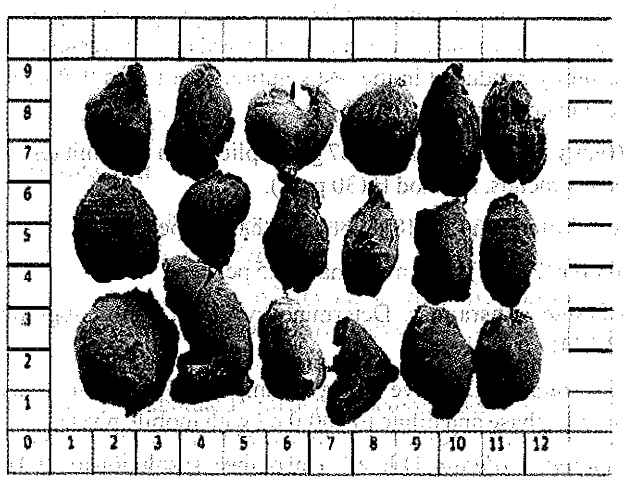
Inject the reference solution and the test solution.

Calculate the content of $C_{20}H_{18}ClNO_4$ in the sample.

Storage. Store protected from light and moisture, at a temperature not exceeding 30°.

Bhibhitaki

Bahera; Baheda; Belliric Myrobalan; *Terminalia bellirica*



Bhibhitaki consists of the dried fruit pericarp of *Terminalia bellirica* (Gaertn.) Roxb. (Fam. Combretaceae).

Bhibhitaki contains not less than 0.3 per cent w/w of ellagic acid and 0.75 per cent w/w of gallic acid, calculated on the dried basis.

Category. Purgative, expectorant, antihyperlipidaemic, svarabheda.

Description. The dried pericarp appears as curved pieces of irregular shapes, the external surface is velvety, wrinkled grey to brown in colour and has astringent taste.

Identification

A. *Macroscopic* — The dried pericarp of the ripe fruit occurs as curved pieces of irregular shapes with convex external surface. The external surface appears velvety, slightly wrinkled grey to brown in colour. Internal surface is pale yellow. The cut surface is with occasional projecting threads, representing the vascular bundles.

B. *Microscopic* — The cells of the epidermis has a characteristic and a slightly bulged based with a hair like prolongation. Several vascular strands traverse the mesocarp in various directions. Peripheral layers of mesocarp have tangentially elongated cells, devoid of starch grains, containing rosettes of calcium oxalate crystals and few small stone cells.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 20 volumes of *toluene*, 45 volumes of *ethyl acetate* and 20 volumes of *glacial acetic acid* and 5 volumes of *formic acid*.

Test solution. Reflux 2 g of the coarsely powdered substance under examination with 50-75 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further for two times with 75 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 50 ml.

Reference solution. Reflux 0.4 g of the coarsely powdered *bhibhitaki IPBRS* with 50-75 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further for two times with 75 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 100° for 5-10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

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Ethanol-soluble extractive (2.6.2). Not less than 25.0 per cent.

Water-soluble extractive (2.6.3). Not less than 35.0 per cent by Method I.

Total ash (2.3.19). Not more than 8.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh 0.5 g of coarsely powdered substance under examination, add 50 ml of *water*, sonicate for 3 minutes and heat on a boiling water bath for 15 minutes, cool and dilute to 100.0 ml with *water* and filter.

Reference solution. A solution containing 0.01 per cent w/v each of *gallic acid IPRS* and *ellagic acid IPRS* in *water.*

NOTE — Use freshly prepared solution and protected from light.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a gradient mixtures of *acetonitrile* and a buffer solution prepared by dissolving 0.136 g of *potassium di-hydrogen orthophosphate* in 500 ml of *water*, add 0.5 ml of *orthophosphoric acid* and make upto 1000 ml with *water,*

B. *acetonitrile*

- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
18	65	35
25	45	55
30	95	5

Inject the reference solution. The relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of gallic acid and ellagic acid.

Storage. Store protected from light, heat, moisture and against attack by insects and rodents.

Bhibhitaki Aqueous Extract

Bhibhitaki Aqueous Extract is obtained by extracting Bhibhitaki (*Terminalia bellirica* (Gaertn.) Roxb., Fam. Combretaceae) fruit with *water.*

Bhibhitaki Aqueous Extract contains not less than 90.0 per cent w/w and not more than 120.0 per cent w/w of the stated amount of gallic acid and ellagic acid.

Category. Purgative, expectorant, antihyperlipidaemic, svarabheda.

Usual strength. 7.0 per cent w/w.

Description. A light brown to dark brown powder.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 35 volumes of *benzene*, 10 volumes of *methanol*, 4 volumes of *acetone* and 1 volume of *water.*

Test solution. Dissolve 0.5 g of the extract under examination in 20 ml of *methanol* under reflux at 80° on a water bath and filter.

Reference solution. A 0.01 per cent w/v solution of *gallic acid IPRS* in *methanol.*

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Acid insoluble ash (2.3.19). Not more than 4.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss of drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 0.2 g of the extract under examination or a quantity containing 10 mg of *gallic acid* and 1 mg of *ellagic acid* in 80 ml of *methanol* and dilute to 100.0 ml with *water* and filter.

Reference solution (a). A 0.01 per cent w/v solution of *gallic acid IPRS* in 80 per cent v/v *methanol* in *water.*

Reference solution (b). A 0.001 per cent w/v solution of *ellagic acid IPRS* in 80 per cent v/v *methanol* in *water.*

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Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A mixture of *acetonitrile* and a 0.1 per cent v/v *orthophosphoric acid* in water,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 252 nm for *ellagic acid* and at 271 nm for *gallic acid*,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
8	95	5
25	0	100
30	0	100
32	95	5

Inject reference solutions (a) and (b). The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

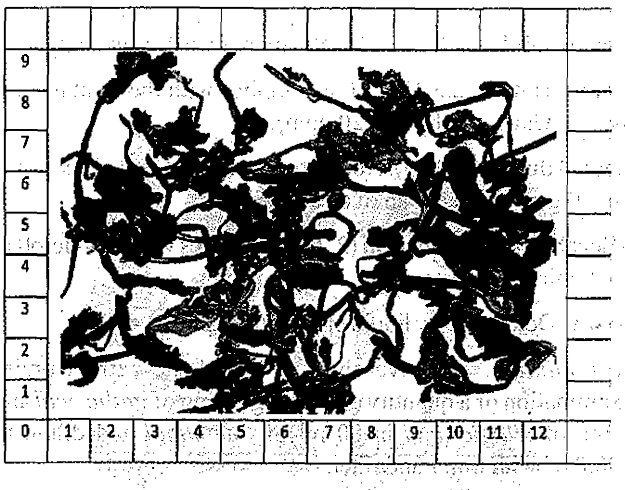
Inject reference solutions (a), (b) and the test solution.

Calculate the content of gallic acid and ellagic acid in the extract

Storage. Store protected from heat and moisture.

Bhringraj

Eclipta prostrata



Bhringraj consists of the dried whole plant of *Eclipta prostrata* Linn. (Fam. Asteraceae).

Bhringraj contains not less than 0.1 per cent of wedelolactone, calculated on the dried basis.

Category. Hepatoprotective, cardiotonic, aphrodisiac, in alopecia, yakrdroga.

Description. A green to greenish brown colour when completely dry.

Identification

A. *Macroscopic* — Root. Well developed, a number of secondary branches arise from main root up to about 7 mm in diameter, cylindrical, greyish.

Stem. Herbaceous, branched occasionally rooting at nodes, cylindrical or flat, rough due to appressed white hairs, node distinct, greenish, occasionally brownish.

Leaf. Opposite, sessile to sub sessile, usually oblong, lanceolate, sub-entire, sub-acute or acute, strigose with appressed hairs on both surfaces.

Flower. 1 or 2, together on unequal axillary peduncles, involucral bracts about 8, ovate, obtuse or acute, herbaceous, strigose with appressed hairs; ray flowers ligulate, ligule small, spreading, scarcely as long as bracts, not toothed; white disc flowers tubular, corolla often 4 toothed; pappus absent, except occasionally very minute teeth on the top of achene; stamen 5, filaments epipetalous, free, anthers united into a tube with base obtuse; pistil bicarpellary; ovary inferior; unilocular with one basal ovule.

Fruit. Achenial cypsella, one seeded, cuneate, with a narrow wing, covered with warty excrescences, brown.

Seed. Dark brown, hairy and non endospermic.

B. *Microscopic* — Powder. Dark green; shows vessels in large groups or single broken pieces with pitted walls, numerous fibres entire or in pieces, trichomes entire or in pieces, warty, a few attached with epidermal and subsidiary cells, anomocytic and anisocytic stomata.

Root. The cells of outer one or two rows of secondary cortex, elongated or rounded with air cavities, while cells of inner secondary cortex, elongated to irregular in shape. Stone cells scattered in secondary cortex. Phloem rays broader towards the periphery, cells rounded. Xylem rays distinct, run straight in tangential section, rarely uniseriate and biseriate, cells pitted.

Stem. A few epidermal cells elongate to form characteristic non-glandular trichomes. Secondary cortex composed of large, rounded parenchymatous cells having wide air space. Vascular bundle in a ring, collateral, endarch, of varying size. Vessels barrel-shaped, some elongated with simple perforations, pitted with spiral thickening. A few xylem fibres bifurcate. Xylem rays uniseriate or biseriate.

Leaf. Anomocytic and anisocytic stomata and non-glandular hairs are present on both surface, more abundant on lower side. Vascular bundle, fine in mid rib, central one largest while four other small flanking either side of central bundle.

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C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 9 volumes of *toluene*, 6 volumes of *acetone* and 1 volume of *formic acid*.

Test solution. Reflux 1 g of the coarsely powdered substance under examination with 25 ml of *methanol* for 30 minutes, cool and filter. Reflux the residue further with 3 x 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Reference solution. Reflux 0.5 g *bhringraj IPBRS* with 25 ml of *methanol* for 30 minutes, cool and filter. Reflux the residue further with 3 x 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 100° for 5-10 minutes and examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2 .0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 5.0 per cent.

Water- soluble extractive (2.6.3). Not less than 15.0 per cent by Method I.

Total ash (2.3.19). Not more than 22.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 11.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 15.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 5 g of the coarsely powdered substance under examination with 30 ml of *methanol* on a water-bath for 30 minutes, cool and filter. Reflux the residue further with *methanol* till the last extract turns colourless, cool and filter. Combine all the filtrates and concentrate to 100 ml.

Reference solution. 0.01 per cent w/v solution of *wedelolactone IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: 35 volumes of *acetonitrile* and 60 volumes of 0.1 per cent v/v solution of *orthophosphoric acid* prepared by diluting 1ml of *orthophosphoric acid* to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 249 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of wedelolactone.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Bhuiamla Dry Extract

Phyllanthus amarus Schum. and Thonn.

Bhuiamla Dry Extract is obtained by extracting Bhuiamla with *aqueous ethanol* or *ethanol* or any other suitable solvent and evaporation of solvent.

Bhuiamla Dry Extract contains not less than 90.0 per cent w/w and not more than 120.0 per cent w/w of the stated amount of sum of phyllanthin and hypophyllanthin, calculated on the dried basis. It may contain suitable added substances.

Category. Antiviral, hepatoprotective, antidiabetic, kasaaya.

Description. A greenish brown to green powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 2 volumes of *toluene* and 1 volume of *ethyl acetate*.

Test solution. Dissolve 500 mg of the extract under examination with 50 ml *methanol* and filter.

Reference solution. A solution containing 0.05 per cent w/v each of *phyllanthin IPRS* and *hypophyllanthin IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *methanolic sulphuric acid* (10.0 per cent, v/v). Heat the plate at 120° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

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Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 5.0 g by drying in oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 500 mg of the extract under examination in *methanol* by gently heating, cool and make up the volume to 100 ml and filter.

Reference solution (a). A 0.05 per cent w/v solution of *phyllanthin IPRS* in *methanol*.

Reference solution (b). A 0.05 per cent w/v solution of *hypophyllanthin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 65 volumes of *methanol* and 35 volumes of *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 230 nm,
- injection volume: 20 µl.

Inject reference solutions (a) and (b). The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

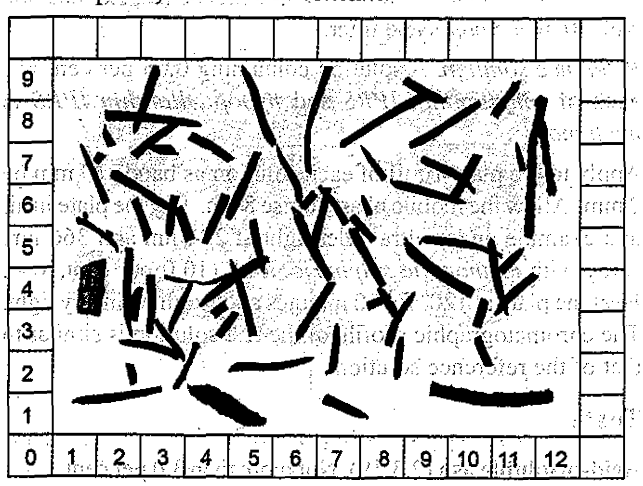
Inject reference solutions (a), (b) and the test solution.

Calculate the contents of phyllanthin and hypophyllanthin.

Storage. Store protected from heat, moisture and against attack by insects and rodents

Birmi

Chalisa patra; *Taxus wallichiana*



Birmi consists of dried leaves of *Taxus wallichiana* Zucc. (Fam. Taxaceae).

Birmi contains not less than 0.04 per cent of paclitaxel, calculated on the dried basis.

Category. Anticancer, analgesic, anti inflammatory, anti convulsant and anti pyretic.

Description. Dorsal surface of leaf is greenish brown and ventral surface is greenish yellow in colour; odour is characteristic and agreeable; taste astringent and bitter.

Identification

A. *Macroscopic* — Leaves are entire or broken pieces of needles; which are simple linear and narrow, 1.5 to 4.0 cm in length and 1.5 to 2.0 cm in breadth. Margin is entire or recurved, base twisted narrowing into a short petiole, apex acute and midrib prominent on upper side.

B. *Microscopic* — Transverse section of the needle shows dorsiventral lamina; upper epidermis consisting of polygonal tubular cells filled with dark brown contents and covered with thick striated cuticle; lower epidermis of smaller cells with dark brown contents and papillose cuticle; bilayered palisade continuing over the midrib with more developed cells in the first row; meristele consisting of radiate xylem, phloem and an arc of compact sclerenchymatous tissue over xylem encircled by a parenchymatous layer containing dark brown contents; loosly packed spongy mesophyll containing air cavities and cells filled with brown contents.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile pahse. A mixture of 4 volumes of *n-butanol*, 1 volume of *water* and 5 volumes of *acetic acid*.

Test solution. Reflux 2 g of coarsely powdered substance under examination with 50 ml of *methanol* for 10 minutes. Cool and filter. Concentrate to 10 ml.

Reference solution. Reflux about 2 g of *birmi IPBRS* with 50 ml of *methanol* for 10 minutes. Cool and filter. Concentrate under vacuum to 10 ml. Take 1 ml of the concentrate and dilute with *methanol* up to 10 ml.

Apply to the plate 10 µl of both test and reference solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. Spray with the *anisaldehyde sulphuric acid reagent*. Heat the plate at 110° for 10 minutes. Examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 11.0 per cent.

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Water-soluble extractive (2.6.3). Not less than 17.0 per cent.

Total ash (2.3.19). Not more than 4.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.7 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 7.0 per cent determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. To 5 g of coarsely powdered substance under examination, add 50 ml of *methanol* and macerate for 24 hours, filter and evaporate to dryness and dissolve in 50 ml of *methanol*.

Reference solution. A 0.1 per cent w/v solution of *paclitaxel IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 35°,
- mobile phase: a mixture of 11 volumes of *water* and 9 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 227 nm,
- injection volume: 10 µl.

Inject the reference solution. The relative standard deviation for the replicate injection is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of paclitaxel.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Black Pepper Oil

Kali Mirchi ka tail

Black Pepper Oil is the essential oil obtained by steam distillation of the dried unripe berries of *Piper nigrum* Linn.(Fam. Piperaceae).

Black Pepper Oil contains not less than 10.0 per cent and not more than 17.0 per cent of limonene, and not less than 12.0 per cent and not more than 29.0 per cent of β-caryophyllene.

Description. A clear, colourless to bluish green liquid, free from sediment and suspended matter.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *black pepper oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described in the Assay.

The chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 46.0° to 50.0°.

Tests

Relative density (2.4.29). 0.864 to 0.880.

Refractive index (2.4.27). 1.478 to 1.487.

Optical rotation (2.4.22). –18.0° to –7.0°.

Acid value (2.3.23). Not more than 1.0.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *limonene IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *β-caryophyllene IPRS* in *ethanol (95 per cent)*.

Chromatographic system
- a capillary column 30 m x 0.25 mm x 0.25 µm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute, and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio : 1 : 25.

Inject reference solutions (a), (b) and the test solution.

Calculate the content of limonene and β-caryophyllene in the oilusing area normalisation procedure.

Storage. Store protected from light and moisture.

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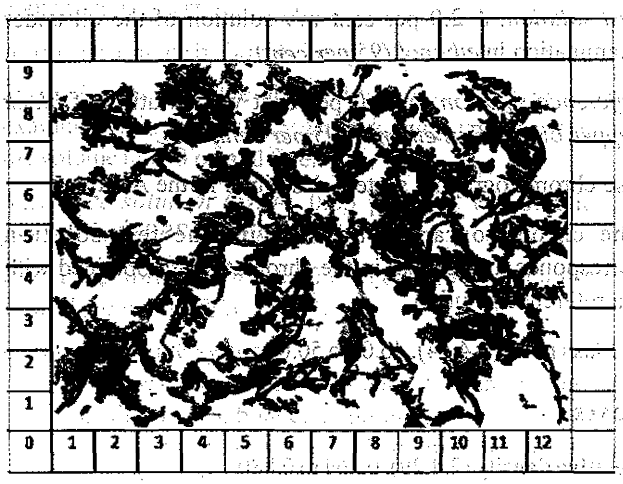
Brahmi

Bacopa monnieri



Brahmi consists of the dried whole plant, preferably leaves and stem of *Bacopa monnieri* (Linn.) Wettst. (Fam. Plantaginaceae).

Brahmi contains not less than 2.5 per cent w/w of bacoside A, calculated on the dried basis.

Category. Braintonic, antiinflammatory, adaptogenic, kustha.

Description. A brown to reddish brown colour when completely dried or green colour when partially dried with slightly bitter taste.

Identification

A. *Macroscopic* — Herbaceous comprising of stems, runner stems and leaves. Stems glabrous, leafless towards the base; internodes long. Leaves spatulate-obovate, sessile, and glabrous.

B. *Microscopic* — Cortex in stem composed of parenchyma cells enclosing large air spaces; xylem vessels radially arranged xylem rays uniseriate; pith parenchyma collapsed. In leaf, midrib indistinct, mesophyll isobilateral of spongy cells, a few prismatic crystals of calcium oxalate in mesophyll; stomata anomocytic on both the surfaces of leaf.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *chloroform* and 30 volumes of *methanol*.

Test solution. Reflux 2 g of coarsely powdered substance under examination with 25 ml *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 x 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 25 ml.

Reference solution. Reflux 0.4 g of coarsely powdered *brahmi IPBRS* with 5 ml of *methanol* for 15 minutes, cool and filter.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *methanolic sulphuric acid* (20 per cent v/v). Heat the plate at 100° for 5-10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

D. In the Assay, the chromatogram obtained with test solution corresponds to the chromatogram obtained with reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 6.0 per cent.

Water-soluble extractive (2.6.3). Not less than 22.0 per cent by Method I.

Total ash (2.3.19). Not more than 18.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 6.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 2 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water bath for 15 minutes, cool and filter. Reflux the residue further with *methanol* till the last extract turns colorless, cool and filter. Combine all the filtrates and concentrate to 100.0 ml.

Reference solution. A 0.2 per cent w/v solution of *bacoside A IPRS* in *methanol*, prepared by heating gently on a water bath.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of *acetonitrile* and a buffer solution prepared by dissolving 0.5 g *phosphoric acid* in 800 ml of *water*, adjusted to pH 2.8 with *dilute phosphoric acid* and dilute to 1000 ml with *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

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Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
25	60	40
35	40	60
36	70	30
45	70	30

Inject the reference solution. The relative retention times are 1.00 for bacoside A3, about 1.04 for bacopaside II, 1.13 for jujubogenin isomer of bacopasaponine C and 1.19 for bacopasaponine C as bacoside A. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of bacoside A.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Brahmi Extract

Brahmi Extract is obtained by extracting Brahmi (*Bacopa monnieri* (Linn.) Wettst., Fam. Plantaginaceae) from the dried leaves and stems of with aqueous ethanol or any other suitable solvent.

Brahmi Extract contains not less than 90.0 per cent w/w and not more than 120.0 percent w/w of the stated amount of bacoside-A (sum of bacoside-A_3, bacopaside-II, bacopasaponin-C, jujubogenin isomer of bacopasaponin-C).

Category. Braintonic, antiinflammatory, adaptogenic, kustha.

Usual strengths. 10 per cent w/w; 20 per cent w/w.

Description. Light green to dark green powder with characteristic odour and bitter taste.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 7 volumes of *Ethyl acetate,* 2 volumes of *methanol* and 1 volume of *water.*

Test solution. Shake well 0.5 g of the extract under examination with 50 ml *methanol* and filter.

Reference solution. A 0.1 per cent w/v solution of *bacoside-A IPRS* in *methanol.*

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. Spray the plate with *vanillin sulphuric acid* solution. Heat the plate at 60° to70° for 10 minutes and examine the plate at 366 nm and in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 2.0 g by drying in an oven at 105° for 3 hours.

Total ash (2.3.19). Not more than 5 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, (Method B) 20 ppm.

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 0.5 g of the extract in *methanol* by gentle heating, dilute to 100 ml and filter.

Reference solution. A 0.1 per cent w/v solution of *bacoside-A IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of *acetonitrile* and a buffer solution prepared by dissolving 0.5 g *phosphoric acid* in 800 ml of *water*, adjusted to pH 2.8 with *dilute phosphoric acid* and dilute to 1000 ml with *water*,

  B. *acetonitrile*
- a gradient programme using the conditions given below,
- flow rate 1.5 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

Time (in min)	mobile phase A (per cent v/v)	mobile phase B (per cent v/v)
0	70	30
25	60	40
35	40	60
36	70	30
45	70	30

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent. The relative retention time with reference to bacoside-A_3 for Bacopaside II is about 1.04, for jujubogenin isomer of bacopasaponin C is about 1.13 and for bacopasaponin C is about 1.19.

Inject the reference solution and the test solution.

Calculate the content of bacoside-A.

Storage. Store protected from heat and moisture.

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Caraway Oil

Shah Jeera ka tail

Caraway Oil is the essential oil obtained by steam distillation of the ripe fruits (dried and crushed) of *Carum carvi* L. (Fam. Umbelliferae).

Caraway Oil contains not less than 35.0 per cent and not more than 45.0 per cent of limonene and not less than 48.0 per cent and not more than 60.0 per cent of carvone.

Description. A clear, colourless or amber yellow liquid, free from sediment and suspended matter.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

Reference solution. A 2.0 per cent w/v solution of *caraway oil IPRS* in *ethanol (95 per cent).*

Use chromatographic system described in the Assay.

The chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 54.0° to 58.0°.

Tests

Relative density (2.4.29). 0.901 to 0.920.

Refractive index (2.4.27). 1.484 to 1.489.

Optical rotation (2.4.22). +67.0° to +80.0°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

Reference solution (a). A 2.0 per cent w/v solution of *limonene IPRS* in *ethanol (95 per cent).*

Reference solution (b) A 2.0 per cent w/v solution of *carvone IPRS* in *ethanol (95 per cent).*

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio : 1 : 25.

Inject reference solutions (a), (b) and the test solution.

Calculate the content of limonene, and carvone in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Cardamom Oil

Elaichi ka tail

Cardamom oil is the essential oil obtained by steam distillation of the fruits *Elletaria cadamomum* (Fam. Zingiberaceae).

Cardamom oil contains not less than 23.0 per cent and not more than 33.0 per cent of 1,8-cineole, and not less than 32.0 per cent and not more than 42.0 per cent of terpinyl acetate.

Description. A clear, colourless to pale yellow liquid, characteristic spicy and anise-like odour.

Identification

Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

Reference solution. A 2.0 per cent w/v solution of *cardamom oil IPRS* in *ethanol (95 per cent).*

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

Tests

Relative density (2.4.29). 0.919 to 0.936.

Refractive index (2.4.27). 1.462 to 1.468.

Optical rotation (2.4.22). +22.0° to + 44.0°.

Acid value (2.3.23). Not more than 1.0.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol* (95 per cent).

Reference solution (a). A 2.0 per cent w/v solution of *1, 8 - cineole IPRS* in ethanol (95 per cent).

Reference solution (b) A 2.0 per cent w/v solution of *terpinyl acetate IPRS* in ethanol (95 per cent).

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,

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– column temperature:
column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
– inlet port at 250° and detector at 280°,
– detection by flame ionization detector,
– flow rate 1 ml per minute using nitrogen as carrier gas,
– injection volume: 1.0 µl,
– split ratio : 1 : 25.

Inject reference solutions (a), (b) and the test solution.

Calculate the content of 1, 8 - cineole, and terpinyl acetate in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Cassia Oil

Cassia oil is the essential oil obtained by steam distillation of the leaves and young branches of *Cinnamomum cassia* Blume (*C. aromaticum* Nees) (Fam. Lauraceae).

Cassia oil contains not less than 70.0 per cent and not more than 90.0 per cent of *trans*-cinnamic aldehyde, not less than 1.0 per cent and not more than 6.0 per cent of cinnamyl acetate, not more than 0.5 per cent of eugenol, not less than 1.5 per cent and not more than 4.0 per cent coumarin, not less than 3.0 per cent and not more than 15 per cent of *trans*-2-methoxycinnamaldehyde.

Category. Antidiarrhoeal, antiviral and antibacterial.

Description. A clear, yellow or reddish brown liquid, characteristic odour reminiscent of cinnamic aldehyde.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *acetone*.

Reference solution. A 2.0 per cent w/v solution of *cassia oil IPRS* in *acetone*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). Not less than 90°.

Tests

Relative density (2.4.29). 1.052 to 1.070.

Refractive index (2.4.27). 1.600 to 1.614.

Optical rotation (2.4.22). - 1° to + 1°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *acetone*.

Reference solution. Dissolve 100 µl of *trans-cinnamic aldehyde IPRS*, 10 µl of *cinnamyl acetate IPRS*, 10 µl of *eugenol IPRS*, 20 mg of *coumarin IPRS* and 10 µl of *trans-2-methoxycinnamaldehyde IPRS* in 1 ml of *acetone*.

Chromatographic system
– a capillary column 60 m x 0.25 mm, coated with *macrogol 20000*,
– temperature:
column. 60° for 10 minute, increase from 60° to 190° at a rate of 2° per minute and maintain at this temperature for 85 minutes,
– inlet port at 200° and detector at 240°,
– detection by flame ionization detector,
– flow rate: 1.5 ml per minute using helium as carrier gas,
– injection volume: 0.2 µl.
– split ratio: 1:100

Inject the reference solutions and the test solution.

The test is not valid unless the resolution between the peaks corresponding to *coumarin* and *trans-2-methoxycinnamaldehyde* is at least 1.5.

Calculate the content of *trans*-cinnamic aldehyde, cinnamyl acetate, eugenol, coumarin and *trans*-2-methoxycinnamaldehyde in the oil using area normalization procedure.

Storage. Store protected from light and moisture.

Castor Oil (Refined grade)

Arandi ka tail

Castor Oil is the fixed oil obtained by cold expression from the seeds of *Ricinus communis* Linn. (Fam. Euphorbiaceae). It is then refined. It may contain suitable antioxidants.

Category. Irritant puragative, antirheumatic, amavata.

Description. A pale yellowish or almost colourless, transparent, viscid liquid; odour, slight and characteristic.

Tests

Light absorption (2.4.7). Absorbance of 1.0 per cent w/v solution in *ethanol* (95 per cent) at the maximum at about 270 nm is between 0.7 and 1.5.

Weight per ml (2.4.29). 0.945 g to 0.965 g.

Refractive index (2.4.27). 1.476 to 1.480.

Optical rotation (2.4.22). + 3.5° to + 6.0°.

Peroxide value (2.3.35). Not more than 5.0.

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Acid value (2.3.23). Not more than 2.0.

Acetyl value (2.3.22). Not less than 143.

Hydroxyl value (2.3.27). Not less than 150.

Saponification value (2.3.37). 176 to 187.

Iodine value (2.3.28). 82 to 90.

Foreign fatty substances. A. A mixture of 2 ml of the substance under examination and 8 ml of *ethanol (95 per cent)* is clear.

B. Shake 10.0 ml with 20.0 ml of *light petroleum (60° to 80°)* and allow to separate; the volume of the lower layer is not less than 16.0 ml.

Storage. Store protected from moisture.

Labelling. The label states (1) the name and quantity of any added antioxidant; (2) whether the contents are suitable for use in the manufacture of parenteral preparations.

Castor Oil (Virgin grade)

Arandi ka tail

Castor Oil is the fixed oil obtained by cold expression from the seeds of *Ricinus communis* Linn. (Fam. Euphorbiaceae). It may contain suitable antioxidants.

Category. Irritant purgative, antirheumatic, amavata.

Description. A pale yellowish or almost colourless, transparent, viscid liquid; odour, slight and characteristic.

Tests

Light absorption (2.4.7). Absorbance of a 1.0 per cent w/v solution in *ethanol (95 per cent)* at the maximum at about 270 nm, not more than 0.7.

Weight per ml (2.4.29). 0.945 g to 0.965 g.

Refractive index (2.4.27). 1.476 to 1.480.

Optical rotation (2.4.22). +3.5° to +6.0°.

Peroxide value (2.3.35). Not more than 5.0.

Acid value (2.3.23). Not more than 2.0.

Acetyl value (2.3.22). Not less than 143.

Hydroxyl value (2.3.27). Not less than 150.

Saponification value (2.3.37). 176 to 187.

Iodine value (2.3.28). 82 to 90.

Foreign fatty substances. A. A mixture of 2 ml of the substance under examination and 8 ml of *ethanol (95 per cent)* is clear.

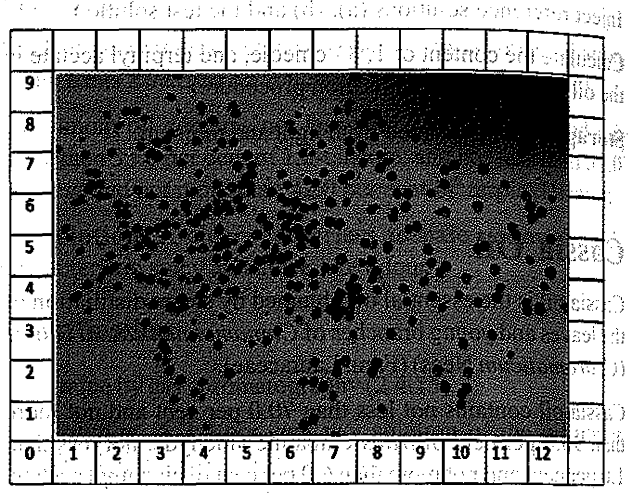
B. Shake 10.0 ml with 20.0 ml of *light petroleum (60° to 80°)* and allow to separate; the volume of the lower layer is not less than 16.0 ml.

Storage. Store protected from moisture.

Labelling. The label states (1) the name and quantity of any added antioxidant; (2) whether the contents are suitable for use in the manufacture of parenteral preparations.

Chaulai

Ramdana; *Amaranthus tricolor*



Chaulai consists of dried seeds of *Amaranthus tricolor* L. (Fam. Amaranthaceae). Chaulai seed contain not less than 2.0 per cent of squalene, calculated on the dried basis.

Category. Antioxidant, astringent, antipyretic and demulcent.

Description. Seeds are small, yellowish to brown colour.

Identification

A. *Macroscopic* — Seeds are white, golden, brown or red to black. Seed is lenticular and tremendously small (0.9 to 1.7 mm diameter).

B. *Microscopic* — Transverse section of the seed shows an outer epidermis of radially elongated rod like thickened cells, 2 to 3 rows of parenchymatous zone lie below this followed by a layer of inner epidermis with spiral-reticulate thickenings, wide zone of perisperm loaded with small sized starch grains is present.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel* GF_{254}.

Mobile phase. A mixture of 7 volumes of *hexane*, 2.4 volumes of *petroleum ether* and 0.5 volume of *diethyl ether* and 0.1 volume of *glacial acetic acid*.

Test solution. Macerate about 2.5 g of coarsely powdered substance under examination with 50 ml of *hexane* and filter the extract and macerate the residue further for 2 times with

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50 ml of *hexane* and filter. Combine all the filtrate and concentrate under vacuum upto 25.0 ml. Add 25 ml of *methanol* to the concentrate. Keep reflux under examination for 10 minutes, cool and filter. Reflux the residue with another 20 ml of *methanol*, cool and filter.

Reference solution. Macerate about 2.5 g of *chaulai IPBRS* powder under examination with 50 ml of *hexane* and filter the extract and macerate the residue further for 2 times with 50 ml of *hexane* and filter. Combine all the filtrate and concentrate under vacuum upto 25.0 ml. Add 25 ml of *methanol* to the concentrate. Keep reflux under examination for 10 minutes, cool and filter. Reflux the residue with another 20 ml of *methanol*, cool and filter.

Apply to the plate 10 µl of each solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with *iodine vapours* and examine the plate under day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 9.0 per cent.

Water-soluble extractive (2.6.3). Not less than 7.0 per cent.

Total ash (2.3.19). Not more than 5.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.5 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 10.0 per cent determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Macerate about 5.0 g of coarsely powdered substance under examination with 100 ml of *hexane* and filter the extract and macerate the residue further for 2 more times with 100 ml of *hexane* and filter. Combine all the filtrates and concentrate under vacuum to dryness. Dissolve the residue in 50 ml of *methanol*.

Reference solution. A 0.01 per cent w/v solution of *squalene IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: *acetonitrile*,
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

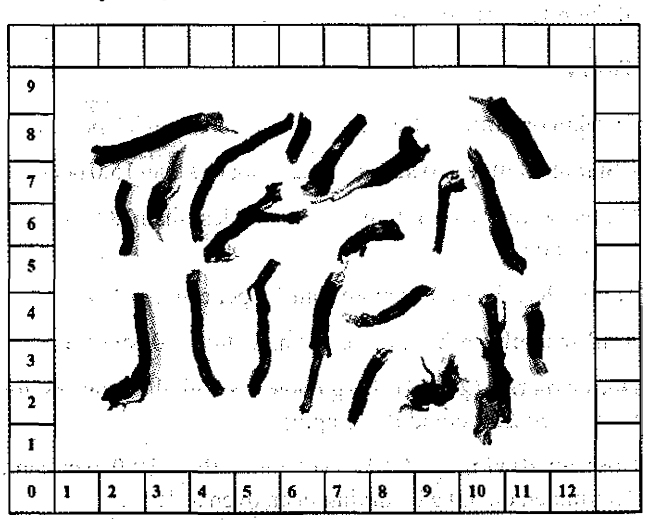
Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution. Calculate the content of squalene.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Chitrak

Plumbago zeylanica



Chitrak consists of dried roots of *Plumbago zeylanica* Linn. (Fam. Plumbaginaceae). Chitrak contains not less than 0.1 per cent w/w of plumbagin, 0.01 per cent w/w of lawsone and 0.001 per cent w/w of juglone calculated on the dried basis.

Category. Antibacterial, antidiabetic, cardiotonic, anti-convulasant, neuroprotective.

Description. Root is cylindrical, straight long, and sometime irregular with reddish brown colour and bitter taste.

Identification

A. *Macroscopic* — Root 30 cm or more in length, 6 mm or more in diameter, straight, unbranched or slightly branched with or without secondary root with uniform and smooth texture, stout, irregular sometimes regular.

B. *Microscopic* — Transverse section of root is circular in outline, cork narrow, central wood width 3-4 growth ring uncirculated by narrow phloem, cortex wider transverse with fibre, xylem vessels radially arranged, lignified medullary rays.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

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Mobile phase. A mixture of 7 volumes of *toluene* and 3 volumes of *ethyl acetate.*

Test solution. Reflux about 5.0 g coarsely powdered substance with 50 ml *methanol.* Reflux the residue further with 3 x 25 ml of *methanol,* cool and filter. Combine all the filtrates and concentrate under vacuum to 5.0 ml.

Reference solution. A 0.1 per cent w/v solution of *plumbagin IPRS, lawsone IPRS* and *juglone IPRS* in *methanol.*

Apply to the plate 10 il of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 15.0 per cent.

Water-soluble extractive (2.6.3). Not less than 18.0 per cent by Method I.

Total ash (2.3.19). Not more than 3.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 5.0 g coarsely powdered substance with 50 ml *methanol.* Reflux the residue further with 3 x 25 ml of *methanol,* cool and filter. Combine all the filtrates and concentrate under vacuum to 5.0 ml.

Reference solution. A solution containing 0.1 per cent w/v, each of, *plumbagin IPRS, lawsone IPRS* and *juglone IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A: 0.1 per cent v/v *glacial acetic acid* in *water,*
  B: 0.1 per cent v/v *glacial acetic acid* in *methanol,*
- a gradient programme using the condition given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 5 µl

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	20	80
22	30	70
28	20	80
30	20	80

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of plumbagin, lawsone and juglone calculated on the dried basis.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Cinnamon Bark Oil

Daalchini ka tail

Cinnamon Bark Oil is the essential oil obtained by steam distillation of the bark of the shoots of *Cinnamomum zeylanicum* Nees (Fam. Lauraceae)

Cinnamon Bark Oil contains not less than 1.0 per cent and not more than 6.0 per cent of linalool, not less than 55.0 per cent and not more than 75 per cent of trans-cinnamic aldehyde and not less than 1.0 per cent, not more than 4.0 per cent of β-caryophyllene, not more than 7.5 per cent eugenol, not more than 3.0 per cent of cineole, not more than 0.5 per cent of coumarin , not more than 0.5 per cent of safrole, not more than 1.0 per cent of benzyl benzoate and not less than 0.1 per cent and not more than 1.0 per cent of trans-2 methoxy cinnamaldehyde.

Category. Antiemetic, antidiarrhoeal, anti-inflammatory.

Description. A clear, pale yellow to amber liquid, free from sediment and suspended matter, characteristic odour, reminiscent of cinnamic aldehyde.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *acetone.*

Reference solution. A 2.0 per cent w/v solution of *cinnamon bark oil IPRS* in *acetone.*

Use chromatographic system described under Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). Not less than 70°.

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Tests

Relative density (2.4.29). 1.000 to 1.030.

Refractive index (2.4.27). 1.572 to 1.591.

Optical rotation (2.4.22). -2° to +1°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *acetone*.

Reference solution. Dissolve 10 µl of *linalool IPRS*, 100 µl of *trans-cinnamic aldehyde IPRS*, 10 µl of *β-caryophyllene IPRS*, 10 µl of *eugenol IPRS*, 10 µl of *cineole IPRS*, 20 mg of *coumarin IPRS*, 10 µl of *safrole IPRS*, 10 µl of *benzyl benzoate IPRS* and 10 µl of *trans-2-methoxycinnamaldehyde IPRS* in 1 ml of *acetone*.

Chromatographic system
- a capillary column 60 m x 0.25 mm, coated with *macrogol 20000*,
- temperature:
  column. 60° for 10 minutes, increase from 60° to 190° at a rate of 2° per minute and maintain at this temperature for 125 minutes,
- inlet port at 200° and detector at 240°,
- detection by flame ionization detector,
- flow rate: 1.5 ml per minute using helium as carrier gas,
- injection volume: 0.2 µl,
- split ratio: 1:100.

Inject the reference solution and the test solution.

Calculate the content of linalool, trans-cinnamic aldehyde, β-caryophyllene, eugenol, cineole, coumarin, safrole, benzyl benzoate and trans-2 methoxy cinnamaldehyde in the oil using area normalization procedure.

Storage. Store protected from light and moisture.

Cinnamon Leaf Oil

Daalchini Ka Tail

Cinnamon Leaf Oil is the essential oil obtained by steam distillation of the leaves of *Cinnamomum verum* J.S. Presl. (Fam. Lauraceae).

Cinnamon Leaf Oil contains not more than 1.0 per cent of cineole, not less than 1.5 per cent and not more than 3.5 per cent of linalool, not less than 1.5 per cent and not more than 7.0 per cent of β-caryophyllene, not more than 3.0 per cent of safrole, not more than 3.0 per cent of *trans*-cinnamic aldehyde, not more than 2.0 per cent of cinnamyl acetate, not less than 70 per cent not more than 85.0 per cent of eugenol and not more than 1.0 per cent of coumarin.

Category. Antiemetic, antidiarrhoeal, anti-inflammatory.

Description. A clear, pale yellow to amber liquid, free from sediment and suspended matter, characteristic odour, reminiscent of eugenol.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *acetone*.

Reference solution. A 2.0 per cent w/v solution of *cinnamon leaf oil IPRS* in *acetone*.

Use chromatographic system described under Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). Not less than 60°.

Tests

Relative density (2.4.29). 1.030 to 1.059.

Refractive index (2.4.27). 1.527 to 1.540.

Optical rotation (2.4.22). -2.5° to +2.0°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *acetone*.

Reference solution. Dissolve 10 µl of *cineole IPRS*, 10 µl of *linalool IPRS*, 10 µl of *β-caryophyllene IPRS*, 10 µl of *safrole IPRS*, 10 µl of *trans-cinnamic aldehyde IPRS*, 10 µl of *cinnamyl acetate IPRS*, 100 µl of *eugenol IPRS* and 10 mg of *coumarin IPRS* in 1 ml of *acetone*.

Chromatographic system
- a capillary column 60 m x 0.25 mm, coated with *macrogol 20000*,
- temperature:
  column. 60° for 10 minutes, increase from 60° to 190° at a rate of 2° per minute and maintain at this temperature for 125 minutes,
- inlet port at 200° and detector at 240°,
- detection by flame ionization detector,
- flow rate: 1.5 ml per minute using helium as carrier gas,
- injection volume: 0.2 µl,
- split ratio: 1:100.

Inject the reference solutions and the test solution.

Calculate the content of cineole, linalol, β-caryophyllene, Safrole, trans-cinnamic aldehyde, cinnamyl acetate, eugenol and coumarin in the oil using area normalization procedure.

Storage. Store protected from light and moisture.

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Citronella Oil (Geraniol Type)

Cymbopogon nardus

Citronella oil is essential oil obtained by steam distillation of fresh or partially dried aerial parts of *Cymbopogon nardus* (Fam. Cardiopteridaceae).

Citronella oil contains not less than 1.0 per cent and not more than 5.0 per cent of limonene, not more than 0.1 per cent citronellal, not more than 0.1 per cent citronellyl acetate, not less than 2.0 per cent and not more than 5.0 per cent of neral, not less than 2.0 per cent and not more than 5.0 per cent of geranial, not less than 5.0 per cent and not more than 10.0 per cent of geranyl acetate, not less than 1.0 per cent and not more than 5.0 per cent of citronellol and not less than 50.0 per cent of geraniol.

Category. Astringent, anti spasmodic, anti inflammatory.

Description. A clear, pale yellow to brownish yellow, mobile liquid, free from sediment and suspended matter. It has fresh, floral-rose type odour.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *citronella oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described under Assay.

The peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). Not more than 93.3°.

Tests

Relative density (2.4.29). 0.880 to 0.900.

Refractive index (2.4.27). 1.460 to 1.480.

Optical rotation (2.4.22) .15.0° to + 5.0°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *limonene IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *citronellal IPRS* in *ethanol (95 per cent)*.

Reference solution (c). A 2.0 per cent w/v solution of *citronellyl acetate IPRS* in *ethanol (95 per cent)*.

Reference solution (d). A 2.0 per cent w/v solution of *neral IPRS* in *ethanol (95 per cent)*.

Reference solution (e). A 2.0 per cent w/v solution of *geranial IPRS* in *ethanol (95 per cent)*.

Reference solution (f). A 2.0 per cent w/v solution of *geranyl acetate IPRS* in *ethanol (95 per cent)*.

Reference solution (g). A 2.0 per cent w/v solution of *citronellol IPRS* in *ethanol (95 per cent)*.

Reference solution (h). A 2.0 per cent w/v solution of *geraniol IPRS* in *ethanol (95 per cent)*.

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with fused silica 5 per cent phenylpolysiloxane,
- temperature:
  column. 60° for 1 minute, increase from 60° to 230° at a rate of 4° per minute, and maintain at this temperature for 10 minutes.
- inlet port at 230° and detector at 240°,
- detection by flame ionization detector,
- flow rate: 1 ml per minute using helium as carrier gas,
- injection volume: 0.5 µl,
- split ratio: 1: 200

Inject reference solutions (a), (b), (c), (d), (e), (f), (g), (h) and the test solution.

Calculate the content of limonene, citronellal, citronellal acetate, neral, geranial, geranyl acetate, citronellol, and geraniol in the oil using area normalization procedure.

Storage. Store protected from light and moisture.

Citronella Oil (Java Type)

Cymbopogon winterianus

Citronella oil is essential oil obtained by steam distillation of fresh or partially dried aerial parts of *Cymbopogon winterianus* (Fam. Poaceae).

Citronella oil contains not less than 1.0 per cent and not more than 5.0 per cent of limonene, not less than 30.0 per cent and not more than 45.0 per cent citronellal, not more than 3.0 per cent of citronellyl acetate, not more than 1.0 per cent of neral, not more than 1.5 per cent of geranial, not less than 2.0 per cent and not more than 5.0 per cent of geranyl acetate, not less than 9.0 per cent and not more than15.0 per cent of citronellol and not less than 20.0 per cent and not more than 30.0 per cent of geraniol.

Category. Astringent, anti spasmodic, anti inflammatory.

Description. A clear, pale yellow to brownish yellow, mobile liquid, free from sediment and suspended matter. It has fresh, sweet and woody odour.

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Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

Reference solution. A 2.0 per cent w/v solution of *citronella oil IPRS* in *ethanol (95 per cent).*

Use chromatographic system described under Assay.

The peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). Not more than 93.3°.

Tests

Relative density (2.4.29). 0.878 to 0.892.

Refractive index (2.4.27). 1.461 to 1.473.

Optical rotation (2.4.22). -4.0° to +1.5°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

Reference solution (a). A 2.0 per cent w/v solution of *limonene IPRS* in *ethanol (95 per cent).*

Reference solution (b). A 2.0 per cent w/v solution of *citronellal IPRS* in *ethanol (95 per cent).*

Reference solution (c). A 2.0 per cent w/v solution of *citronellyl acetate IPRS* in *ethanol (95 per cent).*

Reference solution (d). A 2.0 per cent w/v solution of *neral IPRS* in *ethanol (95 per cent).*

Reference solution (e). A 2.0 per cent w/v solution of *geranial IPRS* in *ethanol (95 per cent).*

Reference solution (f). A 2.0 per cent w/v solution of *geranyl acetate IPRS* in *ethanol (95 per cent).*

Reference solution (g). A 2.0 per cent w/v solution of *citronellol IPRS* in *ethanol (95 per cent).*

Reference solution (h). A 2.0 per cent w/v solution of *geraniol IPRS* in *ethanol (95 per cent).*

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with fused silica 5 per cent phenylpolysiloxane,
- temperature:
  column. 60° for 1 minute, increase from 60° to 230° at a rate of 4° per minute, and maintain at this temperature for 10 minutes,
- inlet port at 230° and detector at 240°,
- detection by flame ionization detector,
- flow rate: 1 ml per minute using helium as carrier gas,
- injection volume: 0.5 µl,
- split ratio: 1: 200.

Inject reference solutions (a), (b), (c), (d), (e), (f), (g), (h) and the test solution.

Calculate the content of limonene, citronellal, citronellal acetate, neral, geranial, geranyl acetate, citronellol, and geraniol in the oil using area normalization procedure.

Storage. Store protected from light and moisture.

Clove Bud Oil

Laung ka tail

Clove Bud Oil is the essential oil obtained by steam distillation from the dried flower buds of *Syzygium aromaticum* L., Merr. and Perry syn. *Eugenia caryophyllus* (Sprengel) Bullock and S. Harrison (Fam. Myrtaceae).

Clove bud oil contains not less than 75.0 per cent and not more than 85.0 per cent of eugenol, not less than 8.0 per cent and not more than 15.0 per cent of eugenyl acetate and not less than 5.0 per cent and not more than 14.0 per cent β-caryophyllene.

Category. Used in toothache, headache, kasa.

Description. A clear, yellow to brown liquid, free from sediment and suspended matter.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

Reference solution. A 2.0 per cent w/v solution of *clove bud oil IPRS* in *ethanol (95 per cent).*

Use chromatographic system described in the Assay.

The chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 110° to 114°.

Tests

Relative density (2.4.29). 1.042 to 1.063.

Refractive index (2.4.27). 1.528 to 1.538.

Optical rotation (2.4.22). – 1.5° to 0.0°.

Assay. Determine by gas chromatography (2.4.13).

Test solution . A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

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Reference solution (a). A 2.0 per cent w/v solution of *eugenol IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *eugenyl acetate IPRS* in *ethanol (95 per cent)*.

Chromatographic system
- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio : 1 : 25.

Inject reference solutions (a), (b) and the test solution.

Calculate the content of eugenol and eugenyl acetate in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Clove Leaf Oil

Clove Leaf Oil is the essential oil obtained by steam distillation of the leaves of *Syzygium aromaticum* L. Merr. and Perry syn. *Eugenia caryophyllus* (Sprengel) Bullock and S. Harrison) of the (Fam. Myrtacae).

Clove Leaf Oil contains not less than 80.0 per cent and not more than 92.0 per cent of eugenol, not less than 0.5 per cent and not more than 4.0 per cent of eugenyl acetate.

Description. A clear, yellow to light brown liquid, free from sediment and suspended matter, spicy and characteristic of eugenol-like odour.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *clove leaf oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 110° to 114°.

Tests

Relative density (2.4.29). 1.039 to 1.049.

Refractive index (2.4.27). 1.528 to 1.535.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *eugenol IPRS* in *ethanol (95 per cent)*.

Reference solution (b) A 2.0 per cent w/v solution of *eugenyl acetate IPRS* in *ethanol (95 per cent)*.

Chromatographic system
- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

Inject reference solutions (a), (b) and the test solution.

Calculate the contents of eugenol and eugenyl acetate in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Clove Stem Oil

Clove Stem Oil is the essential oil obtained by steam distillation from the dried stems of *Syzygium aromaticum* L. Merr. and Perry syn. *Eugenia caryophyllus* (Sprengel) Bullock and S. Harrison (Fam. Myrtacae).

Clove Stem oil contains not less than 83.0 per cent and not more than 92.0 per cent of eugenol, not less than 0.5 per cent and not more than 4.0 per cent of eugenyl acetate.

Description. A clear, yellow to brown liquid, free from sediment and suspended matter, spicy and characteristic eugenol-like odour.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

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Reference solution. A 2.0 per cent w/v solution of *clove stem oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system as described under Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 118° to 122°.

Tests

Relative density (2.4.29). 1.041 to 1.059.

Refractive index (2.4.27). 1.531 to 1.536.

Optical rotation (2.4.22). −1.0° to + 1.0°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *eugenol IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *eugenyl acetate IPRS* in *ethanol (95 per cent)*.

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  - column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
  - inlet port at 250° and detector at 280°,
  - detection by flame ionization detector.
- flow rate 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

Inject reference solutions (a), (b) and the test solution.

Calculate the contents of eugenol, and eugenyl acetate in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Coconut Oil

Nariyal ka tail

Coconut Oil is the refined fixed oil obtained from the dried, solid part of the endosperm of *Cocos nucifera* L. (Fam. Arecaceae).

Coconut oil contains not less than 0.5 per cent w/w of caproic acid, not less than 5.0 per cent and not more than 11.0 per cent w/w of caprylic acid, not less than 4.0 per cent and not more than 9.0 per cent w/w of capric acid, not less than 40.0 per cent and not more than 50.0 per cent w/w of lauric acid, not less than 15.0 per cent and not more than 20.0 per cent w/w of myristic acid, not less than 7.0 per cent and not moe than 12.0 per cent w/w of palmitic acid, not less than 1.5 per cent and not more than 5.0 per cent w/w of stearic acid, not less than 4.0 per cent and not more than 10.0 per cent w/w of oleic acid, not less than 1.0 per cent and not more than 3.0 per cent w/w of linoleic acid, not less than 0.2 per cent w/w and not more than 3.0 per cent w/w of linolenic acid, not less than 0.06 per cent w/w and not more than 0.2 per cent w/w of arachidic acid and not less than 0.07 per cent w/w and not more than 0.2 per cent w/w of eicosenoic acid.

Category. Antieczematous, antipsoriatic, Kesya.

Description. White to light yellow mass or colourless to light yellow, clear oil.

Identification

A. It complies with the test for melting range (2.4.21).

B. It complies with the test for composition of fatty acids.

Tests

Melting range (2.4.21). 23° to 26°.

Refractive index (2.4.27). About 1.449, at 40°.

Peroxide value (2.3.35). Not more than 5.0.

Acid value (2.3.23). Not more than 0.5, determined on 20.0 g.

Unsaponifiable matter (2.3.39). Not more than 1.0 per cent, determined on 5.0 g.

Iodine value (2.3.28). 82 to 90.

Composition of fatty acids

Test Solution. Refined coconut oil is melted under gentle heating to a homogeneous liquid.

Reference solution. Dissolve 15 mg of *tricaproin IPRS*, 80 mg of *tristearin IPRS*, 0.15 g of *tricaprin IPRS*, 0.2 g of *tricaprylin IPRS*, 0.45 g of *trimyristin IPRS* and 1.25 g of *trilaurin IPRS* in a mixture of 2 volumes of *dichloromethane* and 8 volumes of *heptane*, then dilute to 50 ml with the same mixture of solvents heat at 45°to 50°. Transfer 2 ml of this mixture to a 10 ml centrifuge tube with a screw cap and evaporate the solvent in a current of *nitrogen*. Dissolve with 1 ml of *heptane* and 1 ml of *dimethyl carbonate* and mix vigorously under gentle heating (50° to 60°). Add, while still warm, 1 ml of a 1.2 per cent w/v solution of *sodium* in *anhydrous methanol*, prepared with the necessary precautions, and mix vigorously for about 5 minutes. Add 3 ml of *distilled water* and mix vigorously for about 30 seconds. Centrifuge for 15 minutes at 1500 rpm. Inject 1 µl of the organic phase.

Storage. Store protected from light in well-filled containers.

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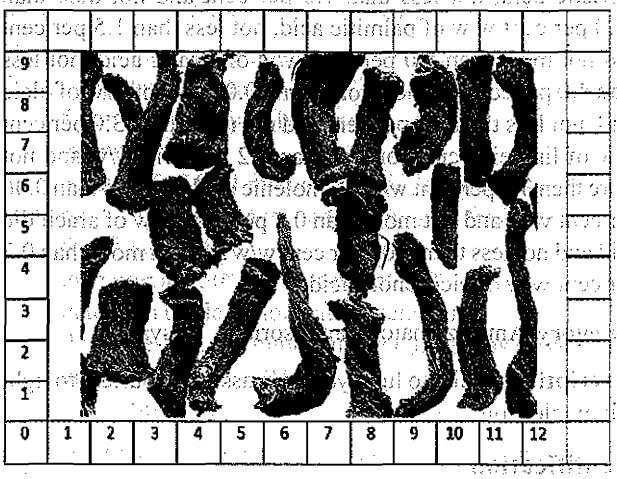
Coleus

Coleus forskohlii



Coleus consists of the whole or cut dried roots of *Coleus forskohlii* Briq. (Fam. Lamiaceae).

Coleus contains not less than 0.4 per cent w/w of forskolin, calculated on the dried basis.

Category. Cardiac stimulant, hypotensive, spasmolytic.

Description. The roots are light brown in color, generally long and radially spread. They have an aromatic characteristic odour and the taste is slightly pungent.

Identification

A. *Macroscopic* — Roots are brown, longitudinally wrinkled, fracture short, cut surface yellowish white.

B. *Microscopic* — The outermost layer consists of rectangular cork cells, cork cambium, rectangular parenchymatous region containing sclereids and calcium oxalate crystals. Vascular cambium is present in the form of a continuous ring. The tracheids and tracheidal fibres have bordered pits.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 75 volumes of *benzene*, and 25 volumes of *ethyl acetate*.

Test solution. To 5 g of the coarsely powdered substance under examination, add 50 ml of *acetonitrile* and reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 50 ml of *acetonitrile*, cool and filter. Combine all the filtrates and concentrate under vacuum to 100 ml.

Reference solution. To 1 g of *coleus IPBRS* add 50 ml of *acetonitrile* and reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 50 ml of *acetonitrile*, cool and filter. Combine all the filtrates and concentrate under vacuum to 20 ml.

Apply to the plate 20 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *vanillin glacial acetic acid reagent*. Heat the plate at 100° for 5-10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 15.0 per cent.

Water-soluble extractive (2.6.3). Not less than 18.0 per cent by Method I.

Total ash (2.3.19). Not more than 15.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 5.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh 3 g of coarsely powdered substance under examination, add 50 ml of *acetonitrile* and reflux on a water bath for 15 minutes, cool and filter. Reflux the residue two times with 75 ml of *acetonitrile*, cool and filter. Concentrate the filterate to 100.0 ml.

Reference solution. A 0.1 per cent w/v solution of *forskolin IPRS* in *acetonitrile*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: filtered and degassed mixture of 45 volumes of *acetonitrile* and 55 volumes of *water*,
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Inject the reference solution. The relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of forskolin.

Storage. Store protected from moisture and against attack by insects and rodents.

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Coleus Dry Extract

Coleus Dry Extract is obtained by extracting Coleus (*Coleus Forskohlii* Willd. Briq., Fam. Lamiaceae) roots with *methanol* or any other suitable solvent and evaporation of solvent.

Coleus Dry Extract contains not less than 90 per cent w/w to not more than 120 per cent w/w of the stated amount of forskolin, calculated on the dried basis. It may contain suitable added substances.

Category. Cardiac stimulant, hypotensive, spasmolytic.

Usual strengths. 10 per cent w/w; 20 per cent w/w.

Description. A light brown to brown colour powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 40 volumes of *ethyl acetate* and 60 volumes of *hexane*.

Test solution. Dissolve about 0.5 g of the extract under examination with 10 ml of *methanol*, filter.

Reference solution. A 0.1 per cent w/v solution of *forskolin IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and spray the plate with a *anisaldehyde-sulphuric acid reagent*. Heat the plate at 110° for 10 minutes and examine under ultraviolet light at 366 nm and in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Total ash (2.3.19). Not more than 10.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 6.0 per cent, determined on 1 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve a quantity of the extract under examination containing about 50 mg of forskolin in 50.0 ml of *methanol and* filter.

Reference solution. A 0.1 per cent w/v solution of *forskolin IPRS* in the *methanol*.

Chromatographic system

- a stainless steel column, 25 cm x 0.46 mm packed with octadecylsilane bonded to porous silica (5µm),
- mobile phase: a mixture of 50 volumes of *water* and 50 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the the test solution.

Calculate the content of the forskolin in the extract.

Storage. Store protected from heat and moisture.

Coriander Oil

Dhania ka tail

Coriander Oil is the essential oil obtained by steam distillation of the fruits of *Coriandrum sativum* (Fam. Apiaceae).

Coriander Oil contains not less than 65.0 per cent and not more than 78.0 per cent of linalool, not less than 3.0 per cent and not more than 7.0 per cent of α-pinene, and not less than 4.0 per cent and not more than 6.0 percent of camphor.

Category. Carminative, jvara, deepan-pacahn.

Description. A clear, colourless pale yellow liquid, free from sediment, suspended matter, characteristic, spicy and odour recalling that of linalool.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *coriander oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution

B. Flash point (2.4.44). + 54.0° to 58.0°.

Tests

Relative density (2.4.29). 0.862 to 0.878.

Refractive index (2.4.27). 1.462 to 1.470.

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Optical rotation (2.4.22). +7.0° to +13.0°.

Acid value (2.3.23). Not more than 3.0.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *linalool IPRS* in *ethanol (95 per cent)*.

Reference solution (b). 2.0 per cent w/v solution of *α-pinene IPRS* in *ethanol (95 per cent)*.

Chromatographic system
- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

Inject reference solutions (a), (b) and the test solution.

Calculate the contents of linalool and α-pinene in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Cumin Oil

Jeera ka tail

Cumin Oil is the essential oil obtained by steam distillation of the fruits or seeds of *Cuminum cyminum* L. (Fam. Apiaceae).

Cumin Oil contains not less than 18.0 per cent and not more than 30.0 per cent of cuminic aldehyde, not less than 14.0 per cent and not more than 18.0 per cent of *p*-mentha-1,3-diene-7-al, not less than 1.1 per cent and not more than 2.0 per cent of *p*-mentha-1,4-dieneo-7-al, not less than 12.0 per cent and not more than 16.0 per cent of β-pinene, not less than 20.0 per cent and not more than 28.0 per cent of γ-terpinene and not less than 10.0 per cent and not more than 15.0 per cent of *p*-cymene.

Description. A clear, light yellow to dark yellow liquid, free from sediment and suspended matter.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *cumin oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system as described under Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 54.0° to 58.0°.

Tests

Relative density (2.4.29). 0.905 to 0.932.

Refractive index (2.4.27). 1.490 to 1.51.

Optical rotation (2.4.22). +1.0° to + 8.0°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *cuminic aldehyde IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *p-mentha-1,3-diene-7-al IPRS* in *ethanol (95 per cent)*.

Reference solution (c). A 2.0 per cent w/v solution of *p-mentha-1,4-dieneo-7-al IPRS* in *ethanol (95 per cent)*.

Reference solution (d). A 2.0 per cent w/v solution of *γ-terpinene IPRS* in *ethanol (95 per cent)*.

Chromatographic system
- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

Inject reference solutions (a),(b),(c), (d) and the test solution.

Calculate the content of cuminic aldehyde, p-mentha-1,3-diene-7-al, p-mentha-1,4-dieneo-7-al and γ-terpinene in the oil using area normalisation procedure.

Storage. Store protect from light and moisture.

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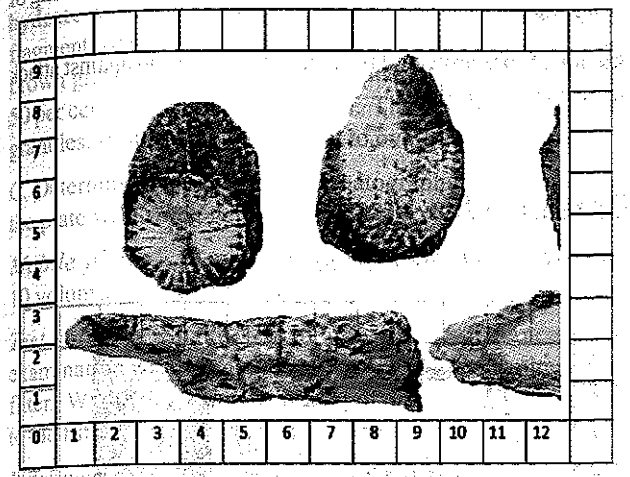
Daruharidra Roots

Berberis, *Berberis aristata*



Daruharidra Roots consist of the dried roots of *Berberis aristata* DC (Fam. Berberidaceae).

Daruharidra Roots contain not less than 0.70 per cent w/w of berberine, calculated on the dried basis.

Category. Hepatoprotective, antiinflammatory, anticancer, amatisara.

Description. The roots are cylindrical, yellowish brown in colour. They have a slightly characteristic odour and the taste is bitter.

Identification

A. Macroscopic — Root cylindrical, more or less knotty, strongly branched, usually cut into pieces of varying length and up to 45 mm in diameter; externally light yellowish-brown, longitudinally wrinkled and short scaly; fracture hard and tough; bark 1 mm. in thickness, easily separable into layers; wood yellow.

B. *Microscopic* — The powder is yellowish-brown; composed chiefly of fragments of wood fibers associated with a few tracheae and medullary rays; wood fibers yellowish, scarcely giving any reaction with *phloroglucinol* and *hydrochloric acid*, and with large, simple, transverse pores; trachea chiefly scalariform with bordered pits, occasionally reticulate; medullary rays one to twelve cells wide, and in very long rows; starch grains simple or two- to three-compound, the individual grains being irregularly spherical.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 68 volumes of *chloroform*, 35 volumes of *methanol* and 8 volumes of *water*.

Test solution. Weigh about 1 g of the coarsely powdered substance under examination, add 40 ml of *methanol*, reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vaccum to 10 ml.

Reference solution. A 0.1 per cent w/v solution of *berberine chloride IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm, 366 nm and also under day light. In the chromatographic profile one of the principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 2.0 per cent.

Water-soluble extractive (2.6.3). Not less than 6.0 per cent by Method I.

Total ash (2.3.19). Not more than 5.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 10.0 per cent determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Perform the Assay by following Method I or by Method II.

Method I

Determine by liquid chromatography (2.4.14).

Test solution. Weigh 2 g of the coarsely powdered substance under examination, add about 20 ml of *2 M hydrochloric acid* and 30 ml of *methanol*, reflux on a water-bath for 15 minutes, cool and filter. Reflux the residue further with *2 M hydrochloric acid* and *methanol*, till the extract turns colourless, cool and filter. Combine all the filtrates and concentrate to a volume slightly less than 50 ml. Dilute to 50.0 ml with *methanol*.

Reference solution. A 0.01 per cent w/v solution of *berberine hydrochloride IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution pH 2.5 prepared by dissolving 0.136 g of *potassium dihydrogen*

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orthophosphate in 500 ml of *water*, add 0.5 ml of *orthophosphoric acid* and make upto 1000 ml with *water*,

B. *acetonitrile*,

- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 346 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
25	50	50
26	80	20
30	80	20

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of berberine.

1 mg of berberine hydrochloride is equivalent to 0.9045 mg of berberine.

Method II

Determine by liquid chromatography (2.4.14.).

Test solution. Boil under reflux 1.0 g of the coarsely powdered substance under examination with 40 ml of 0.1 per cent v/v solution of *strong ammonia solution* in *methanol* for 30 minutes, cool and filter. Boil under reflux the residue with further 2 quantities, each of 30 ml, of 0.1 per cent v/v *strong ammonia solution* in *methanol*, cool and filter. Combine all the filtrates and dilute to 100 ml with 0.1 per cent v/v solution of *strong ammonia solution* in *methanol*. Dilute 5.0 ml of this solution to 50 ml with 0.1 per cent v/v *strong ammonia solution* in *methanol*.

Reference solution. A 0.002 per cent w/v solution of *berberine hydrochloride IPRS* in 0.1 per cent v/v *strong ammonia solution* in *methanol*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 73 volumes of 1.36 per cent w/v solution of *potassium dihydrogen orthophosphate* in *water* and 27 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 235 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

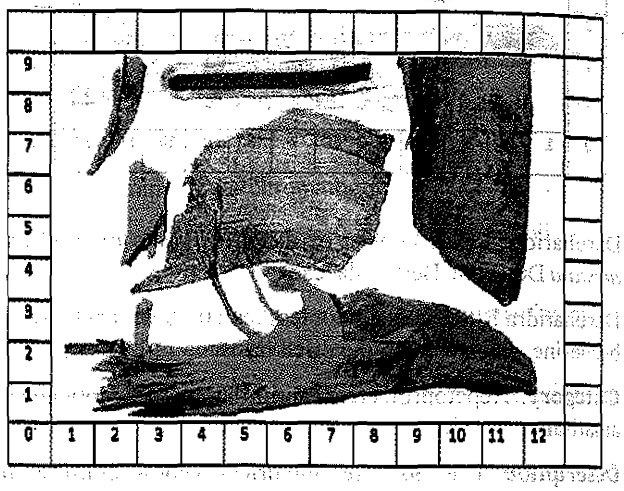
Calculate the content of berberine.

1 mg of berberine hydrochloride is equivalent to 0.9045 mg of berberine.

Storage. Store protected from heat, moisture and against attack of insects and rodents.

Daruharidra Stems

Berberis; *Berberis aristata*



Daruharidra Stems consist of cut dried stems of *Berberis aristata*, DC (Fam. Berberidaceae).

Daruharidra Stems contain not less than 0.5 per cent w/w of berberine, calculated on the dried basis.

Category. Hepatoprotective, antiinflammatory, anticancer, amatisara.

Description. Yellowish-brown, cut chips of varying sizes, fracture fibrous, and taste bitter.

Identification

A. *Macroscopic* — Cut tortuous pieces of varying length and thickness. Bark thick, yellowish to brownish grey, wrinkled irregularly, deeply furrowed; surface hard, rough, fracture short and fibrous.

B. *Microscopic* — Cork consists of rectangular to squarish yellow coloured thin walled cells, radially arranged; sieve elements irregularly shaped, few cells containing yellowish brown to orange brown masses; phloem fibers thick walled arranged tangentially; phloem ray cells have calcium oxalate crystals, scattered stone cells; secondary phloem broad, sieve elements arranged as tangential bands, phloem fibers short, thick walled; secondary xylem broad, consisting of small to

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medium sized xylem vessels, tracheids, xylem fibers.

Powder is greenish yellow and shows the following diagnostic characters when observed under a microscope using chloral hydrate solution. Abundant thin walled parenchyma, fragments of yellowish-brown cork; ovoid or spherical orange brown granular masses. Examine under a microscope using 50 per cent v/v solution of *glycerol*. The powder shows starch granules, simple, small, spherical to ovoid.

C. Determine by thin-layer chromatography (2.4.17) coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *n-butanol*, 10 volumes of *glacial acetic acid* and 20 volumes of *water*.

Test solution. Shake 1 g of powdered substance under examination with 10 ml of *methanol* for 10 to 15 minutes and filter. Wash the residue with 5 ml of *methanol*, add washing to the filtrate.

Reference solution. Shake 1 g of powdered *daruharidra IPBRS* with 10 ml of *methanol* for 10-15 minutes and filter. Wash the residue with 5 ml of *methanol* and add washing to the filtrate.

Apply to the plate, 10 µl of each solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and observe under ultraviolet light at 254 nm and 366 nm and also under day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 2.0 per cent.

Water-soluble extractive (2.6.3). Not less than 5 per cent by Method I.

Total ash (2.3.19). Not more than 5 per cent.

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Perform the Assay by following Method I or by Method II.

Method I

Determine by liquid chromatography (2.4.14).

Test solution. Boil under reflux 1 g of the coarsely powdered substance under examination with 40 ml of 0.1 per cent v/v solution of *strong ammonia solution* in *methanol* for 30 minutes, cool and filter. Boil under reflux the residue with further 2 quantities, each of 30 ml, of 0.1 per cent v/v *strong ammonia solution* in *methanol*, cool and filter. Combine all the filtrates and dilute to 100 ml with 0.1 per cent v/v solution of *strong ammonia solution* in *methanol*. Dilute 5.0 ml of this solution to 50 ml with a 0.1 per cent v/v *strong ammonia solution* in *methanol*.

Reference solution. A 0.002 per cent w/v solution of *berberine hydrochloride IPRS* in 0.1 per cent v/v *strong ammonia solution* in *methanol*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane chemically bonded to porous silica (5 µm),
- mobile phase: a mixture of 73 volumes of 1.36 per cent w/v solution of *potassium dihydrogen orthophosphate* in *water* and 27 volumes of *acetonitrile*,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 235 nm,
- injection volume: 10 µl.

Method II

Determine by liquid chromatography (2.4.14.).

Test solution. Weigh 2 g of the coarsely powdered substance under examination, add about 20 ml of *2 M hydrochloric acid* and 30 ml of *methanol*, reflux on a water-bath for 15 minutes, cool and filter. Reflux the residue further with *2 M hydrochloric acid* and *methanol*, till the extract turns colourless, cool and filter. Combine all the filtrates and concentrate to a volume slightly less than 50 ml. Dilute to 50.0 ml with *methanol*.

Reference solution. A 0.01 per cent w/v solution of *berberine hydrochloride IPRS* in *methanol*.

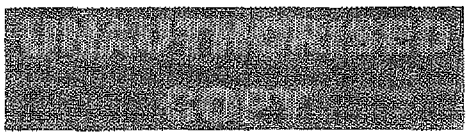
Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution pH 2.5 prepared by dissolving 0.136 g of *potassium dihydrogen orthophosphate* in 500 ml of *water*, add 0.5 ml of *orthophosphoric acid* and make upto 1000 ml with *water*,

B. *acetonitrile*,

- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 346 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
25	50	50
26	80	20
30	80	20



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Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of berberine.

1 mg of berberine hydrochloride is equivalent to 0.9045 mg of berberine.

Storage. Store protected from heat, moisture and against attack of insects and rodents.

Dill Seed Oil

Soa ka tail

Dill Seeds Oil is the essential oil obtained by steam distillation of the mature crushed seeds of *Anethum sowa* (Fam. Umbelliferae).

Dill seed oil contains not less than 40.0 per cent of limonene and not less than 43.0 per cent and not more than 63.0 per cent of carvone.

Description. A clear, pale yellow liquid, free from sediment, suspended matter and fresh green spicy with a strong camphoraceous odour.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *dill seed oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system as described under Assay.

The chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). Not less than 100.0°.

Tests

Relative density (2.4.29) .0.925 to 0.980.

Refractive index (2.4.27). 1.4860 to 1.4950.

Optical rotation (2.4.22). +84.0° to +95.0°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *cis-dihydrocarvone IPRS* in *ethanol (95 per cent)*.

Reference solution (b) A 2.0 per cent w/v solution of *L- carvone IPRS* in *ethanol (95 per cent)*.

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column: 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

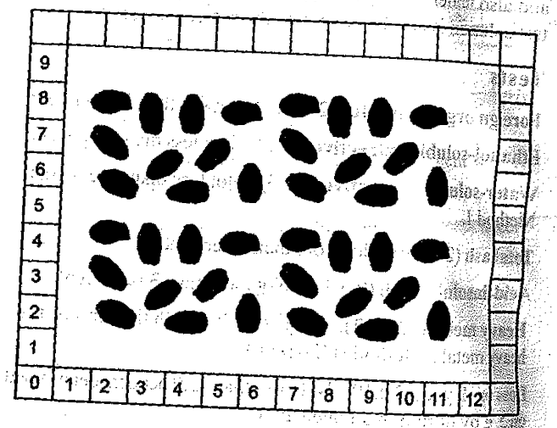
Inject reference solutions (a), (b) and the test solution.

Calculate the cis-dihydrocarvone, and L- carvone contents in the oil under examination using the ratios.

Storage. Store protected from light and moisture.

Draksha

Draksha, *Vitis vinifera*



Draksha consists of dried mature fruits of *Vitis vinifera* Linn. (Fam.Vitaceae).

Draksha contains not less than 0.0004 per cent w/w of resveratrol, calculated on the dried basis.

Category. Laxative, demulcent, cardiotonic, haematinic, aphrodisiac.

Description. Fruits are sticky and pulpy, dark brown to black; oblong or oval, sometimes spherical. Odour is sweetish and pleasant; taste sweet.

Identification

A. *Macroscopic* — Mature dried fruit dark brown to black in colour, 1.5 to 2.5 cm long and 0.5 to 1.5 cm wide; outer skin is irregularly wrinkled forming ridges and furrows; seeds, generally 1 to 4 in number, 4 to 7 mm long, ovoid-rounded to triangular or simply ovoid, brown or black.

B. *Microscopic* — A single layered epidermis cells filled with reddish-brown contents; mesocarp pulpy, made up of thin-walled, irregular cells containing prismatic crystals of calcium oxalate; some fibro-vascular bundles also present in this region.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 25 volumes of *chloroform*, 10 volumes of *ethyl acetate* and 1 volume of *formic acid*.

Test solution. Extract 5 g of the substance under examination with 10 ml of *ethanol* for 15 minutes. Cool and filter.

Reference solution. Extract 2 g of *draksha IPBRS* with 10 ml of *ethanol* for 15 minutes. Cool and filter.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. Spray with the *anisaldehyde sulphuric acid reagent* . Heat the plate at 110° for 10 minutes. Examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 20.0 per cent.

Water-soluble extractive (2.6.3). Not less than 40.0 per cent by Method I.

Total ash (2.3.19) Not more than 7.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.7 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20ppm).

Water (2.3.43). Not more than 20.0 per cent, determined on 0.2 g.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Extract 2 g of the substance under examination with 10 ml of *ethanol* by cold percolation for 30 minutes, cool and filter. Extract the residue further with two quantities of 10 ml of *ethanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10.0 ml.

Reference solution. A 0.005 per cent w/v solution of *resveratrol IPRS* in *ethanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with silica gel consisting of porous spherical particles with chemically bonded nitrile groups (5 µm),
- mobile phase: A. a solution prepared by dissolving 0.136 g of *potassium di-hydrogen orthophosphate* in 500 ml of *water*, add 0.5 ml of *orthophosphoric acid* and dilute to 1000 ml with *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 310 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
18	100	0
25	55	45
30	20	80
33	100	0

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

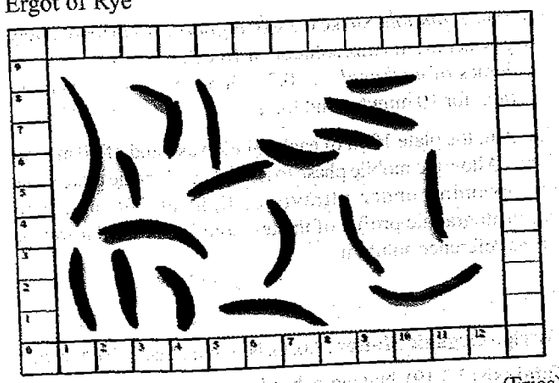
Inject the reference solution and the test solution.

Calculate the content of resveratrol.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Ergot

Ergot of Rye



Ergot is the dried selerotia of *Claviceps purpurea* (Fries) tulasne. (Fam. Hypocreaceae) developed on rye plants *Secale cereale (lin)*.

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It contains not less than 0.1 per cent of total alkaloids of Ergot calculated as ergotoxine.

Description. Blackish violet, odour disagreeable and faint, taste unpleasant.

Identification

A. *Macroscopic*— Dark violet to nearly black, usually from about 1 to 3 cm long and from 1 to 5 mm broad, fusiform, obscurely 3-angled usually tapering towards both ends, often with a longitudinal furrow on each face; fracture short internally whitish or pinkish white and showing darker lines radiating from the center.

B. *Microscopic*—The powder is purplish brown. When examined under a microscope the powder shows the following diagnostic characters: thin layers of dark purple to dark brown rectangular cells in longitudinal rows, dense pseudo parenchyma with small rounded or oval cells varying in size containing abundant oil globules with thin and colourless highly refractive chitinous walls, calcium oxalate crystals and vascular tissues are absent.

C. Shake 1g in closed flask for about five minutes with 20 ml of *solvent ether* and about 15 drops of *sulphuric acid* (20 per cent). Filter and shake the filtrate with 15 drops of cold saturated solution of *sodium bicarbonate*. The aqueous layer turns red or violet.

D. Determine by thin layer chromatography (2.4.17) coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 95 volumes of *chloroform* and 5 volumes of *ethanol (96 per cent)*.

Test solution. Shake 0.2 g of substance in moderately fine powder in 5 ml mix solution of 18 volumes of *chloroform*, 2 volumes of *methanol* and 0.2 volume of *strong ammonia* solution for 10 minutes and filter.

Reference solution. Shake 0.2 g of *ergot IPBRS* in moderately fine powder in 5 ml mix solution of 18 volumes of *chloroform*, 2 volumes of *methanol* and 0.2 volume of *strong ammonia solution*, for 10 minutes and filter.

Apply to the plate 10 µl of each solution as band of 10 mm by 2 mm. Allow the mobile phase to rise 12 cm. Dry the plate in air and examine under ultraviolet light at 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign Organic Matter (2.6.1). Not more than 2.0 per cent.

Total ash (2.3.19). Not more than 5.0 per cent.

Loss on drying (2.4.19). Not more than 8.0 per cent determined on 1 g of the drug dried at 105° for three hours.

Assay. Shake vigorously about 5 gm accurately weighed, moderately fine powder with 60 ml of mixture (23 *methanol*, 4 ml *strong ammonia solution* and 223 ml *chloroform*). Add few drops of *water*. Shake, allow to separate and filter the chloroformic extract through cotton wool. Repeat the extraction using three successive 60 ml portions of the *methanol, strong ammonia, chloroform* mixture and filtering the extract through the same cotton wool. Combine all *extracts* in 250 ml volumetric flask. Dilute up to the mark with same solvent mixture. Evaporate 50 ml extract to dryness in vacuum or under nitrogen at a temperature not exceeding 40°. Dissolve the residue in 5 ml of alcohol (95 per cent) and transfer to a separating funnel with the aid of 80 ml of anesthetic ether.

Extract the anesthetic ether layer with 25 ml portion, followed by three successive 20 ml portion of N/5 *sulphuric acid* washing each acid extract with the same 25 ml of *anesthetic ether* in another separator. Collect all *sulphuric acid* extract and remove traces of ether in vacuum or under *nitrogen*. Transfer to 100 ml volumetric flask and dilute up to the mark with N/5 *sulphuric acid*. Filter the extract through ordinary filter paper. Take three 50 ml conical flask. In first flask take 5 ml of standard {0.0125 g *ergometrine maleate* in 0.1 per cent w/v *tartaric acid* diluted to 500 ml (25 ppm)}. In second 5 ml of sample solution and in third 5 ml of N/5 *sulphuric acid*. Cool all three solutions in ice. Add 10 ml of PABA (0.252 g *p-dimethyl amino benzaldehyde* in 130 ml conc. cold *sulphuric acid* + 70 ml cold water + 0.2 ml 5 per cent solution of *ferric chloride*) solution to all three solutions dropwise shaking vigorously. Measure the absorbance at 590 nm (2.4.7) Each mg of ergometrine maleate is equivalent to 1.370 mg of total alkaloids calculated as ergotoxine.

Calculate the content of total alkaloids of ergot as ergotoxine.

Storage. Store protected from light, heat and moisture and against attack by insects and rodents.

Prepared Ergot

Prepared Ergot is ergot, which has been powdered and immediately deprived of most of its fat and adjusted to contain total alkaloids of required strength. Prepared Ergot contains 90 to 110 per cent of the labeled amount of total alkaloids of ergot calculated as ergotoxine.

Usual strengths. 0.15 per cent and 0.2 per cent.

Description. Purplish brown coloured powder, odour and taste characteristic.

Identification

A. Shake 1 g in closed flask for about five minutes with 20 ml of solvent *ether*; add about 15 drops of *sulphuric acid* (20 per

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cent). Filter and shake the filtrate with 15 drops of cold saturated solution of *sodium bicarbonate*. The aqueous layer turns red or violet.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 95 volumes of *chloroform*, 5 volumes of 96 per cent *alcohol*.

Test solution. Dissolve 0.2 g of substance in a 5 ml of mixture of 18 volumes of *chloroform*, 2 volumes of *methanol* and 0.2 volumes of *strong ammonia solution*.

Reference solution. Dissolve 10 mg of *ergometrine maleate IPRS* in 1 ml mixture of 18 volumes of *chloroform*, 2 volumes of *methanol* and 0.2 volumes of *strong ammonia solution* (solution A). Dissolve 10 mg of *ergotamine tartrate IPRS* in 1 ml of the same mixture (solution B). Mix solution A and solution B and use this as reference solution.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 12 cm. Dry the plate in air and examine under ultraviolet light at 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Fat content. Not more than 5.0 per cent. Extract 5 gm sample in a continuous extraction apparatus for 4 hours using petroleum ether (b.p. 40° to 60°) as solvent. Evaporate the petroleum ether and dry for 1 hour at 80°C. Calculate the percentage of fat in the sample.

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Total ash (2.3.19). Not more than 5.0 per cent.

Assay. Weigh accurately the substance under examination equivalent to 10 mg of total alkaloids, shake vigorously with 60 ml of mixture (23 ml *methanol*, 4 ml *strong ammonia solution*, and 23 ml *chloroform*), shake for 20 minutes. Add few drops of water, shake, allow to separate and filter the chloroform extract through cotton wool. Repeat the extraction using three successive 60 ml portions of the *methanol*, strong *ammonia*, *chloroform* mixture and filtering the extract through the same cotton wool. Combine all extracts in 250 ml volumetric flask. Dilute up to the mark with same solvent mixture. Evaporate the 50 ml extract to dryness in vacuum or under nitrogen at a temperature not exceeding 40°. Dissolve the residue in 5 ml of alcohol (95 per cent) transfer to a separating funnel with the aid of 80 ml of anesthetic ether.

Extract the *anesthetic ether* layer with 25 ml portion, followed by three successive 20 ml portion of N/5 *sulphuric acid* washing each acid extract with the same 25 ml of anesthetic ether in another separator. Collect all *sulphuric acid* extract and remove traces of ether in vacuum or under *nitrogen*. Transfer to 100 ml volumetric flask and dilute up to the mark with N/5 *sulphuric acid*. Filter the extract through ordinary filter paper. Take three 50 ml conical flask. In first flask take 5 ml of standard {0.0125 gm *ergometrine maleate* in 0.1 per cent w/v *tartaric acid* diluted to 500 ml (25 ppm)}, in second 5 ml of sample solution & in third 5 ml of N/5 *sulphuric acid*. Cool all three solutions in ice. Add 10 ml of PABA (0.252 gm *p-dimethyl amino benzaldehyde* in 130 ml conc. cold *sulphuric acid* + 70 ml cold water + 0.2 ml 5 per cent solution of *ferric chloride*) solution to all three solutions dropwise shaking vigorously. Measure the absorbance of solution at 590 nm (2.4.7).

1 mg of ergometrine maleate is equivalent to 1.370 mg of total alkaloid calculated as ergotoxine.

Calculate the content of total alkaloids of ergot as ergotoxine.

Storage. Store in air tight container protected from heat and moisture.

Eucalyptus Oil

Nilgiri Oil

Eucalyptus Oil is the essential oil obtained by steam distillation of the leaves of *Eucalyptus globulous* (Fam. Myrtaceae).

Eucalyptus Oil contains not less than 60 per cent and not more than 80 per cent of 1, 8 cineol, and not less than 0.5 per cent and not more than 10 per cent of α-pinene.

Category. Counterirritant, antiseptic, expectorant, antirheumatic.

Description. A colourless or pale yellow liquid; free from sediment, suspended matter, odour aromatic and camphoraceous; taste pungent followed by a sensation of cold.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *eucalyptus oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 52.0° to 56.0°.

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Tests

Relative density (2.4.29). 0.905 to 0.925, at 20°

Refractive index (2.4.27). 1.457 to 1.470, at 20°

Optical rotation (2.4.22). + 0° to +10°, at 20°

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *1,8-cineole IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *α-pinene IPRS* in *ethanol (95 per cent)*.

Chromatographic system

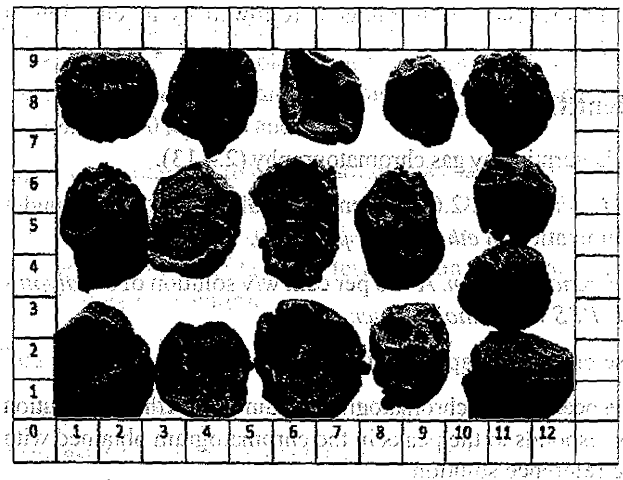
- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

Inject reference solutions (a), (b) and the test solution.

Calculate the content of 1, 8-cineole, and α-pinene contents in the oil using area normalisation procedure.

Garcinia

Vilayati Imli; *Garcinia cambogia*



Garcinia is the dried deseeded fruit of *Garcinia cambogia* Desr. {*Garcinia gummi-gutta* (L.) N: Robson} (Fam. Guttiferae). Garcinia contains not less than 1.0 per cent of garcinol, 12.0 per cent of total hydroxycitric acid and hydroxycitric acid lactone calculated on dried basis.

Category. Antiulcer, sthoulyo, medohara.

Description. Dark brown to blackish brown fruits. Taste, acidic.

Identification

A. *Macroscopic* — Dark brown to blackish brown fruits, ovoid, longitudinally grooved.

B. *Microscopic* — Mesocarp very wide composed of parenchymatous cells of various sizes and shapes. Compound starch grains and prismatic crystals of calcium oxalate traverse throughout the parenchymatous cells of the mesocarp.

C. In the Assay, the principal peak in the chromatogram obtained with test solution has a retention time similar to that of the peak due to hydroxycitric acid in the chromatogram obtained with reference solution.

D. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 4 volumes of *toluene*, 1 volume of *ethyl acetate* and 0.5 volume of *formic acid*.

Test solution. Weigh about 2 g of coarsely powdered substance under examination, add 10 ml of *methanol*, reflux for 10 minutes, cool and filter. Reflux the residue further with 2 x 10 ml *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Reference solution. A solution of 0.1 per cent w/v of *garcinol IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as band length 8 mm. Allow the mobile phase to rise 8 cm. Dry the plate at 105° for 10 minutes, and examine under ultra violet light 254 nm and 366 nm. In the chromatographic profile one of the principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution.

Tests

Citric Acid. Not more than 2.0 per cent.

Determine by liquid chromatography (2.4.14).

Test solution, reference solutions (a), (b) and chromatographic system as described under Assay.

Inject test solution and reference solution (b).

Calculate the content of citric acid.

Foreign organic matter (2.6.1). Not more than 5.0 per cent.

Water-soluble extractive (2.6.3). Not less than 40.0 per cent by Method I.

Total ash (2.3.19). Not more than 8.0 per cent.

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Acid-insoluble ash (2.3.19). Not more than 1.5 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

For hydroxycitic acid and hydroxycitic acid lactone —

Test solution. Weigh about 5 g of the coarsely powdered substance under examination and transfer to a 250 ml beaker. Add 5 ml of *dilute ortho phosphoric acid* and 100 ml of *water*, concentrate to half the volume by heating, cool and filter. Extract the residue further with water till the last extract turns colorless, cool and filter. Combine all the filtrates and concentrate under vacuum to 250.0 ml.

Reference solution (a). A 0.8 per cent w/v solution of *hydroxycitric acid calcium salt IPRS* in *dilute ortho phosphoric acid.*

Reference solution (b). A 0.1 per cent w/v solution of *citric acid* in *dilute ortho phosphoric acid.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen phosphate* in 900 ml of *water*, adjusted to the pH to 2.5 with *dilute ortho phosphoric acid* and diluting to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject the reference solution (a). The test is not valid unless the relative retention times are about 0.8 for *hydroxycitric acid lactone*, 1.0 for *hydroxycitric acid* and 2.0 for *citric acid*, the resolution factor between *hydroxycitric acid lactone* and *hydroxycitric acid* is not less than 1.8, the tailing factor is not more than 1.5 and the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the sum of the contents of hydroxycitric acid and hydroxycitric acid lactone.

For Garcinol —

Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 5 gm coarsely powdered substance under examination with 50 ml *methanol* for 20 minutes, cool and filter. Reflux the residue further with four extracts of 50.0 ml each of *methanol*, cool and filter. Combine all filtrates and concentrate to 100.0 ml.

Reference solution. A solution of 0.1 per cent w/v of *garcinol IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: 0.1 per cent *orthophosphoric acid* in *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of the garcinol in the sample.

Storage. Store protected from heat and moisture.

Garcinia Aqueous Extract

Garcinia Aqueous Extract is obtained by extracting Garcinia (*Garcinia cambogia* Desr., *Garcinia gummi-gutta* (L.) N: Robson, Fam. Guttiferae) fruit rinds with *water* as calcium salts.

Garcinia Aqueous Extract contains not less than 90.0 per cent w/w to not more than 120.0 per cent w/w of the stated amount of hydroxy citric acid, and calcium not less than 15.0 per cent w/w calculated on the dried basis. It may contain suitable added substances.

Category. Antiulcer, sthoulyo, medohara.

Usual strengths. 50 per cent; 60 per cent.

Description. A pale brown to brown powder.

Tests

Total ash (2.3.19). Not more than 10.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 6.0 per cent determined on 1 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. *hydroxy citric acid* — Determine by liquid chromatography (2.4.14).

Test solution. Shake well 0.2 g of the extract under examination add 2 ml of *3 M hydrochloric acid* and add 10 ml of *water*, sonicate it to dissolve, make up to volume 100.0 ml with *water* and mix.

Reference solution. A 0.1 per cent w/v solution of *hydroxy citric acid IPRS* with 2 ml of *3 M hydrochloric acid* in *water.*

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Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5µm),
- mobile phase: 0.1 per cent *orthophosphoric acid* in *water*, filter and degas,
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of the hydroxy citric acid in the extract.

Calcium —

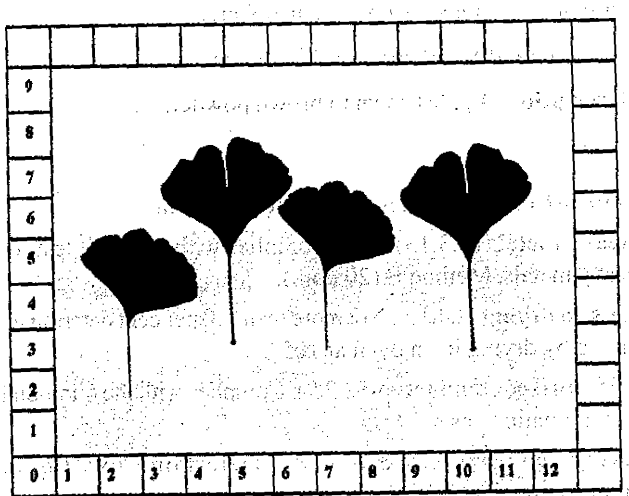
Test solution. Weigh about 0.2 g of the extract and transfer into a 500 ml conical flask. Dissolve in 2 ml of *3 M hydrochloric acid* and add 200 ml of *water*. Add 15 ml of *1 M sodium hydroxide* and titrate with *0.05 M EDTA* using *hydroxy naphthol blue* as indicator until deep blue colour persist.

1 ml of *0.05 M EDTA* solution is equivalent to 0.002004 g of calcium.

Storage. Store in air-tight containers, protected from light.

Ginkgo Leaf

Maidenhair tree; Kew tree; Fossil tree; Temple tree; Pankha plant; *Ginkgo biloba*.



Ginkgo consists of dried leaves of *Ginkgo biloba* L. (Fam. Taxaceae).

Ginkgo leaves contains not less than 0.5 per cent w/w of *flavone glycosides*, calculated on dry basis.

Category. Brain tonic.

Description. Leaves are green in colour; odour is characteristic and agreeable; taste astringent and bitter.

Identification

A. *Macroscopic* — The upper surface of ginkgo leaf is slightly darker than the lower surface. The petioles of the leaf are about 4 cm to 9 cm long. The lamina is about 4 cm to 10 cm wide, fan-shaped, usually bilobate or sometimes undivided. Both surfaces are smooth, and the venation dichotomous, the veins appearing to radiate from the base; they are equally prominent on both surfaces. The distal margin is incised, irregularly to different degrees, and irregularly lobate or emarginate. The lateral margins are entire and taper towards the base.

B. *Microscopic* — Transverse section of the leaves shows a single layer of upper epidermis and lower epidermis surrounded or subsquare cells, covered with thin cuticle. Collenchyma arranged in at the inner sides of upper and lower epidermis of midrib; only visible in mature and old leaves, palisade tissues are present underneath the upper epidermis, elongated at right angles to the surface. The cells of mesophyll are smaller than the palisade cells, and separated by large intercellular spaces. Vascular bundles arranged at regular interval along the width of the lamina with adjacent clusters of calcium oxalate; collateral, with xylem dorsal, phloem ventral, surrounded by fibers. The secretory canals are large and distinct, present in the mesophyll between vascular bundles.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 17.5 volumes of *water*, 67.5 volumes of *ethyl acetate*, 7.5 volumes of *acetic acid* and 7.5 volumes of *anhydrous formic acid*.

Test solution. Reflux 2 g of coarsely powdered substance under examination with 10 ml of *methanol* for 10 minutes, cool and filter. Reflux the residue further with 2 x 10 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Reference solution. Reflux 2 g of *ginkgo IPBRS* with 10 ml of *methanol* for 10 minutes, cool and filter. Reflux the residue further with 2 x 10 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Apply to the plate 10 µl of each solution as band length 8 mm. Allow the mobile phase to rise 8 cm. Dry the plate at 105° for 10 minutes, and examine under ultraviolet light at 254 nm and 366 nm.

The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

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Ethanol-soluble extractive (2.6.2). Not less than 11.0 per cent.

Water-soluble extractive (2.6.3). Not less than 17.0 per cent.

Total ash (2.3.19). Not more than 4.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.7 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 11.0 per cent determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 2.5 g coarsely powdered substance under examination with 50 ml of *methanol* for 20 minutes, cool and filter. Reflex the residue further with 50 ml of *methanol*, cool and filter. Combine all filtrates and concentrate to dryness then add 20 ml of *methanol* and add 20 ml of *1.5 M hydrochloric acid*, and sonicate for 10 minutes then dilute to 50 ml with *methanol*, centrifuge for 10 minutes and take 10 ml of supernatant liquid in a 10 ml brown glass vial. Close with a rubber seal and aluminum cap and heat on a water-bath for 25 minutes. Allow to cool to room temperature.

Reference solution (a). Dissolve about 10 mg *quercetin IPRS* in 25 ml of *methanol* and sonicate for 10 minutes, dilute to 50.0 ml with *methanol.*

Reference solution (b). Dissolve about 10 mg *kaempferol IPRS* and 2.5 mg *isorhamnetin IPRS* in 25 ml of *methanol* and filter.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm)
- mobile phase: A. *water* and adjust to pH 2.0 with *orthophosphoric acid.*
  B. *methanol*
- a gradient programme using the conditions given below,
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 370 nm,
- injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	60	40
1	60	40
20	45	55
21	0	100
25	60	40
27	60	40

Inject reference solution (a). The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject reference solution (b) for peak identification.

Inject reference solution (a) and the test solution.

Calculate the percentage content of flavonoids, expressed as flavones glycoside, using the following expression:

$$FG = \frac{F_1 \times M_1 \times 2.514 \times P}{F_2 \times M_2}$$

Where, F_1 = Sum of the areas of all the peaks from the peak due to *quercetin* to the peak due to *isorhamnetin* (RRT of about 1.5) in the chromatogram obtained with the test solution.

F_2 = Area of the peak due to *quercetin* in the chromatogram obtained with the reference solution.

M_1 = Mass of *quercetin IPRS* in the reference solution, in grams.

M_2 = Mass of the extract to be examined used to prepare the test solution, in grams.

P = Percentage content of *quercetin IPRS.*

Storage. Store in air tight amber colour containers protected from light, heat and moisture.

Ginkgo Dry Extract

Ginkgo Dry Extract is obtained from whole or cut dried leaves of *Ginkgo biloba L.* by extraction with a suitable solvent using an appropriate procedure.

Ginkgo Dry Extract contains not less than 22.0 per cent w/w and not more than 27.0 per cent w/w of *flavonoids*, calculated on dry basis.

Category. Brain tonic.

Description. Yellowish to orange coloured powder, hygroscopic.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 17.5 volumes of *water*, 67.5 volumes of *ethyl acetate*, 7.5 volumes of *acetic acid* and 7.5 volumes of *anhydrous formic acid.*

Test solution. Dissolve 100 mg of the extract in 10 ml of *methanol* then sonicate for 10 minutes.

Reference solution. Reflux 2 g of *ginkgo IPBRS* with 10 ml of *methanol* for 10 minutes, cool and filter. Reflux the residue further with 2 x 10 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Apply to the plate 10 µl of each solution as band length 8 mm. Allow the mobile phase to rise 8 cm. Dry the plate at 105° for

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10 minutes, and examine under ultraviolet light at 254 nm and 366 nm.

The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Heavy Metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 2.0 g by drying in an oven at 105° for 3 hours.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 0.300 gm of extract under examination with 100 ml of extraction solvent containing 25 volumes of *methanol*, 3 volumes of conc. *hydrochloric acid* and 12 volumes of *water* in a water bath for 135 minutes. Allow to cool at room temperature and make final volume up to 100.0 ml with *methanol*.

(*NOTE — The solution will turn deep red. The colour of the solution is not a definitive indication of reaction completeness.*)

Reference solution (a). Dissolve 12.5 mg *quercetin IPRS* in 50.0 ml *methanol* and sonicate for 10 minutes, dilute to 100.0 ml with *methanol* and filter.

Reference solution (b). Dissolve 12.5 mg *kaempferol IPRS* and 3 mg *isorhamnetin IPRS* in 50 ml *methanol* and sonicate for 10 minutes, dilute to 100.0 ml with *methanol* and filter.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm)
- mobile phase: A. *water* and adjust to pH 2.0 with *orthophosphoric acid.*
  B. *methanol*
- a gradient programme using the conditions given below,
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 370 nm,
- injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	60	40
1	60	40
20	45	55
21	0	100
25	60	40
27	60	40

Inject reference solution (a). The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject reference solution (b) for peak identification.

Inject reference solution (a) and the test solution.

Calculate the percentage content of flavonoids, expressed as flavones glycoside, using the following expression:

$$FG = \frac{F_1 \times M_1 \times 2.514 \times P}{F_2 \times M_2}$$

Where, F_1 = Sum of the areas of all the peaks from the peak due to *quercetin* to the peak due to *isorhamnetin* (RRT of about 1.5) in the chromatogram obtained with the test solution,

F_2 = Area of the peak due to *quercetin* in the chromatogram obtained with the reference solution.

M_1 = Mass of *quercetin IPRS* in the reference solution, in grams.

M_2 = Mass of the extract to be examined used to prepare the test solution, in grams.

P = Percentage content of *quercetin IPRS.*

Storage. Store in air tight amber colour containers protected from light, heat and moisture.

Ginkgo Tablet

Ginkgo biloba tablet

Ginkgo tablet contains not less than 90.0 per cent and not more than 110.0 per cent of stated amount of flavone glycosides. The tablet may be coated.

Category. Brain tonic.

Usual strength. 120 mg

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 17.5 volumes of *water,* 67.5 volumes of *ethyl acetate,* 7.5 volumes of *acetic acid* and 7.5 volumes of *anhydrous formic acid.*

Test solution. Dissolve 100 mg of the extract in 10 ml of *methanol* then sonicate for 10 minutes.

Reference solution. Reflux 2 g of *ginkgo IPBRS* with 10 ml of *methanol* for 10 minutes, cool and filter. Reflux the residue further with 2 x 10 ml of *methanol,* cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Apply to the plate 10 µl of each solution as band length 8 mm. Allow the mobile phase to rise 8 cm. Dry the plate at 105° for

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20 minutes, and examine under ultraviolet light at 254 nm and 366 nm.

The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 2.0 gm by drying in an oven at 105° for 3 hours.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh and powder 20 tablets. Weigh accurately a quantity of powder equivalent to about 50 mg of flavones glycosides in a 50.0 ml volumetric flask. Add 20 ml of *methanol*, and sonicate for 3 minutes. Add 20 ml of *1.5 M hydrochloric acid* and 12 volumes of *water* in a water-bath for 135 minutes. Allow to cool at room temperature and make final volume upto 50.0 ml with *methanol*.

(*NOTE—The solution will turn deep red. The colour of the solution is not a definitive indication of reaction completeness.*)

Reference solution. Dissolve about 2.0 mg *quercetin IPRS*, 2.0 mg of *kaempferol IPRS* and 0.5 mg of *isorhamnetin IPRS* in 5.0 ml of *methanol* and sonicate for 10 minutes, dilute to 10.0 ml with *methanol* and filter.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm)
- mobile phase: A. *water* and adjust to pH 2.0 with *orthophosphoric acid*.
  B. *methanol*
- a gradient programme using the conditions given below,
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 370 nm,
- injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	60	40
1	60	40
20	45	55
21	0	100
25	60	40
27	60	40

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the percentage content of flavonoids, expressed as flavones glycoside, using the following expression:

$$FG = \frac{5\,(F_1 \times M_1 \times 2.514 \times P)}{F_2 \times M_2}$$

Where, F_1 = Sum of the areas of all the peaks from the peak due to *quercetin* to the peak due to *isorhamnetin* (RRT of about 1.5) in the chromatogram obtained with the test solution.

F_2 = Area of the peak due to *quercetin* in the chromatogram obtained with the reference solution.

M_1 = Mass of *quercetin IPRS* in the reference solution, in grams.

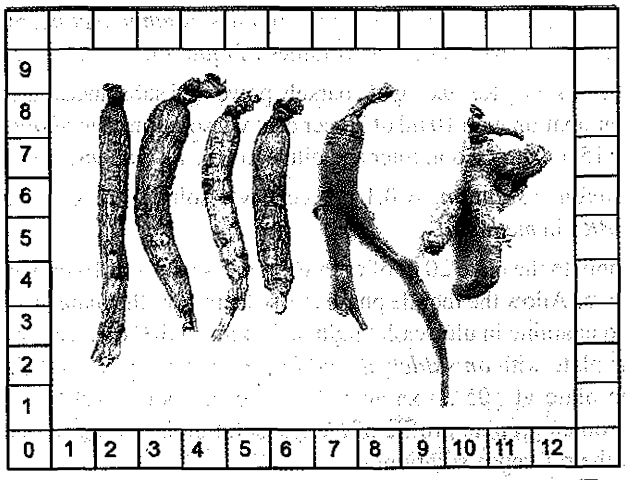
M_2 = Mass of the extract to be examined used to prepare the test solution, in grams.

P = Percentage content of *quercetin IPRS*.

Storage. Store in air tight amber colour containers protected from light, heat and moisture.

Ginseng

Korean ginseng, *Panax ginseng*



Ginseng consists of dried roots of *Panax ginseng* (Fam. Araliaceae).

Ginseng contains not less than 0.2 per cent of ginsenosides Rg1 and not less than 0.1 per cent of ginsenosides Rb1, calculated on dried basis.

Category. Aphrodisiac, anticancer, adoptogenic, antidiabetic and antiviral.

Description. Roots are longitudinally striated, transversely cracked and pale yellow in colour. Fracture short and fibrous, odour characteristic and taste bitter.

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Identification

A. *Macroscopic* — The dried root is cylindrical, sometimes branched, up to about 20 cm long and 2.5 cm in diameter, and may be curved or markedly re-curved. The surface is pale yellow to cream in white ginseng, brownish-red in red ginseng and shows longitudinal ridges. Stem scars may be seen at the crown. The fracture is short. The transversely-cut surface shows a wide outer zone with scattered orange-red resin canals and a finely radiate inner region. The rootlets, numerous in the lower part of white ginseng, are normally absent in red ginseng.

B. *Microscopic* — The powder is light yellow. When examined under a microscope it shows the following diagnostic characters: abundant fragments of thin-walled parenchymatous cells and fragments of large secretory canals containing yellowish-brown resin, non- lignified tracheids and partially-lignified vessels with spiral or reticulate thickening, isolated or in groups; scattered cluster crystals of calcium oxalate. Examine under a microscope using a mixture of equal volumes of *glycerol* and *water*. The starch granules are very abundant, simple or 2 or 3 compound, and range from 1-10 µm in diameter. In red ginseng the starch granules are often deformed and destroyed by treating with steam, or may be absent.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 25 volumes of *ethyl acetate,* 50 volumes of *water* and 100 volumes of *butanol.*

Test solution. Reflux 1 g of coarsely powdered substance under examination with 10 ml of 70 per cent v/v solution of *methanol* for 15 minutes. Cool, filter and dilute to 10.0 ml with *methanol.*

Reference solution. A 0.1 per cent w/v solution of *ginseng IPBRS* in *methanol.*

Apply to the plate 20 µl of each solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in ultraviolet light at 254 nm and 366 nm. Spray the plate with *anisaldehyde-sulphuric acid reagent* and heat the plate at 105°. Examine the plate in visible light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol- soluble extractives (2.6.2). Not less than 22.0 per cent.

Water soluble extractive (2.6.3). Not less than 27.0 per cent.

Total ash (2.3.19). Not more than 7.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Loss on drying (2.4.19). Not more than 10.0 per cent determined on 1.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by liquid chromatography (2.4.14)

Solvent mixture. 4 volumes of *ethanol* and 6 volumes of *water.*

Test solution. Reflux 1 g of coarsely powdered substance under examination two times with 50 ml of solvent mixture for 1 hour, cool and filter. Combine the filtrate and evaporate to dryness under reduced pressure. Dissolve the residue in 10 ml of solvent mixture.

Reference solution. Dissolve 2.0 mg, each of, *ginsenoside Rg1 IPRS* and *ginsenoside Rb1 IPRS* in 10.0 ml of solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5µm) (Such as Eclipse Plus C18),
- Guard column 4.6 mm x 12.5 mm x 5 µm (Such as Zorbax ODS)
- column temperature: 25°
- mobile phase: A. *water*
  B. 4 volumes of *acetonitrile and* 1 volume of *water,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 203 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injection is not more than 2.0 per cent of Rg1 and Rb1.

Inject the reference solution and the test solution.

Calculate the content of ginsenosides Rg1 and ginsenosides Rb1.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Ginseng Dry Extract

Ginseng Dry Extract is obtained from dried Ginseng root of *Panax ginseng* (Fam. Araliaceae) with aqueous ethanol or ethanol or any other solvent and evaporation of solvent.

Ginseng Dry Extract contains not less than 4.0 per cent of total ginsenosides, calculated on the dried basis.

Category. Aphrodisiac, anticancer, adoptogenic, antidiabetics.

Description. A light brownish-yellow, hygroscopic powder or brittle mass.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 25 volumes of *ethyl acetate,* 50 volumes of *water* and 100 volumes of *butanol.*

Test solution. Dissolve 0.15 g of the extract under examination in 10 ml of *methanol.*

Reference solution. A 0.1 per cent w/v solution of *ginseng IPBRS* in *methanol.*

Apply to the plate 10 µl of each solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in ultraviolet light at 254 nm and 366 nm. Spray the plate with *anisaldehyde-sulphuric acid reagent* and heat the plate at 105° for 10 minute. Examine the plate in visible light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 7.0 per cent determined on 1.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by liquid chromatography (2.4.14)

Solvent mixture. 4 volumes of *ethanol* and 6 volumes of *water.*

Test solution. Dissolve 50 mg of extract under examination with 5 ml of *solvent mixture* and sonicate for 20 minutes and filter through 0.45µ nylon filter.

Reference solution. Dissolve 50 mg of *ginseng IPBRS* in 5 ml of *solvent mixture* and sonicate for 20 minutes and filter through 0.45µ nylon filter.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5µm) (Such as Eclipse Plus C18),
- Guard column 4.6 mm x 12.5 mm x 5 µm (Such as Zorbax ODS)
- column temperature: 25°
- mobile phase: A. *water*
  B. 4 volumes of *acetonitrile and* 1 volume of *water,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 203 n
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	M (p
0	76	
12	76	
28	65	
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

The relative retention time with reference to Ginsenosides Rg1 (Retention time is about 18.44 minutes)

Name	Relative retention time
Ginsenosides Re	1.04
Ginsenosides Rf	1.63
Ginsenosides Rb1	2.15
Ginsenosides Rc	2.27
Ginsenosides Rb2	2.38
Ginsenosides Rd	2.61

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injection is not more than 2.0 per cent Rg1 and Rb1.

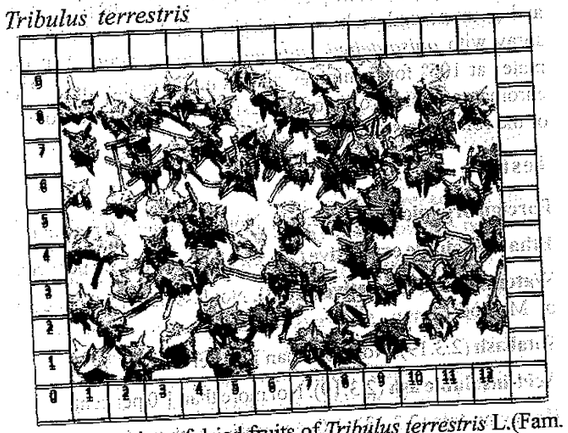
Inject the reference solution and the test solution.

Calculate the total content of ginsenosides.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Gokhru

Tribulus terrestris



Gokhru consists of dried fruits of *Tribulus terrestris* L.(Fam. Zygophyllaceae).

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Gokhru contains not less than 0.5 per cent of diosgenin, calculated on the dried basis.

Category. Diuretic, antiinflammatory, anthelmintic, potent aphrodisiac, kasa.

Description. Pedicellate and globose fruits, having wedge-shaped cocci, covered with short and stiff spines. Possesses faintly aromatic smell and acrid taste.

Identification

A. *Macroscopic*— Fruit is pedicellate, having wedge-shaped cocci, covered with spines. Surface of schizocarp is rough.

B. *Microscopic* — Pericarp is differentiated into epicarp, mesocarp and endocarp. Epicarp is surrounded by non-glandular trichomes.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 8 volumes of *toluene* and 2 volumes of *ethyl acetate*.

Test solution. Reflux 5 g of coarsely powdered substance under examination with 50 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 50 ml of *methanol*, cool and filter. Combine both the filtrates and concentrate under vacuum to dryness. Extract the dried residue with 10 ml of *methanol* at 50° for 10 minutes, filter the solution and use filtrates for analysis.

Reference solution. Reflux 2.5 g of *gokhru IPBRS* with 50 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 50 ml of *methanol*, cool and filter. Combine both the filtrates and concentrate under vacuum to dryness. Extract the dried residue with 5 ml of *methanol* at 50° for 10 minutes, filter the solution and use the filtrates for analysis.

Apply to the plate 20 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 105° for 5 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 3.0 per cent.

Water-soluble extractive (2.6.3). Not less than 15.0 per cent by Method I.

Total ash (2.3.19). Not more than 11.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 5.0 g of the substance under examination with 50 ml of *sulphuric acid (10 per cent)* for 4 hours. Cool and transfer to separating funnel. Extract with 50 ml of *ethyl acetate*. Repeat the extraction 3 times. Pass the *ethyl acetate* layer through *sodium sulphate* and evaporate. Dissolve the residue with 50 ml of *methanol*.

Reference solution. A 0.1 per cent w/v solution of *diosgenin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: 80 volumes of *acetonitrile* and 20 volumes of *methanol*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the test solution.

Calculate the content of diosgenin.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Green Coffee Bean Extract

Coffea arabica

Green Coffee Bean Extract is obtained by extracting green coffee bean (*Coffea arabica* Linn. Gaertner Fam. Asteraceae) with *methanol* or any other suitable solvent.

Green Coffee Bean Extract contains not less than 4.0 per cent w/w of chlorogenic acid, calculated on the dried basis.

Category. Central Nervous System Stimulant.

Description. A greenish brown dry powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 10 volumes of *ethyl acetate*, 2.5 volumes of *dichloromethane*, 1 volume of *formic acid*, 1 volume of *acetic acid* and 1.1 volume of *water*.

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Test solution. Dissolve about 500 mg of the extract under examination in *methanol*, sonicate for 5 minutes and dilute to 50 ml with same solvent and filter.

Reference solution. A 0.02 per cent mix solution of *theophylline IPRS* and *chlorogenic acid IPRS* in the *methanol*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm, allow the mobile phase to rise 8 cm. Dry the plate in air and examine the plate under ultraviolet light at 254 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Total ash (2.3.19). Not more than 10.0 per cent .

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 1.0 g by drying in an oven at 105° for 3 hours.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 50 mg of the substance under examination in 25 ml of *methanol* then sonicate for 5 minutes and dilute to 50.0 ml with same solvent, and filter.

Reference solution. Dissolve about 10 mg *chlorogenic acid IPRS* in 50 ml of *methanol*, sonicate for 5 minutes, dilute 5.0 ml of this solution to 25.0 ml with same solvent, and filter.

Chromatographic system

- a stainless steel column 250 cm x 4.0 mm packed with octadecylsilane bound to porous silica (5 µm),
- mobile phase: A. 0.1 per cent v/v *trifluoroacetic acid* in *water*,

  B. 0.1 per cent v/v *trifluoroacetic acid* in *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 340 nm,
- injection volume: 20 µl.

Time (in min.)	mobile phase A (per cent v/v)	mobile phase B (per cent v/v)
0.02	90	10
21.0	73	27
26.0	60	40
31.0	90	10
40.0	90	10

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of chlorogenic acid.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Guar Gum

Guar Gum is a gum obtained from the ground endosperms of the seeds of *Cyamopsis tetragonolobus* (Linn.) Taub or other species of *Cyamopsis* (Fam. Leguminosae). It consists mainly of a high molecular weight hydrocolloidal polysaccharide, composed of galactan and mannan units combined through glycosidic linkages.

Category. Laxative, antidiabetic, antiatherosclerotic.

Description. An almost white to pale yellowish white powder; odour, characteristic.

Identification

A. When mounted in *lactophenol* and examined under a microscope, irregular, angular particles of various sizes and shapes are seen.

B. To 0.1 g add 1 ml of *0.2 M iodine*; the mixture does not acquire an olive-green colour.

C. Dissolve 0.1 g in 20 ml of *water* by shaking and add 0.5 ml of *hydrogen peroxide solution (20 vol)* and 0.5 ml of a 1 per cent w/v solution of *benzidine* in *ethanol (90 per cent)*, shake and allow to stand; no blue colour is produced (distinction from acacia).

D. Mount a small quantity in *ruthenium red solution* and examine under a microscope; the particles do not acquire a pink colour (distinction from sterculia gum and agar).

E. To 2 ml of a 0.5 per cent w/v solution add 2 ml of a 20 per cent w/v solution of *lead acetate*; a flocculent precipitate is produced (distinction from acacia, ghatti gum and sterculia).

Tests

Acidity or alkalinity. A 0.5 per cent w/v solution is neutral to *litmus paper*.

Tannin. To 5 ml of a 0.5 per cent w/v solution add 0.1 ml of *ferric chloride test solution*; no bluish black colour is produced.

Arsenic (2.3.10). Mix 3.3 g with 3 g of *anhydrous sodium carbonate*, add 10 ml of *bromine solution* and mix thoroughly. Evaporate to dryness on a water-bath, gently ignite and dissolve the cooled residue in a mixture of 16 ml of *brominated hydrochloric acid* and 45 ml of *water*. Remove the excess of bromine with 2 ml of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (3 ppm).

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Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Protein. Not more than 5.0 per cent, determined by the following method. Carry out the determination of nitrogen (2.3.30), using about 3.5 g, accurately weighed, and multiplying the percentage of nitrogen determined by 6.25 to obtain the percentage of protein.

Acid-insoluble matter. Not more than 3.0 per cent, determined by the following method. Weigh about 1.5 g and disperse in 150 ml of *water* and 1.5 ml of *sulphuric acid*. Warm on a water-bath for 6 hours, replacing the water lost by evaporation. Add about 0.5 g of a suitable filter-aid, accurately weighed, and filter through a suitable ashless filter paper. Wash the residue several times with hot *water*, dry the filter and its contents at 105° for 3 hours. Cool in a desiccator, weigh and subtract the weight of the filter aid.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 5×10^3 CFU per g. 1 g is free from *Escherichia coli* and 10 g is free from *Salmonella*.

Total ash (2.3.19). Not more than 2.0 per cent, determined on 1.0 g.

Loss on drying (2.4.19). Not more than 13.0 per cent, determined on 0.5 g by drying in an oven at 105°.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Gudmar Dry Extract

Gymnema sylvestre

Gudmar Dry Extract is obtained by extracting Gudmar with *aqueous ethanol* or *ethanol* or any other suitable solvent and evaporation of solvent.

Gudmar Dry Extract contains not less than 90.0 per cent w/w and not more than 120.0 per cent w/w of stated amount of *gymnemic acids* (calculated as *gymnemagenin*), calculated on the dried basis.

Category. Antidiabetic, febrifuge, antiinflammatory, diuretic, meha.

Description. A greenish brown to green powder

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 8 volumes of *chloroform*, 15 volumes of *methanol* and 5 volumes of *formic acid*.

Test solution (a). Reflux 1 g of the extract under examination with 25 ml of *ethanol (50 per cent v/v)* for 1 hour, cool and add 10 per cent *hydrochloric acid* until the precipitation is complete. Centrifuge and collect the precipitate. Dissolve the precipitate in 50 ml of *ethanol (50 per cent v/v)* and filter.

Test solution (b). Dissolve about 500 mg of the extract under examination in 20 ml of *ethanol (50 per cent v/v)* with the aid of ultrasound. Add 2 ml of 12 per cent w/v *potassium hydroxide solution* and reflux for 1 hour. Cool and add 5.5 ml of *4 M hydrochloric acid* and reflux for 1 hour. Cool and adjust the pH to 7.5 - 8.5 with 12 per cent w/v *potassium hydroxide solution*. Dilute the solution with *ethanol (50 per cent v/v)* to 100 ml and filter.

Reference solution. A 0.01 per cent w/v solution of *gymnemagenin IPRS* in *ethanol (50 per cent v/v)*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with 10.0 per cent *methanolic sulphuric acid*. Heat the plate at 120° for 20 minutes and examine in day light. The chromatogram obtained with test solution (b) after saponification followed by hydrolysis shows band corresponding to the band obtained using reference solution indicating the presence of *gymnemagenin* and the chromatogram obtained with test solution (a) does not show *gymnemagenin* however few other bands are visible.

Tests

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent determined on 5.0 g by drying in oven at 105° .

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 250 mg of the extract under examination in 20 ml of *ethanol (50.0 per cent v/v)* by sonication. Add 2 ml of 12 per cent w/v *potassium hydroxide solution* and reflux for 1 hour. Cool and add 5.5 ml of *4 M hydrochloric acid* and reflux for 1 hour. Cool and adjust the pH 7.5 to 8.5 with 12 per cent w/v *potassium hydroxide solution*. Dilute the solution with *ethanol (50 per cent v/v)* in a 100.0 ml of volumetric flask and filter.

Reference solution. A 0.01 per cent w/v solution of *gymnemagenin IPRS* in *ethanol* (50.0 per cent v/v).

Chromatographic system

– a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),

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mobile phase: A. a buffer solution prepared by dissolving 0.136 g of *potassium dihydrogen orthophosphate* in 500 ml *water* and 0.5 ml of *orthophosphoric acid*, dilute to 1000 ml with *water*.

B. *acetonitrile*,

a gradient programme using the conditions given below,

flow rate: 1.6 ml per minute,

spectrophotometer set at 210 nm,

injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	75	25
15	45	55
20	40	60
25	40	60
30	45	55
35	75	25
40	75	25

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

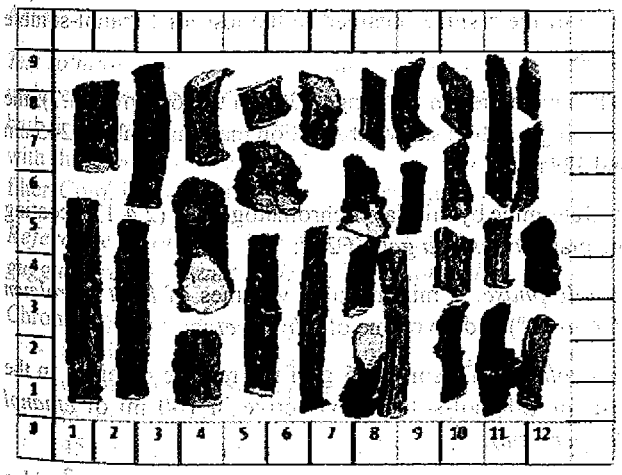
Inject the reference solution and the test solution.

Calculate the content of gymnemic acid as gymnemagenin.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Guduchi

Giloe; Amrita; *Tinospora cordifolia*



Guduchi consists of the dried, mature pieces of stem of *Tinospora cordifolia* (Willd.) Miers (Fam. Menispermaceae). Guduchi contains not less than 0.02 per cent w/w of cordifolioside A, calculated on the dried basis.

Category. Immunomodulator, hepatoprotective, antipyretic, analgesic, kustha, rasayana.

Description. A greyish-black in colour, fibrous fracture and no distinct odour with bitter taste.

Identification

A. *Macroscopic* — Stem-pieces glabrous, cylindrical, solid, lenticillate, 5-15 mm in diameter having light brown surface marked with warty protuberances due to circular lenticels. Transversely smoothened surface shows a radial structure with conspicuous medullary rays traversing porous tissues.

B. *Microscopic* — Transverse section of stem shows outermost layer of cork which is differentiated in to outer zone of thick walled, compressed cells and inner zone of thin walled, tangential cells. Cork broken at some places due to lenticels. Cortex consists of 3-5 rows of irregularly arranged tangential, chlorenchymatous cells with numerous intercellular spaces. Inner cortex filled with plenty of starch gains. Vascular zone consists of 10-12 wedge-shaped strips of xylem externally surrounded by semi-circular strips of phloem. Cambium composed of one to two layers of tangentially elongated cells. Primary phloem appears crushed and obliterated; secondary phloem groups are massive. Pith composed of large, thin walled cells mostly containing starch gains.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 85 volumes of *chloroform* and 15 volumes of *methanol*.

Test solution. Reflux 2 g of the coarsely powdered substance under examination with 25 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 × 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 5 ml.

Reference solution. Reflux 2 g of the *guduchi IPBRS* with 25 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 × 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 110° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

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Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 1.5 per cent.

Water-soluble extractive (2.6.3). Not less than 9.0 per cent by Method I.

Total ash (2.3.19). Not more than 10.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 2 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water-bath for 15 minutes, cool and filter. Reflux the residue further with *methanol* till the extract turns colourless, cool and filter. Combine all the filtrates and concentrate to a volume slightly less than 25 ml. Dilute with *methanol* to 25.0 ml.

Reference solution. A 0.004 per cent w/v solution of *cordifolioside A IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. *water*
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	80	20
25	20	80
30	80	20

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

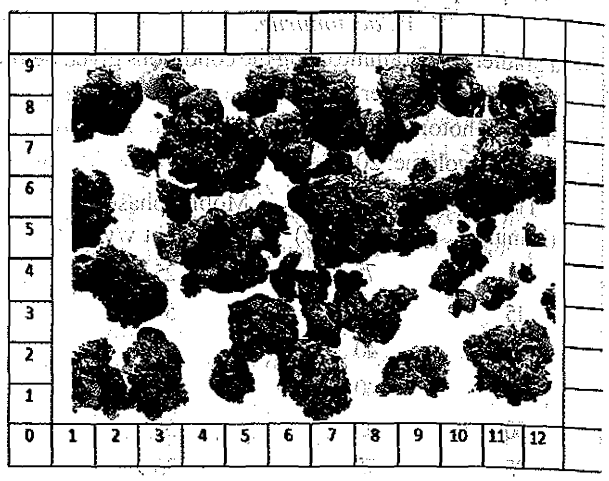
Inject the reference solution and the test solution.

Calculate the content of cordifolioside A.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Guggul Resin

Guggul; *Commiphora wightii*



Guggul Resin is the oleoresin exudation from *Commiphora wightii* (Arnott) Bhandari (*Commiphora mukul* (Arn.) Bhandari, *Balsamodendron mukul* Hook. ex Stocks) (Fam. Burseraceae).

Guggul Resin contains not less than 1.0 per cent w/w and not more than 1.5 per cent w/w of guggulsterones (Z and E).

Category. Antihyperlipidaemic, antirheumatic, aphrodisiac, neuroprotective, vatavyadhi.

Description. Light to dark-brown conglomerates of tears, rounded or irregular, slightly sticky to touch; odour, faintly balsamic.

Identification

A. Prepare a 0.005 per cent w/v solution in *ethanol (95 per cent)* of the residue obtained in the test for Ethanol-soluble extractive.

When examined in the range 230 nm to 360 nm (2.4.7), the resulting solution shows absorption maxima at about 245 nm and 327 nm.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 3 volumes of *light petroleum (60° to 80°)* and 1 volume of *ethyl acetate*.

Test solution. Dissolve 0.5 g of the residue obtained in the test for Ethanol-soluble extractive in 100 ml of *ethanol (95 per cent)*.

Reference solution. A 0.5 per cent w/v solution of the residue obtained similarly from *guggul resin IPBRS* in *ethanol (95 per cent)*.

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Apply to the plate 20 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air until the odour of the solvent is no longer detectable and examine under ultraviolet light at 254 nm and 366 nm, spray with a 10 per cent v/v solution of *sulphuric acid* in *methanol*. Heat the plate at 100° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Ethyl acetate-soluble extractive. Not less than 25.0 per cent, determined by the following method. Crush the substance under examination to a coarse powder. Shake 5.0 g of the powder with 25 ml of *light petroleum (60° to 80°)* for 1 hour and separate the liquid by filtration. Repeat the extraction twice and dry the defatted material over *phosphorus pentoxide* at room temperature at a pressure not exceeding 2.75 kPa for 8 hours. Crush the dried material and extract with four quantities, each of 25 ml, of *ethyl acetate* by shaking each time for 1 hour followed by filtration through a sintered-glass funnel (porosity No. 3) and combining the filtrates. Evaporate the combined filtrates, dry over *phosphorus pentoxide* at room temperature at a pressure not exceeding 2.75 kPa for 12 hours and weigh.

Ethanol-soluble extractive (2.3.46). Not less than 35.0 per cent, determined by the following method. Crush the substance under examination to a coarse powder. Macerate 5.0 g of the powder with 100 ml of *ethanol (95 per cent)* in a closed flask for 24 hours, shaking frequently during the first 6 hours and allowing to stand for 18 hours. Filter rapidly taking care to avoid loss of ethanol, evaporate 25 ml of the filtrate to dryness, dry at 105° and weigh.

Sulphated ash (2.3.18). Not more than 10.0 per cent, determined on 0.5 g.

Assay. Determine by liquid chromatography (2.3.14).

Test solution. Weigh accurately about 3.0 g of the substance under examination, add 50 ml of *acetonitrile*, reflux on a water-bath for 30 minutes, cool and filter. Reflux the residue further with three portions, each of 30 ml, of *acetonitrile*, cool and filter. Combine the filtrates and concentrate to 100.0 ml.

Reference solution. A 0.02 per cent w/v solution of *guggulsterones (Z and E) IPRS* in *acetonitrile*.

Chromatographic system
- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane silica gel (5 µm),
- mobile phase: a filtered and degassed mixture of 45 volumes of *acetonitrile* and 55 volumes of *water*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 242 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative retention times are about 0.69 for guggulsterones E and 1.0 for guggulsterones Z and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the contents of guggulsterones (Z and E).

Storage. Store protected from light at a temperature not exceeding 30°.

Guggulipid

Guggulipid is the ethyl acetate extractive of Guggul Resin.

Guggulipid contains not less than 4.0 per cent w/w and not more than 6.0 per cent w/w of guggulsterones (*Z* and *E*).

Category. Antihyperlipidaemic, antirheumatic, aphrodisiac, vatavyadhi, medohara.

Description. A brown, viscous liquid.

Identification

A. When examined in the range 230 nm to 360 nm (2.4.7), a 0.005 per cent w/v solution in *chloroform* shows absorption maxima at about 245 nm and 327 nm; absorbance at about 245 nm, about 0.87 and at about 327 nm, about 0.52.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 3 volumes of *light petroleum (60° to 80°)* and 1 volume of *ethyl acetate*.

Test solution. Dissolve 0.25 g of the substance under examination in 100 ml of *ethanol (95 per cent)*.

Reference solution. A 0.25 per cent w/v solution of *guggulipid IPBRS* in *ethanol (95 per cent)*.

Apply to the plate 5 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air until the odour of the solvent is no longer detectable and examine under ultraviolet light at 254 nm and 366 nm, spray with a 10 per cent v/v solution of *sulphuric acid* in *methanol*. Heat the plate at 100° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh about 0.75 g of the substance under examination, add 50 ml of *acetonitrile* and warm on a boiling water-bath for 10 minutes. Cool and add sufficient *acetonitrile* to produce 100 ml.

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Reference solution. A solution containing 0.02 per cent w/v of *guggulsterones (Z and E)* in *acetonitrile*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane silica gel (5 µm),
- mobile phase: a filtered and degassed mixture of 45 volumes of *acetonitrile* and 55 volumes of *water*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 242 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative retention times are about 0.69 for guggulsterones E and 1.0 for guggulsterones Z and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the contents of guggulsterones (Z and E).

Storage. Store protected from light at a temperature not exceeding 30°.

Guggulipid Tablets

Guggulipid Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of guggulsterones (*Z* and *E*). The tablets may be coated.

Category. Antihyperlipidaemic, antirheumatic, aphrodisiac, vatavyadhi, medohara.

Identification

Extract a quantity of the powdered tablets containing about 20 mg of guggulsterones (*Z* and *E*) with two quantities, each of 15 ml, of *ethyl acetate*, combine the extracts, filter and evaporate to dryness. The residue complies with the following tests.

A. When examined in the range 230 nm to 360 nm (2.4.7), a 0.005 per cent w/v solution in *chloroform* shows absorption maxima at about 245 nm and 327 nm; absorbance at about 245 nm, about 0.87 and at about 327 nm, about 0.52.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase. A mixture of 3 volumes of *light petroleum (60° to 80°)* and 1 volume of *ethyl acetate*.

Test solution. A 0.25 per cent w/v solution of the substance under examination in *ethanol (95 per cent)*.

Reference solution. A 0.25 per cent w/v solution of *guggulipid IPBRS* in *ethanol (95 per cent)*.

Apply to the plate 5 µl of each solution. After development, dry the plate in air until the odour of the solvent is no longer detectable and spray with 50 per cent w/v solution of *sulphuric acid*. The principal spots in the chromatogram obtained with the test solution correspond to those in the chromatogram obtained with the reference solution.

Tests

Disintegration (2.5.1). Not more than 60 minutes.

Other tests. Comply with the tests stated under Tablets.

Assay. Determine by liquid chromatography (2.4.13).

Test solution. Weigh and powder 20 tablets. Weigh accurately a quantity of the powder containing about 10 mg of guggulsterones (Z and E) and extract with five quantities, each of 20 ml, of *acetonitrile*, with the aid of heat. Combine the extracts and concentrate to 50 ml. Filter the solution through a membrane filter disc with an average pore diameter not greater than 1.0 µm and use the filtrate.

Reference solution. A solution containing 0.02 per cent w/v of *guggulsterones (Z and E)* in *acetonitrile*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilyl silica gel (5 µm),
- mobile phase: a filtered and degassed mixture of 45 volumes of *acetonitrile* and 55 volumes of *water*,
- flow rate: 2 ml per minute,
- spectrophotometer set at 242 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative retention times are about 0.69 for guggulsterones E and 1.0 for gugulsterone Z and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the contents of guggulsterones (Z and E) in the tablets.

Storage. Store protected from light at a temperature not exceeding 30°.

Labelling. The label states the strength in terms of the equivalent amount of guggulsterones (*Z* and *E*).

Haridra Dry Extract

Haridra Dry Extract is a partially purified natural complex of diaryl heptanoid derivatives isolated by extracting Haridra *(Curcuma longa* L., Fam. Zingiberaceae), rhizomes with *acetone* or *ethanol* or any other suitable solvent and evaporation of solvent under vacuum.

Haridra Dry Extract contains not less than 95.0 per cent w/w and not more than 102.0 per cent w/w of the stated amount of total curcuminoids calculated on the dried basis, as the sum of curcumin, demethoxycurcumin and bisdemethoxycurcumin. It contains not less than 70.0 per cent w/w to not more than 80.0 per cent w/w of curcumin, not less than 15.0 per cent w/w to not more than 25.0 per cent w/w of demethoxycurcumin and not less than 2.5 per cent w/w to not more than 6.5 per cent w/w of bisdemethoxycurcumin.

Category. Antiinflammatory, galactogogue, stomachic, spasmolytic, visavikara.

Description. An orange yellow crystalline powder.

Identification

A. Determine by thin-layer chromatography (2.4.17) coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 10 volumes of *chloroform*, 1.0 volume of *methanol*, 0.5 volume of *glacial acetic acid* and 0.5 volume of *formic acid*.

Test solution. Dissolve about 50 mg of the extract under examination with 100.0 ml of *methanol* and filter.

Reference solution. A solution containing 0.01 per cent w/v each of *bisdemethoxycurcumin IPRS, demethoxycurcumin IPRS, curcumin IPRS* in the *methanol*.

Apply to the plate 10 µl of each of solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. Spray the plate with a *anisaldehyde- sulphuric acid reagent*. Heat the plate at 110° for 10 minutes and examine the plate at 366 nm and day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Sulphated ash (2.3.18). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 2.0 per cent, determined on 1 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Total curcuminoids — Determine by liquid chromatography (2.4.14).

Test Solution. Dissolve a quantity of the extract under examination containing about 50 mg of curcumin in 100 ml of *tetrahydrofuran*. Dilute 5.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution. A solution containing 0.04 per cent w/v of *curcumin IPRS*, 0.01 per cent w/v of *demethoxycurcumin IPRS* and 0.002 per cent w/v of *bisdemethoxycurcumin IPRS* in *tetrahydrofuran*. Dilute 5 ml of this solution to 50 ml with the mobile phase.

Chromatographic system
- a stainless steel column, 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5µm),
- mobile phase: a mixture of 56 volumes of 0.1 per cent w/v solution of *citric acid* and 44 volumes of *tetrahydrofuran*,
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 420 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent. The relative retention time with reference to curcumin for bisdemethoxycurcumin is about 1.24 and for demethoxycurcumin is about 1.12.

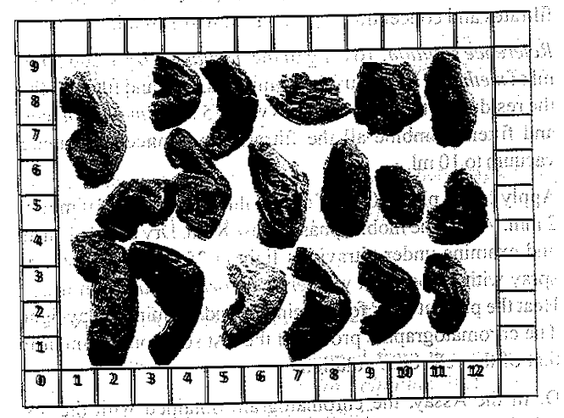
Inject the reference solution and the test solution.

Calculate the content of the bisdemethoxycurcumin, demethoxycurcumin and curcumin in extract.

Storage. Store protected from heat and moisture.

Haritaki

Harad; Chebulic myrobalan; *Terminalia chebula*



Haritaki consists of pericarp of the dried fruit of *Terminalia chebula* Retz. (Fam. Combretaceae).

Haritaki contains not less than 5 per cent w/w of chebulagic acid and not less than 12.5 per cent w/w of chebulinic acid, calculated on the dried basis.

Category. Purgative, astringent, antiageing, resortative, rasayana.

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Description. It has a shine on its external part and has longitudinal ridges. The colour varies from yellowish brown to light black. It has a astringent taste and is also slightly bitter.

Identification

Test C may be omitted if tests A, B and D are carried out and test D may be omitted if tests A, B and C are carried out.

A. *Macroscopic* — The fruit is 2 to 3 cm in length and 1 to 2 cm in diameter with hard stony appearance. Externally it is shining and is adorned with longitudinal ridges. Color of the fruit rind varies from yellowish brown, uniform brown to light black. Internally the fruit is light yellow.

B. *Microscopic* — Epicarp has thick walls covered with cuticle. The mesocarp has many stone cells of various sizes and shapes which forms a reticulum. Large quantity of tannin is present in the mesocarp. Simple starch granules are present in plenty.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 35 volumes of *toluene*, 50 volumes of *acetone*, 15 volumes of *glacial acetic acid* and 5 volumes of *formic acid*.

Test solution. To 1 g of the coarsely powdered substance being examined, add 50-75 ml of *methanol* and reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 75 ml of *methanol*, cool and filter. Combined all the filtrates and concentrate under vacuum to 100 ml.

Reference solution. To 0.1 g of the *haritaki IPBRS*, add 50-75 ml of *methanol* and reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 75 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with 10 per cent w/v *ferric chloride solution* in *water*. Heat the plate at 100° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

D. In the Assay, the chromatogram obtained with the test solution corresponds to the chromatogram obtained with reference solution (a).

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 35.0 per cent.

Water-soluble extractive (2.6.3). Not less than 50 per cent by Method I.

Total ash (2.3.19). Not more than 6.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm)

Loss on drying (2.4.19). Not more than 12 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh 0.5 g of coarsely powdered sample, add 50 ml of *water*, sonicate for 3 minutes and heat on a boiling water bath for 15 minutes, cool and dilute to 100.0 ml with *water* and filter. Dilute 10.0 ml of the solution to 25.0 ml with *water*.

Reference solution (a). Weigh 0.5 g of *haritaki IPRS*, add 50 ml of *water*, sonicate for 3 minutes and heat on a boiling water bath for 15 minutes, cool and dilute to 100.0 ml with *water* and filter. Dilute 10.0 ml of the solution to 25.0 ml with *water*.

Reference solution (b). A 0.1 per cent w/v solution of *chebulagic acid IPRS* in *water*.

Reference solution (c). A 0.1 per cent w/v solution of *chebulinic acid IPRS* in *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution pH 2.5 prepared by dissolving 0.136 g of *potassium dihydrogen orthophosphate* in 500 ml of *water*, add 0.5 ml of *orthophosphoric acid* and make upto 1000 ml with *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
18	65	35
25	45	55
28	45	55
35	95	5

Inject reference solutions (b) and (c). The relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

Calculate the content of chebulagic acid and chebulinic acid.

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Storage. Store protected from heat, moisture and against attack by insects and rodents.

Haritaki Extract

Haritaki extract is obtained by extracting Haritaki (*Terminalia chebula* Retz., Fam. Combretaceae) from the dried fruit pericarp with *ethanol* or any other suitable solvent.

Haritaki extract contains not less than 90.0 per cent w/w and not more than 120.0 percent w/w of the stated amount of chebulinic acid and chebulagic acid.

Category. Purgative, astringent, antiageing, resortative, rasayana.

Usual strength. 15 per cent w/w.

Description. Light yellowish to yellowish brown powder with odour, characteristic; taste, bitter.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 6 volumes of *ethyl acetate*, 2 volumes of *formic* acid, 2 volumes of *toluene* and 1 volume of *methanol.*

Test solution. Dissolve 0.5 g of extract under examination with 100 ml *methanol* and filter.

Reference solution. A. 0.1 per cent w/v solution of *chebulagic acid IPRS and* 0.1 per cent w/v solution of *chebulinic acid IPRS* in *methanol.*

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in ultra-violet light at 254 nm. Spray the plate with *ferric chloride reagent.* Heat the plate at 110° for 10 minutes and examine the plates at 366 nm and in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

B. In the assay, the peaks due to chebulinic acid and chebulagic acid in the chromatogram obtained with test solution corresponds to the peak obtained with the reference solutions.

Tests

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 2.0 g by drying in an oven at 105° for 3 hours.

Total ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, (Method B) 20 ppm.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 0.5 g of the extract or quantity equivalent to 100 mg of polyphenols in water, make up to 100 ml and filter.

Reference solution (a). 0.01 per cent w/v solution of *chebulagic acid IPRS* in *water.*

Reference solution (b). 0.01 per cent w/v solution of *chebulinic acid IPRS* in *water.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase:A. a buffer solution prepared by dissolving 0.136 g of *potassium di-hydrogen orthophosphate* in 500 ml of *water,* add 0.5 ml of *orthophosphoric acid* and dilute to 1000 ml with water,

  B. *acetonitrile,*
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
18	75	25
25	65	35
28	65	35
35	95	5

Inject reference solution (a) and (b). The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of chebulinic acid and chebuliagic acid.

Storage. Store protected from heat and moisture.

Haritaki Aqueous Extract

Haritaki Aqueous Extract is obtained from the dried fruit pericarp of Haritaki (*Terminalia chebula* Retz., Fam. Combretaceae) by extraction with water.

Haritaki Aqueous Extract contains not less than 90.0 per cent w/w and not more than 120.0 percent w/w of the stated amount of total polyphenols (sum of chebulagic acid, chebulic acid, gallic acid, corlagin, 1,3,6 trigalloyl glucose and ellagic acid).

Category. Purgative, astringent, antiageing, resortative, rasayana.

Usual strength. 40 per cent w/w.

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Description. Brown to brown powder; with characteristic odour and bitter taste.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 6 volumes of *ethyl acetate*, 2 volumes of *formic* acid, 2 volumes of *toluene* and 1 volume of *methanol*.

Test solution. Dissolve 0.5 g of extract under examination with 100 ml *methanol* and filter.

Reference solution. A 0.1 per cent w/v solution of *chebulagic acid IPRS* and 0.1 per cent w/v solution of *gallic acid* in *methanol*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in ultra-violet light at 254 nm. Spray the plate with *ferric chloride reagent* . Heat the plate at 110° for 10 minutes and examine the plates under 365nm and under day light. The chromatographic profile of the test solution is similar to that of the reference solution.

B. In the assay the peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the chebulinic acid and gallic acid.

Tests

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 2.0 g by drying in an oven at 105° for 3 hours.

Total ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, (Method B) 20 ppm.

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 0.5 g of the extract containing 100 mg of polyphenols in 100 ml of water,and filter.

Reference solution (a). 0.01 per cent w/v solution of *chebulagic acid IPRS* in *water*.

Reference solution (b). 0.01 per cent w/v solution of *gallic acid IPRS* in *water*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution prepared by mixtures dissolving 0.136 g of *potassium dihydrogen orthophosphate* in 500 ml of *water*, add 0.5 ml of *orthophosphoric acid* and dilute to 1000 ml with *water*.
  B. *acetonitrile*
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
18	75	25
25	65	35
28	65	35
35	95	5

Inject reference solutions (a) and (b). The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject reference solution (a) and the test solution.

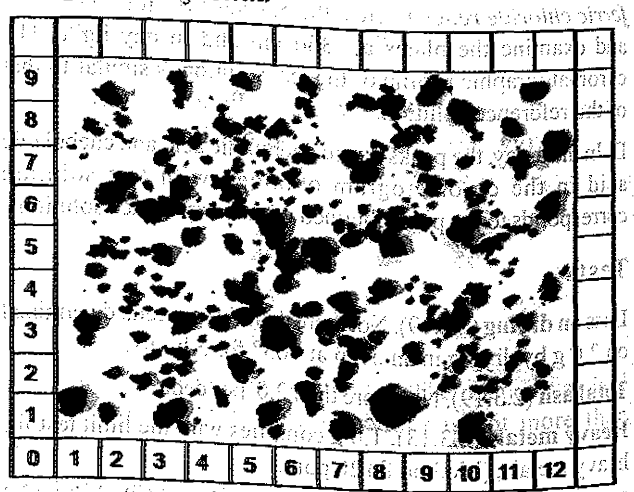
Calculate the content of total polyphenols by summing the peak areas of chebuliagic acid, ellagic acid, 1,3,6 tri galloyl glucose, corilagin, gallic acid and chebulic acid using chebulagic acid. The relative retention times of various polyphenols with reference to gallic acid and chebulagic acid are as follows.

Chebulic acid	0.80	0.25
Gallic acid	1.00	0.32
Corlagin	2.57	0.81
1, 3, 6 Trigalloyl glucose	2.81	0.89
Chebulagic acid	3.17	1.00
Ellagic acid	3.49	1.10
Chebulinic acid	3.64	1.15

Storage. Store protected from heat and moisture.

Hingu

Heeng, *Ferula foetida*



Hingu consists of oleo-gum-resin obtained from rhizomes and roots of *Ferula foetida* Regel. (Fam. Umbelliferae).

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Hingu contains not less than 0.10 per cent w/w of trans-ferulic acid, calculated on the dried basis.

Category. Anti-spasmodic, carminative, expectorant, laxative and sedative.

Description. Externally dark brown, internally yellow when fresh, slowly becoming pink to red, finally reddish brown, odour strongly characteristic and persistent; taste bitter and acrid.

Identification

A. *Macroscopic* — Rounded, flattened or masses of agglutinated tears; yellowish and translucentor milky white, opaque, slowly becoming pink, red, finally reddish brown, soft in texture.

B. Freshly broken surface when treated with sulphuric acid, a bright red or reddish brown colour is produced, changing to violet when acid washed off with water.

C. *Absence of colophony resin* — Triturate 1 g with 10 ml of light petroleum (b.p. 40-60°) for 2 minutes, filter into a test tube and add to the filtrate 10 ml of a fresh 0.5 per cent w/v aqueous solution of copper acetate, shake well and allow the liquids to separate, petroleum layer does not show any green colour indicating absence of colophony resin.

D. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *toluene*, 35 volumes of *ethyl acetate* and 5 volumes of *formic acid*.

Test solution. Extract 1 g of the substance under examination with 20 ml of *ethanol* for 10 minutes with slight warming. Filter and use the filtrate.

Reference solution. Extract 1 g *hingu IPBRS* with 20 ml of *ethanol* for 10 minutes with slight warming. Cool and filter.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, Spray with the *anisaldehyde sulphuric acid reagent* and heat the plate at 105°. Examine the plate in visible light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 12.0 per cent.

Water-soluble extractive (2.6.3). Not less than 15.0 per cent by Method I.

Total ash (2.3.19). Not more than 10.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not more than 10.0 per cent, determined on 0.2 g.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Extract 1 g of the substance under examination with 10 ml of *ethanol* with slight warming for 10 minutes, cool and filter. Extract the residue further with two quantities of 10 ml of *ethanol*, cool and filter. Combine all the filtrates and concentrate to 20.0 ml.

Reference solution. A 0.005 per cent w/v solution of *trans-ferulic acid IPRS* in *ethanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with silica gel consisting of porous spherical particles with chemically bonded nitrile group (5 µm),
- mobile phase: a mixture of 25 volumes of *acetonitrile* and 75 volumes of 10 per cent v/v solution of *acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 319 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of trans-ferulic acid.

Storage. Store protected from light and moisture.

Horse Chestnut Dry Extract

Horse chestnut; *Aesculus hippocastanum*

Horse Chestnut Dry Extract is obtained by extracting fruit of Horse Chestnut (*Aesculus hippocastanum* Fam. Sapindaceae) with methanol or any other suitable solvent.

Horse Chestnut Dry Extract contains not less than 45.0 per cent of the stated amount of total aescin, calculated on the dried basis.

Category. Nervine Antioxidant.

Description. Brown coloured powder, hygroscopic.

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Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 12.8 volumes of *chloroform*, 6.4 volumes of *glacial acetic acid*, 2.4 volumes of *methanol* and 1.6 volumes of *water*.

Test solution. Reflux 1.0 g of coarasely powdered substances under examination with 50 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 x 50 ml of *methanol*, cool and filter. Combined all filtrates and concentrate to 10 ml.

Reference solution. Reflux 0.5 g of *horse chestnut IPBRS* under examination with 50 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 x 50 ml of *methanol*, cool and filter. Combine all filtrates and concentrate to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and spray with *anisaldehyde sulphuric acid* reagent. Heat the plate at 110° for 10 minutes and examine the plate under ultraviolet light at 254 nm and 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Total ash (2.3.19). Not more than 6.0 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 0.05 g of the extract under examination with 50 ml of *methanol*, sonicate for 5 minutes and dilute to 100.0 ml with same solvent.

Reference solution. Dissolve about 0.001 g of *aescin IPRS* in 50 ml of *methanol*, sonicate for 5 minutes and dilute to 100.0 ml with same solvent.

Chromatographic system

- a stainless steel column 250 cm x 4.0 mm packed with octadecylsilane bound to porous silica (5 µm),
- mobile phase: A. 0.3 per cent v/v *orthophosphoric acid* in *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 205 nm;
- injection volume: 20 µl.

Time (in min.)	mobile phase A (per cent v/v)	mobile phase B (per cent v/v)
0.01	75	25
48.0	25	75
50.0	75	25
60.0	75	25

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Calculate the content of total aescin in the extract.

Storage. Store protected from heat and moisture.

Hydrogenated Castor Oil

Castor Wax; Opalwax

Hydrogenated Castor Oil is refined, bleached, hydrogenated and deodorised castor oil. It consists mainly of the triglyceride of hydroxystearic acid.

Category. Pharmaceutical aid.

Description. A white to yellow powder of uniform consistency and texture. It may have a hard, waxy consistency.

Tests

Melting range (2.4.21). 85° to 88°, determined by Method II.

Free fatty acids. Weigh about 20 g, melt on a water-bath, add 75 ml of hot *ethanol (95 per cent)*, previously neutralised to *phenolphthalein solution* with *0.1 M sodium hydroxide*, swirl, add 1 ml of *phenolphthalein solution* and titrate with *0.1 M sodium hydroxide*, swirling vigorously until the solution remains faintly pink after being shaken for 60 seconds; not more than 11.0 ml of *0.1 M sodium hydroxide* is required.

Heavy metals (2.3.13). 2.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Acetyl value (2.3.22). Not less than 143.

Acid value (2.3.23). Not more than 2.0.

Hydroxyl value (2.3.27). 154 to 162.

Iodine value (2.3.28). Not more than 5.0.

Peroxide value (2.3.35). Not more than 5.0.

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Saponification value (2.3.37). 176 to 182.

Storage. Store at a temperature not exceeding 30°. Avoid exposure to excessive heat.

Ipecac Tincture

Cephaelis ipecacuanha

Ipecac Tincture obtained from roots and rhizomes of *Cephaelis ipecacuanha* A. Rich (Fam. Rubiaceae).

Ipecac Tincture contains not less than 90.0 per cent w/v and not more than 110.0 per cent w/v of the stated amount of alkaloids of Ipecac calculated as *emetine*.

Category. Emetic, antiamoebic.

Usual strengths. 0.1 per cent w/v; 0.2 per cent w/v.

Description. A yellowish brown liquid.

Identification

Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 2 volumes of *ammonia*, 15 volumes of *methanol*, 18 volumes of *ethyl acetate* and 65 volumes of *toluene*.

Test solution. Shake 2.0 ml of tincture under examination with 2 ml of water and 0.1 ml of *ammonia*. Add 10 ml of *ether* and shake. Separate the ether layer, dry it over about 2 g of *anhydrous sodium sulphate* and filter.

Reference solution. Dissolve 2.5 mg of *emetine hydrochloride IPRS* in *methanol* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise to 10 cm. Dry the plate in air. Spray the plate with 0.5 per cent w/v solution of *iodine* in *ethanol* (95 per cent v/v) solution. Heat the plate at 60° for 10 min and examine the plate under ultraviolet light at 366 nm and in day light. An intense yellow, fluorescent zone of emetine is observed in chromatogram of test solution corresponding to intense yellow fluorescent zone of emetine in chromatogram of reference solution.

Tests

Ethanol. Not less than 24.0 per cent and not more than 28.0 per cent.

Determine by gas chromatography (2.4.13).

Test solution. A 0.8 per cent v/v solution of the sample under examination in *water* and 0.2 per cent v/v solution of *acetone* as internal standard.

Reference solution. A 0.2 per cent v/v solution of *ethanol* in *water* and 0.2 per cent v/v solution of *acetone* as internal standard.

Chromatographic system

- a stainless steel column 1.8 m x 3 mm, packed with copolymer of ethylvinylbenzene and divinylbenzene (100 to 150 mesh),
- temperature:
  column. 120°,
  inlet port. 210°,
- a flame ionization detector at 210°,
- flow rate: Adjust the carrier flow so that *acetone* internal standard, elutes in 5 to 10 minutes.

Calculate the percentage v/v of ethanol.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Transfer 5 ml of sample into a 50 ml volumetric flask. Dilute to volume with 0.01M *hydrochloric acid* and mix.

Reference solution. A 0.01 per cent w/v solution of *emetine hydrochloride heptahydrate IPRS* in *0.01M hydrochloric acid*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 50 volumes of *methanol* and 50 volumes of buffer prepared by dissolving 2.0 g of *1-heptane sulfonate* in 500 volumes *water*, adjust the pH to 4.0 with *acetic acid*,
- column temperature: 50°,
- flow rate: Adjust the flow rate so that the retention time of *emetine* is about 14 minutes,
- spectrophotometer set at 283 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of cephaeline and emetine as total alkaloids in the sample.

1 mg of *emetine hydrochloride* contains 0.868 mg of *emetine* and correction factor for *cephaeline* is 0.971.

Storage. Store protected from light and moisture.

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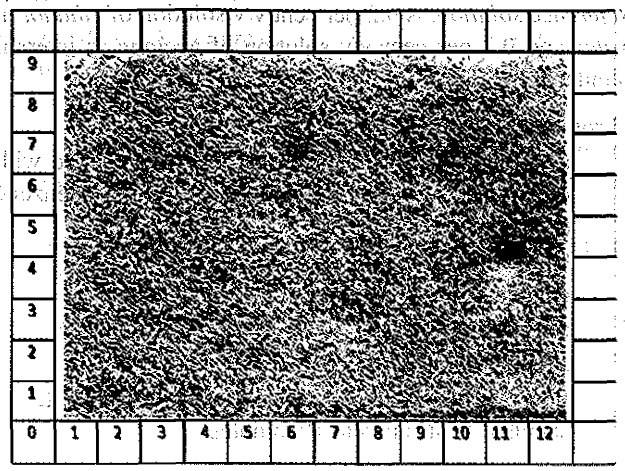
Ispaghula Husk

Isapgol Husk; *Plantago ovata*



Ispaghula Husk consists of the epidermis and collapsed adjacent layers removed from the dried ripe seeds of *Plantago ovata* Forssk. (Fam. Plantaginaceae).

Category. Bulk laxative, antidiarrhoeal.

Description. Pale buff, brittle flakes, more or less lanceolate, up to 2 mm long and 1 mm wide at the centre, much broken into smaller fragments; many of the flakes having a small, brownish, oval spot, about 0.8 to 1.0 mm long, in the centre; the material swells rapidly in water, forming a stiff mucilage.

Identification

When mounted in *cresol* and examined under a microscope, the particles are found to be transparent and angular, the edges straight or curved and sometimes rolled. They are composed of polygonal prismatic cells with four to six straight or slightly curved walls; the cells vary in size in different parts of the seed coat, from about 25 mm to 60 mm at the summit of the seed, that is, near and over the brown spot, to 25 mm to 100 mm for the remainder of the epidermis except at the edges of the seed, where the cells are smaller, about 45 mm to 70 mm. When mounted in *ethanol (95 per cent)* and irrigated with *water*, the mucilage in the outer part of the epidermal cells swells rapidly and goes into solution, while the two inner layers of mucilage are more resistant and swell to form rounded papillae. When mounted in *0.005 M iodine*, occasional simple and two- to four-compound starch granules, about 2 mm to 10 mm, can be seen in some of the cells. Occasional fragments of thick-walled, reddish brown endosperm, cells with pitted walls and elongated fragments of grey embryo may be present.

Swelling power. Transfer 1 g to a 100 ml stoppered cylinder containing 90 ml of *water*, shake well for 30 seconds and allow to stand 24 hours, shaking gently on three occasions during this period. Add sufficient *water* to produce 100 ml, mix gently for 30 seconds, avoiding the entrapment of air, allow to stand for 5 hours and measure the volume of mucilage. Repeat the determination three times. The average of four determinations is not less than 40 ml.

Total ash (2.3.19). Not more than 4.5 per cent, determined on 1 g.

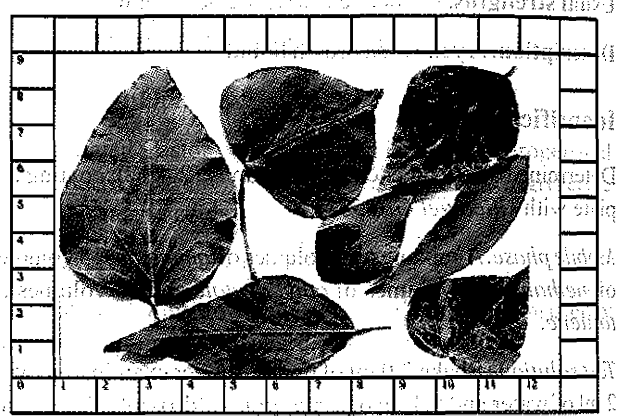
Acid-insoluble ash (2.3.19). Not more than 0.45 per cent.

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 0.5 g by drying in an oven at 105° for 5 hours.

Storage. Store protected from moisture and from attack by insects and rodents.

Ivy Leaf

Hedera helix



Ivy Leaf consists of whole or cut dried leaves of *Hedera helix* Linn. (Fam. Araliaceae)

Ivy leaf contains not less than 3.0 per cent of hederacoside C ($C_{59}H_{96}O_{26}$) calculated on dried basis.

Description. Green to greyish green coloured leaves, broken or whole

Identification

A. *Macroscopic* — Whole leaves are 4-10 cm in length and width cordate at the base. The lamina is palmately 3-5 lobed the lobes more or less triangular with entire margins. The upper surface is dark green with radiate venation the lower surface more grayish green and the venation is distinctly raised. The petioles are long cylindrical about 2 mm in diameter and grooved longitudinally.

B. *Microscopic* — Powder-green: shows diagnostic characters: fragments of the lamina in surface view showing the cells of both the upper and lower epidermises with

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thickened walls and a thick cuticle, numerous stomata occur in the lower epidermis and some of the surrounding cells may show faint cuticular striations and a very porous spongy mesophyll. Cluster crystals of calcium oxalate, present.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

Mobile phase: A mixture of 4 volumes of *anhydrous formic acid*, 20 volumes of *acetone*, 20 volumes of *methanol* and 30 volumes of *ethyl acetate*.

Test solution. To 0.5 gm of the coarsely powdered substance under examination add 5 ml of *methanol*. Sonicate for 15-20 minutes and filter.

Reference solution. To 0.5 gm of *ivy leaf IPBRS* add 5 ml *methanol*. Sonicate for 15-20 minutes and filter.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise to 12 cm. Dry the plate at 100 to 105°. Spray with *alcoholic solution* of *sulphuric acid* (10 per cent). Heat at 110° for 10 minutes and examine in day light.

The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent of other foreign matter.

Total ash (2.3.19). Not more than 10.0 per cent.

Loss on drying (2.4.19). Not more than 10.0 per cent determined on 1gm dried at 105° for 2 hours.

Assay. Determine by Liquid Chromatography (2.4.14).

Test solution. Reflux 1 g of coarsely powdered substance under examination with 50 ml of a mixture of 80 parts of *methanol* and 20 parts of *water* for 1 hour, cool and filter. Reflux the residue further with same solvent mixture for 30 minutes, cool and filter. Make volume up to 100 ml with the same solvent mixture.

Reference solution. Dissolve an amount of *ivy standardized tincture IPRS* corresponding to 3.0 mg of *hederacoside C IPRS* in *methanol* and dilute to 5.0 ml with the same solvent.

Chromatographic system

- a stainless steel column 12.5 cm x 4.0 mm packed with octadecylsilane bonded to porous silica (5 µm)
- mobile phase: A. a mixture of 140 volumes of *acetonitrile* and 880 volumes of *water* and adjust to pH 2.0 with *orthophosphoric acid*,

  B. a mixture of 2 volumes of *orthophosphoric acid* and 998 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	100	0
6	94	6
40	60	40
41	0	100
55	0	100

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of hederacoside C.

Storage. Store protected from light, heat, moisture and against attack by insects and rodents.

Ivy Leaf Dry Extract

Hedera helix dry extract

Ivy Leaf Dry Extract is obtained from whole or cut dried leaves of *Ivy* by extraction with a suitable solvent using an appropriate procedure.

Ivy Leaf Dry Extract contains not less than 90.0 per cent w/w and not more than 110.0 per cent w/w of the stated amount of hederacoside C, calculated on dry basis.

Usual strength. 10.0 per cent w/w.

Description. Green to greenish brown coloured powder, hygroscopic.

Identification

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 4 volumes of *anhydrous formic acid*, 20 volumes of *acetone*, 20 volumes of *methanol* and 30 volumes of *ethylacetate*.

Test solution. Dissolve 0.1 gm of the extract in 5 ml of *methanol*.

Reference solution. Dissolve *ivy leaf IPBRS* containing about 1 mg of *hederacoside C* in 1 ml *methanol*.

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Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise to 12 cm. Dry the plate at 100-105° spray with *alcoholic solution of sulphuric acid* (10 per cent). Heat at 110° for 10 minutes. and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Loss on drying (2.4.19) Not more than 5 per cent determined on 1 gm of the dry extract dried at 105° to constant weight.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve an accurately weighed quantity of the substance under examination equivalent to about 60 mg of *hederacoside C*, in 80 ml of *methanol* with the aid of ultrasound for 30 minutes and dilute to 100.0 ml with *methanol*, mix and filter.

Reference solution. Dissolve *ivy leaf IPBRS* equivalent to 3.0 mg of hederacoside *C* in *methanol* and dilute to 5.0 ml with the same solvent.

Chromatographic system

- a stainless steel column 12.5 cm x 4.0 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. mix 140 volumes of *acetonitrile* and 880 volumes of *water* and adjusted to pH 2.0 with *orthophosphoric acid*,
  B. a mixture of 2 volumes of *orthophosphoric acid* and 998 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 205 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	100	0
6	94	6
40	60	40
41	0	100
55	0	100

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

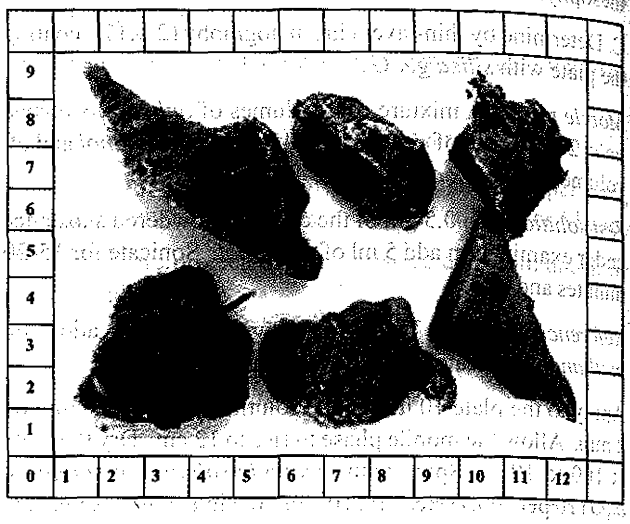
Inject the reference solution and the test solution.

Calculate the content of hederacoside C.

Storage. Store in air tight containers protected from heat and moisture.

Jangali Haldi

Haldi, Vanharidra, *Curcuma aromatica*



Jangali Haldi consists of dried rhizome of *Curcuma aromatica* (Fam. Zingiberaceae).

Jangali Haldi contains not more than 2.1 per cent w/w of total curcuminoids.

Category. Anti-inflammatory, galactogogue, stomachic and spasmolytic.

Description. Rhizome is orange yellow inside, having camphorous odour and taste, warmly bitter.

Identification

A. *Macroscopic* — It is a rhizome, 2-6 cm long, and has brownish outer skin, camphoraceous odour and bitter spicy taste. It has an orange yellow cut surface. It shows palmately attached sessile finger shaped branches with root tubers present, giving it an uneven surface.

B. *Microscopic* — The transverse section of the rhizome shows the presence of trichomes, vascular bundle and parenchymatous cells. The longitudinal section of the rhizome shows the presence of starch grains and fibres. Powdered rhizome shows presence of starch grains, xylem vessels and macerated elements.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 10 volumes of *chloroform*, 1 volume of *methanol*, 0.5 volume of *glacial acetic acid* and 0.5 volume of *formic acid*.

Test solution. Weigh about 2 g of sample and reflux under 100 ml of *methanol* and concentrated the volume upto 10.0 ml and filter.

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Reference solution. A solution containing 0.01 per cent w/v each of *demethoxycurcumin IPRS*, *bisdesmethoxycurcumin IPRS*, and *curcumin IPRS* in the *methanol*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. Spray the plate with *anisaldehyde-sulphuric acid* reagent. Heat the plate at 110° for 10 minutes and examine the plate at 366 nm and day light. In the chromatographic profile one of the principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 8.0 per cent.

Water-soluble extractive (2.6.3). Not less than 12.0 per cent.

Total ash (2.3.19). Not more than 7.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 20.0 per cent.

Microbial contamination (2.2.9) Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 1 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water-bath for 15 minutes, cool and filter. Reflux the residue further with 5 x 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate to 100 ml.

Reference solution. A solution containing 0.04 per cent w/v of *curcumin IPRS* 0.01 per cent w/v of *demethoxycurcumin IPRS* and 0.002 per cent w/v of *bisdemethoxycurcumin IPRS* in *tetrahydrofuran*. Dilute 5.0 ml of this solution to 50.0 ml with mobile phase.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with *octadecylsilane* bonded to porous silica (5 µm),
- mobile phase: a mixture of 56 volumes of 0.1 per cent w/v solution of *citric acid* and 44 volume of *tetrahydrofuran*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 420 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent. The relative retention time with reference to *curcumin* for *bisdemethoxycurcumin* is about 1.24 and for *desmethoxycurcumin* is about 1.12.

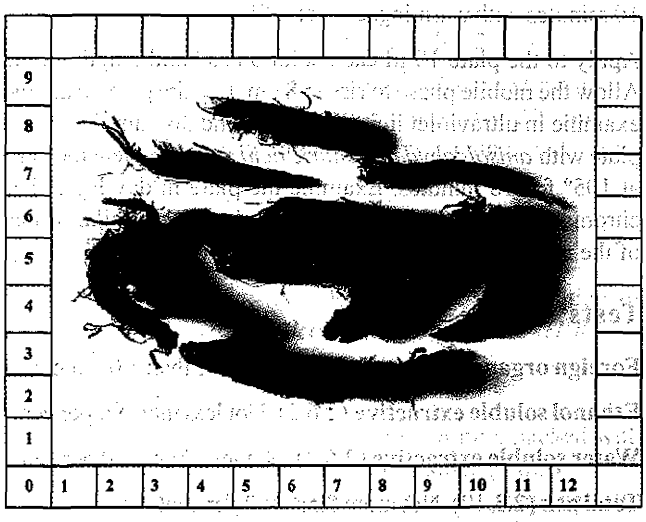
Inject the reference solution and the test solution.

Calculate the content of the *bisdemethoxycurcumin*, *demethoxycurcumin* and *curcumin* in Jangli Haldi.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Jatamansi

Jatamansi, *Valeriana jatamansi*



Jatamansi consists of dried rhizomes of *Valeriana jatamansi* (Fam. Valerianaceae).

Jatamansi contains not less than 2.0 per cent w/v of valproic acid, calculated on the dried basis.

Category. Antispasmodic, tranquillizer and antiepileptic.

Description. The rhizome is dark grey or brown in colour whereas inner layer is slight brown or yellow, odour is agreeable, aromatic, taste is acrid, slightly bitter and aromatic. It breaks easily.

Identification

A. *Macroscopic* — Dried rhizome is elongated, cylindrical in shape, 2.5 - 7.5 cm long and 1.5 cm thick, covered by bundles of fine brown fibres forming a network.

B. *Microscopic* — Transverse section of rhizome circular in out-line. Cork consists of outer most layers of irregular polygonal cells, filled with oil globules. Cortex characterized

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by presence of oleoresin cells. Phloem in the form of patches of small cells. Cambium rings distinct and continuous thickenings. Tracheids linear with scalariform thickenings; fibres few in number. Medullary rays bi to multiserrate, not prominent. Parenchymatous pith present in the centre. Starch grains abundantly present in groups.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 8 volumes of *petroleum ether*, 2 volumes of *methyl ethyl ketone*.

Test solutions. Reflux about 2 g of the coarsely powdered substance under examination with 10 ml of *hexane* for 10 minutes with warming, cool and filter.

Reference solution: Reflux about 2 g of the coarsely powdered *jatamansi IPBRS* under examination with 10 ml of *hexane* for 10 minutes with warming, cool and filter.

Apply to the plate 10 µl each solution as band length 8 mm. Allow the mobile phase to rise to 8 cm. Dry the plate in air and examine in ultraviolet light at 254 nm and 366 nm. Spray the plate with *anisaldehyde sulphuric acid reagent*. Heat the plate at 105° for 10 minutes. Examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 1.0 per cent.

Ethanol soluble extractive (2.6.2). Not less than 6.0 per cent

Water soluble extractive (2.6.3). Not less than 13.0 per cent.

Total ash (2.3.19). Not more than 20.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 6.0 per cent.

Water soluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 8.0 per cent.

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 80 volumes of *petroleum ether* and 20 volumes of *ethyl methyl ketone*.

Test solution. Reflux about 5 g of the coarsely powdered substance under examination with 200 ml of *hexane* by soxhlet apparatus for 2 hours, cool and filter. Concentrate about 10 ml.

Reference solution. 0.001 per cent w/v solution of *valproic acid IPRS* in *hexane*.

Apply to the plate, 10 µl of each solution as band length 8 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and scan the plate in absorbance mode at 366 nm. Record the chromatogram and measure the response for the analyte peak. The chromatographic profile of the test solution is similar to that of the reference solution.

Calculate the content of valproic acid.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Juniper Oil

Dhoopchandan ka tail, *Juniperus communis*

Juniper oil is the essential oil obtained by steam distillation of dried, crushed or slightly dried ripe berries of *Juniperus communis* (Fam. Cupressaceae).

Juniper oil contains not less than 20.0 per cent and not more than 50.0 per cent of α- pinene, and not less than 1.0 per cent and not more than 12.0 per cent of β-pinene and not less than 2.0 per cent and not more than 12.0 per cent of limonene.

Category. Cleansing agent, analgesic, antimicrobial, antiseptic, antispasmodic.

Description. A clear, pale yellow to dark yellow liquid, free from sediment and suspended matter.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *juniper oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described under Assay.

The peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 42.17° to 44.49°.

Tests

Relative density (2.4.29). 0.857 to 0.876.

Refractive index (2.4.27). 1.471 to 1.483.

Optical rotation (2.4.22). -15° to -0.5°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

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Reference solution(a). A 2.0 per cent w/v solution of *α-pinene IPRS* in *ethanol (95 per cent).*

Reference solution (b). A 2.0 per cent w/v solution of *β-pinene IPRS* in *ethanol (95 per cent).*

Reference solution (c). A 2.0 per cent w/v solution of *limonene IPRS* in *ethanol (95 per cent).*

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 60° for 1 minute, increase from 60° to 230° at a rate of 10° per minute and maintain at this temperature for 20 minutes,
- inlet port at 250° and detector at 250°,
- detection by flame ionization detector,
- flow rate: 2 ml per minute using helium as carrier gas,
- injection volume: 0.5µl,
- split ratio: 1:50.

Inject reference solutions (a), (b), (c) and the test solution.

Calculate the content of α-pinene, β-pinene and limonene in the oil using area normalization procedure.

Storage. Store protected from light and moisture.

Kalmegh Dry Extract

Kalmegh Dry Extract is obtained by extracting Kalmegh with *aqueous ethanol* or *ethanol* or any other suitable solvent.

Kalmegh Dry Extract contains not less than 90.0 per cent w/w and not more than 120.0 per cent w/w of the labelled amount of andrographolides (sum of andrographolide, neo-andrographolide and andrograpanin) The content of 14-deoxy-11,12-didehydroandrogapholide content shall not be more than one sixth of the amount of andrographolide.

Category. Hepatoprotective, bittertonic, febrifuge, blood purifier, dipana.

Description. A light green to dark green powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 7 volumes of *chloroform* and 1 volume of *methanol.*

Test solution. Dissolve 200 mg of the extract under examination with 50 ml *methanol* and filter.

Reference solution. A 0.02 per cent w/v solution of *andrographolide IPRS* in *methanol.*

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm and also under day light. Spray the plate with *methanolic sulphuric acid* (20 per cent) and heat at 120° for 10 minutes. The chromatogram obtained with test solution shows a band corresponding to the band obtained using reference solution indicating the presence of andrographolide.

Tests

Acid insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss of drying (2.4.19). Not more than 5.0 per cent.

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 250 mg of the extract under examination or quantity equivalent to 20 mg of *andrographolide* in *methanol* by gently heating, make it up to 100.0 ml and filter.

Reference solution (a). A 0.02 per cent w/v solution of *andrographolide IPRS* in *methanol.*

Reference solution (b). A 0.005 per cent w/v solution of *14-deoxy-11,12-didehydroandrographolide IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution prepared by dissolving 0.136 g of *potassium dihydrogen orthophosphate* in 500 ml of *water* and 0.5 ml of *orthophosphoric acid*, dilute to 1000 ml with *water*,
  B. *acetonitrile*,
- flow rate: 1.5 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 223 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase (per cent v/v)	Mobile phase (per cent v/v)
0	95	5
8	55	45
25	20	80
30	95	5

Inject reference solutions (a) and (b). The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent. The relative retention time with respect to andrographolide and neo-andrographolide is about 1.2 and for andrograpanin is about 1.6.

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Inject reference solutions (a), (b) and the test solution.

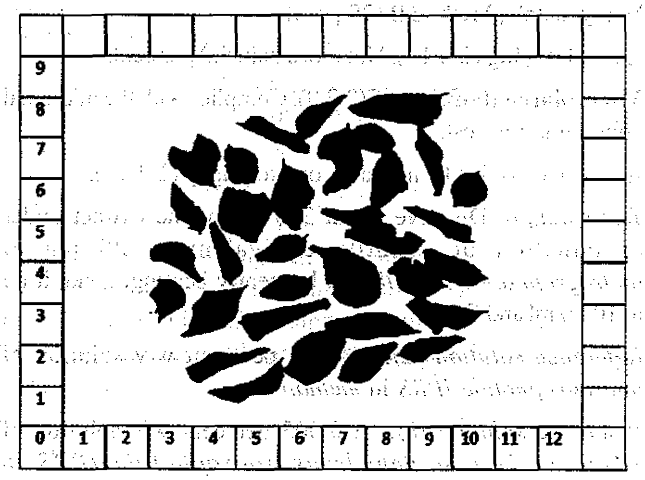
Calculate the content of 14-deoxy-11, 12-didehydroandrographolide and andrographolides in the extract.

Calculate andrographolide by summing the peak areas of andrographolide, neo-andrographolide, and andrograpanin.

Storage. Store protected from heat and moisture.

Karipatta

Curry leaf tree, Mithaneem; *Murraya koenigii*



Karipatta consists of fresh and dried leaflets of *Murraya koenigii* (L.) Spreng (Fam. Rutaceae).

Karipatta contain not less than 0.4 per cent (w/w) mahanimbine, calculated on the dried basis.

Category. Anti-hyperglycemic, Antidiabetic, Antimicrobial, Anti-inflammatory, Immunomodulatory.

Description. Leaf texture is papery to sub coriaceous, slightly brittle when dry. Odour characteristically aromatic, taste acrid.

Identification

A. *Macroscopic* — Compound and imparipinnate 12 - 22 cm in length and 4 - 5.5 cm in breadth; rachis slender and pubescent, petiole slender, terete 1-2 mm in length, leaflet 10 - 20, alternate, ovate, lanceolate or rhomboid, 2 - 4.5 cm in length and 1 to 1.24 cm in breadth, margin crenulated and base obliquely subcutenate, tip obtusely accuminate, minutely notched, dull green dorsally and greenish-white ventrally when dry, with characteristic odour and taste, veins 3 - 6 pairs alternate, reticulate, not prominent on both sides; texture, papery to subcoriaceous and slightly brittle when dry.

B. *Microscopic* — Transverse section shows a dorsi-ventral structure. Epidermis is composed of cubical to slightly tangentially elongated cells. The upper epidermal cells in surface view are polyhedral and straight walled. Trichomes rare, unicellular and found mainly on midrib. Stomata anomocytic, palisade of two layers, irregularly arranged isodiametrical or rectangular cells constitute the spongy parenchyma. Calcium oxalate crystals present in the form of prisms. Secretory canals are large and circular. The midrib shows an arc of radiating xylem with phloem below. Inner to the lower epidermis is present 1 to 3 layers of collenchymas. Ground tissue is composed of thin parenchymatous polygonal cells.

C. Determine by thin layer chromatography (2.4.17) coating the plate with *silica gel GF 254*.

Mobile phase. A mixture of 6.5 volumes of *n-hexane*, 3.5 volumes of *chloroform* and 0.5 volumes of *methanol*.

Test solution. Reflux 5 g of coarsely powdered substance under examination with 50.0 ml *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 x 50.0 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate to 10.0 ml.

Reference solution. Dissolve 10 mg of *mahanimbine* in 10.0 ml of *methanol* and sonicate for 10 minutes, filter.

Apply to the plate 10 µl of each solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air. Spray with *anisaldehyde sulphuric acid reagent* and heat the plate at 105° for 5 minutes. In the chromatographic profile one of the principal spot in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 20.0 per cent.

Water-soluble extractive (2.6.3). Not less than 34.0 per cent.

Total ash (2.3.19). Not more than 12.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.19). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 5 g by drying in an oven at 105° for three hours.

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 10 g of coarsely powdered substance under examination with 100 ml *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2x 100 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate to 10.0 ml.

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Reference solution. Dissolve about 5 mg *mahanimbine IPRS* in 10 ml of *methanol* then further rediluted 1.0 ml to 100.0 ml with mobile phase then sonicate for 5 minutes and filter.

Chromatographic system

– a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bound to porous silica (5 µm),

– mobile phase: A. *methanol*,
B. 0.5 per cent *acetic acid* in *water*,

– a gradient program using the conditions given below,

– flow rate: 1 ml per minute,

– spectrophotometer set at 254 nm,

– injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
21.0	73	27
26.0	60	40
31.0	90	10
40.0	90	10

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

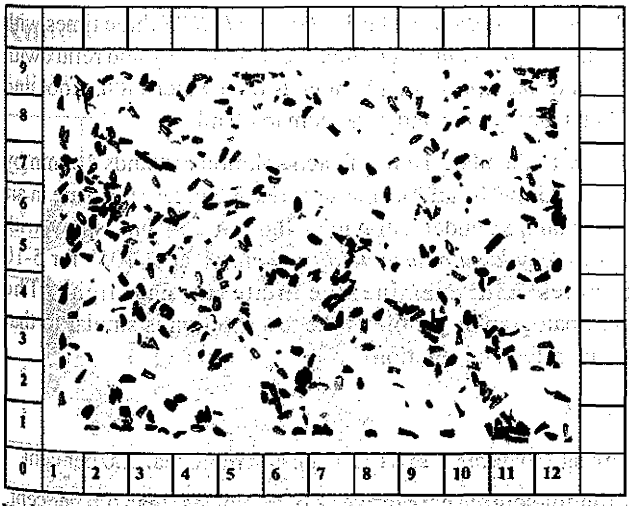
Inject the reference solution and the test solution.

Calculate the content of mahanimbine.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Kasni

Kasani, Hinduba, Kasni, Chicory, *Cichorium intybus*



Kasni consists of fruits and seeds of *Cichorium intybus* Linn (Fam. Asteraceae).

Kasni contains not less than 0.7 per cent w/w of chlorogenic acid calculated on the dried basis.

Category. Hepatoprotective, antidiabetic and hypolipidimic.

Description. The dried fruit and seed is yellowish brown in colour, 1 to 3 mm in length, 1 to 2 mm in breadth, 1 mm in thickness triangle shaped and has characteristic odour.

Identification

A. *Macroscopic — Fruits.* The fruits are achenes which are smooth, 5-angled, pale brown to black, crowned with a ring of pappus scales.

Seed. Brown, minutely puberulous.

B. *Microscopic* — The transverse section of mature fruit shows outer single layered tangentially elongated epidermis, with thick cuticle. Numerous rod shaped unequal epicuticular wax deposits of different size are present on cuticle which are perpendicular to the surface of epidermis. The epidermis is followed by 6 to 10 layers of thick sclerenchymatous cells. Most of sclerenchymatous cells contain prism shaped calcium oxalate crystals.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *toluene*, 30 volumes of *ethyl acetate* and 1 volume of *glacial acetic acid*.

Test solution. Reflux 1 g of coarsely powdered substance under examination with 25 ml of *methanol* on a water bath. Cool and filter. Reflux the residue with 3 x 25 ml of *methanol*. Combine the extracts and concentrate under vacuum to 25 ml.

Reference solution. Reflux 1g of *kasni IPBRS* with 25 ml of *methanol* on a water bath. Cool and filter. Reflux the residue with 3 x 25 ml of *methanol*. Combine the extracts and concentrate under vacuum to 25 ml.

Apply to the plate 10 µl of each solution as band length 8 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in ultraviolet light at 254 and 366 nm. Spray with a *anisaldehyde sulphuric acid reagent*. Heat at 110° for 10 minutes and examine in the visible light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 23.0 per cent.

Water-soluble extractive (2.6.3). Not less than 38.0 per cent.

Total ash (2.3.19). Not more than 7.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.8 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 7.5 per cent.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

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Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 2 g coarsely powdered substance under examination with 100 ml of *methanol* by soxhlet appratus till last extract turn colourless, cool and filter. Concentrate the filtrate to 10 ml.

Reference solution. A 0.04 per cent w/v solution of *chlorogenic acid IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5µm),
- mobile phase: A. a mixture of 40 volumes of *acetonitrile,* 60 volumes of buffer solution and adjusted to pH 2.0 with *orthophosphoric acid,*
  B. *methanol,*
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 327 nm,
- injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
10	80	20
20	70	30
25	90	10
26	90	10

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

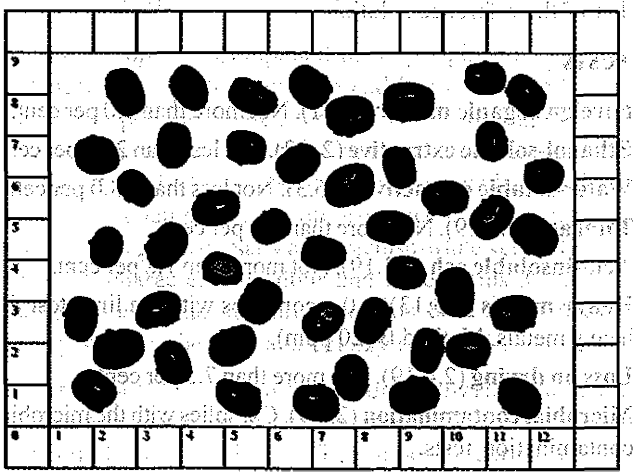
Inject the reference solution and the test solution.

Calculate the content of chlorogenic acid.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Kaunch

Kavach; Atmagupta; Cowhage; *Mucuna pruriens*



Kaunch consists of dried seeds of *Mucuna pruriens* Linn. (Fam. Caesalpiniaceae).

Kaunch contains not less than 3.0 per cent w/w of l-dopa, calculated on the dried basis.

Category. Antiparkinson, aphrodisiac, antidepressant, nervine tonic, dustavrana.

Description. Seeds are hard and reniform in shape, and black in colour.

Identification

A. *Macroscopic* — Black in colour, reniform in shape, 15 to 20 mm long and 7 to 15 mm broad; funicular hilum and cellular pit growth around the hilum; seed coat hard, thick glossy. Embryo fills up the seed and is made up of two large cotyledons.

B. *Microscopic*—Transverse section of the seed shows testa with palisade-like cells with thickened anticlinal walls in epidermis. Hypodermis comprises of I shaped bearer cells, which is followed by parenchyma with tangentially elongated cells. Cotyledons comprise of epidermis, and parenchymatous cells containing oil globules and oval starch grains.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 5 volumes of *chloroform,* 1 volume of *methanol* and 1 volume of *ethyl acetate.*

Test solution. Defat 1 g of coarsely powdered substance under examination three times with 15 ml of *petroleum ether,* filter the residue. Dry and reflux with 20 ml of *methanol* for 15 minutes on a water-bath. Cool and filter. Concentrate under vacuum to 10 ml.

Reference solution. Defat 1 g *kaunch IPBRS* three times with 15 ml of *petroleum ether,* filter the residue. Dry and reflux with 20 ml of *methanol* for 15 minutes on a water-bath. Cool and filter. Concentrate under vacuum to 10 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm, spray with *anisaldehyde sulphuric acid reagent.* Heat at 105° for 5-10 minutes and examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 6.0 per cent.

Water-soluble extractive (2.6.3). Not less than 21.0 per cent.

Total ash (2.3.19). Not more than 4.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.3 per cent.

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Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 7.8 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 1.0 g of the coarsely powdered substance under examination three times with 15 ml of *petroleum ether* 60° to 80° for 15 minutes, cool and filter. Extract the residue further by shaking with 10 ml of *0.01 M hydrochloric acid* and 25 ml *water* for 15 minutes. Adjust the final volume to 100 ml with *water* and filter.

Reference solution. A 0.005 per cent w/v solution of *l-dopa IPRS* in *0.01 M hydrochloric acid.*

Chromatographic system

- a stainless steel column 15 cm x 4 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 45 volumes of *acetonitrile* and 75 volumes of a solution prepared by dissolving 1 g of *sodium lauryl sulphate* and 15 g *tartaric acid* in 1000 ml *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

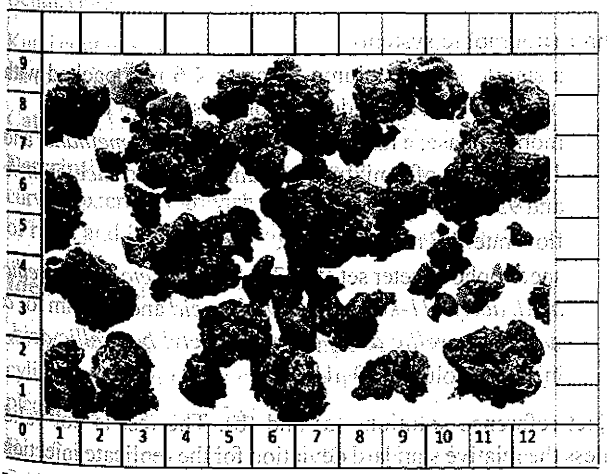
Inject the reference solution and the test solution.

Calculate the content of l-dopa.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Kunduru

Sallaki Gum; Gum of *Boswellia serrata*



Kunduru is the gum-resin from *Boswellia serrata* Roxb. (Fam. Burseraceae).

Kunduru contains not less than 1.0 per cent w/w of total 11-keto-β-boswellic acid and acetyl-11-keto-β-boswellic acid, calculated on the dried basis.

Category. Diuretic, diaphoretic, astringent, antiasthmatic, swasa.

Description. Translucent, brittle, whitish yellow substance, club-shaped, pear-shaped, or irregular tears.

Identification

A. Macroscopic — Fracture dull. Slightly sticky to touch; odour, balsamic; taste slightly mucilaginous, bitter and aromatic.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 7 volumes of *hexane* and 3 volumes of *ethyl acetate.*

Test solution. Reflux 1 g of coarsely powdered substance under examination with 50 ml of *methanol* on a boiling water-bath for 30 minutes, cool and filter. Evaporate the filtrate to dryness and dissolve the residue in 10 ml of *methanol.*

Reference solution. Reflux 1 g of coarsely powdered *kunduru IPBRS* with 50 ml of *methanol* on a boiling water-bath for 30 minutes, cool and filter. Evaporate the filtrate to dryness and dissolve the residue in 10 ml of *methanol.*

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light 254 nm and 366 nm, spray with *methanolic sulphuric acid* (10 per cent, v/v). Heat the plate at 110° for 5-10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 35.0 per cent.

Total ash (2.3.19). Not more than 10.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not more than 12.0 per cent, determined on 0.2 g.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 2 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water bath for 15 minutes, cool and filter. Reflux the residue

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further with *methanol* till the last extract turns colorless, cool and filter. Combine all the filtrates and concentrate to 100.0 ml.

Reference solution (a). A 0.01 per cent w/v solution of *11-keto-β-boswellic acid IPRS* in *methanol.*

Reference solution (b). A 0.05 per cent w/v solution of *acetyl-11-keto-β-boswellic acid IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: 90 volumes of *methanol* and 10 volumes of a mixture containing 5 ml of *acetonitrile* and 95 ml of *water*, adjusting the pH to 2.8 with *dilute orthophosphoric acid,*
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 247 nm,
- injection volume: 20 µl.

Inject the reference solutions (a) and (b). The test is not valid unless the relative retention times are about 0.65 for 11-keto-β-boswellic acid and 1.0 for acetyl-11-keto-β-boswellic acid and the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the test solution.

Calculate the sum of the contents of 11-keto-β-boswellic acid and acetyl-11-keto-β-boswellic acid.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Kunduru Dry Extract

Sallaki Gum; Gum of *Boswellia serrata*

Kunduru Dry Extract is obtained by extracting Kunduru gum-resin with aqueous ethanol or ethanol or any other suitable solvent and evaporation of solvents.

Kunduru Dry Extract contains not less than 90.0 per cent w/w and not more than 120.0 per cent w/w of stated amount of boswellic acids (sum of 11-keto-β–boswellic acid and acetyl-11-keto- β-boswellic acid, α and β boswellic acid and α and β acetyl boswellic acid) calculated on the anhydrous basis. It may contain suitable added substances.

Category. Diuretic, diaphoretic, astringent, antiasthmatic, swasa.

Description. Off white to pale brown or cream colour powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 6 volumes of *hexane* and 3 volumes of *ethyl acetate.*

Test solution. Dissolve 200 mg of the extract under examination with 50 ml of *methanol* and filter.

Reference solution. A 0.05 per cent w/v solution each of *11-keto-β-boswellic acid IPRS* and *acetyl-11-keto-β-boswellic acid IPRS* in *methanol.*

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with 10 per cent v/v solution of *methanolic sulphuric acid.* Heat the plate at 120° for 20 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43) Not more than 5.0 per cent using Method I.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14)

Test solution. Dissolve 400 mg of the extract under examination in *methanol* by gentle heating, dilute to 50.0 ml with *methanol* and filter.

Reference solution (a). A 0.02 per cent w/v solution each of *11-keto- β -boswellic acid IPRS* and *acetyl-11-keto-β-boswellic acid IPRS* in *methanol.*

Reference solution (b). A solution containing 0.1 per cent w/v each of *α and β-boswellic acid IPRS* and *α and β-acetyl boswellic acid IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 90 volumes of *methanol* and 10 volumes of a mixture containing 5 ml of *acetonitrile* and 95 ml of *water,*
- flow rate: 1.8 ml per minute,
- spectrophotometer set at 247 nm for *11-keto- β-boswellic acid, acetyl-11-keto-β-boswellic acid* and 205 nm for *α and β-boswellic acid, α and β-acetyl boswellic acid,*
- injection volume: 20 µl.

Inject reference solutions (a) and (b). The test is not valid unless the relative standard deviation for the replicate injection is not more than 2.0 per cent. The relative retention times are about 0.65 for 11-keto-β -boswellic acid; 1.0 for acetyl-11-keto-

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β-boswellic acid; 2.0 for α-boswellic acid; 2.2 for β-boswellic acid; 3.9 for α-acetyl boswellic acid and 4.4 for β-acetyl boswellic acid.

Inject reference solutions (a), (b) and the test solution.

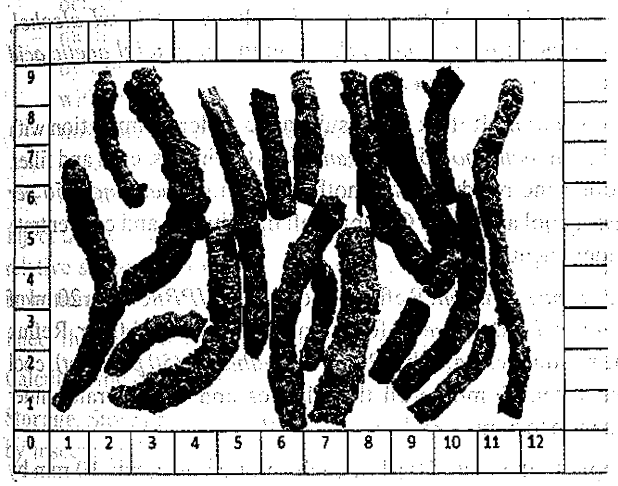
Calculate the contents of 11-keto- β-boswellic acid and acetyl-11-keto- β-boswellic acid, α and β-boswellic acid, α and β-acetyl boswellic acid in the extract.

Calculate the boswellic acids by summing the contents of 11-keto- β-boswellic acid and acetyl-11-keto- β-boswellic acid, α and β-boswellic acid, α and β-acetyl boswellic acid.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Kutki

Picrorhiza kurroa



Kutki consists of dried roots of *Picrorhiza kurroa* Royle ex Benth. (Fam. Scrophulariaceae).

Kutki contains not less than 5 per cent w/w of kutkin, calculated on the dried basis.

Category. Hepatoprotective, bitter tonic, antiulcer, kamala.

Description. Rhizomes are sub cylindrical, straight or slightly curved, externally greyish with wrinkled surfaces, circular scars of roots and bud scales, with cork exposed at places.

Identification

A. *Macroscopic* — 3-6 cm long and about 1 cm thick, sub-cylindrical, straight, grayish brown, wrinkled roots. Odour is pleasant and tastes bitter.

B. *Microscopic* — There is well-developed periderm. About 7-10 layers of cork cells are seen. Cells of phelloderm loosely arranged.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 7.5 volumes of *ethyl acetate*, 2.2 volumes of *methanol* and 0.1 volume *glacial acetic acid.*

Test solution. Reflux 1 g of coarsely powdered substance under examination with 50 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 50 ml of *methanol*, cool and filter. Combine both the filtrates and concentrate under vacuum to dryness. Extract the dried residue with 50 ml of *methanol* at 50° for 10 minutes, filter the solution and use the filtrate for analysis.

Reference solution. Reflux 5 g of *kutki IPBRS* with 50 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 50 ml of *methanol*, cool and filter. Combine both the filtrates and concentrate under vacuum to dryness. Extract the dried residue with 10 ml of *methanol* at 50° for 10 minutes, filter the solution and use the filtrate for analysis.

Apply to the plate 20 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 100° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 15.0 per cent.

Water-soluble extractive (2.6.3). Not less than 25.0 per cent by Method I.

Total ash (2.3.19). Not more than 6.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 100 mg of the substance under examination in 25.0 ml of *methanol*, filter.

Reference solution. A 0.1 per cent w/v solution of *kutkin IPRS* in *methanol*.

Chromatographic system

– a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),

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– mobile phase: 83 volumes of 1 per cent v/v *orthophosphoric acid* in *water* and 17 volumes of *acetonitrile*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 280 nm,
– injection volume: 20 µl.

Inject the reference solution. The relative standard deviation for the replicate injections is not more than 2.0 per cent.

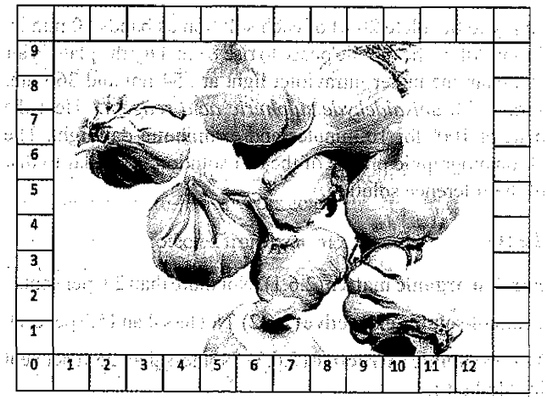
Inject the reference solution and the test solution.

Calculate the content of kutkin.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Lasuna

Garlic; *Allium sativum*



Lasuna consists of the fresh or dried compound bulbs of *Allium sativum* Linn. (Fam. Liliaceae).

Lasuna contains not less than 0.2 per cent w/w of alliin, calculated on the dried basis.

Category. Antihyperlipidaemic, antibacterial, digestant, jirna.

Description. Bulbs made up of cloves and is wrapped in a white papery sheath with pungent taste and odour

Identification

A. *Macroscopic* — Each bulb has several cloves which are arranged in concentric rings and enclosed in a shining white or pinkish papery envelope. The cloves are attached to a flat, circular hard disc with numerous thin wiry roots from its underside and short, cylindrical out growth from the upper surface. Each clove is ovoid and further covered by papery sheath with a tail like structure at one and opposite to its attachment. The cloves in the outer ring are loose and white in colour where as cloves in the inner ring are adherent and pale pinkish in colour. Each clove covered with a white scale leaf and a pinkish white epidermis, easily separated from the solid portion. In the middle of the bulb a hollow, cylindrical, linear remnant of the scape is seen.

B. *Microscopic* — The protective leaf contains an epidermis enclosing a mesophyll free from chlorophyll. The outer epidermis consists of lignified sclereid cells of thick, pitted walls, elongated, covered with thin cuticle. The cortical cells are thick walled, non lignified, tending to collapse on maturity, isodiametric and contain purple pigments. The vascular bundles consist of lignified spiral and annular vessels. The storage leaves show an outer epidermis of thin, delicate cells of variable shape. Stomata are present on the outer epidermis only at extreme tip near the base of the foliage leaves. The mesophyll consists of swollen storage parenchyma cells filled with fine granular reserve material.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 3 volumes of *butyl alcohol*, 1 volume of *propyl alcohol*, 1 volume of *glacial acetic acid* and 1 volume of *water*.

Test solution. Reflux 1 g of substance under examination with 20 ml of *methanol (50 per cent)* for 10 minutes, cool and filter. Reflux the residue with another 20 ml of *methanol (50 per cent)*, cool and filter. Combine all the filtrates and concentrate under vacuum to 5 ml.

Reference solution. Reflux 1 g of *lasuna IPBRS* with 20 ml of *methanol (50 per cent)* for 10 minutes, cool and filter. Reflux the residue with another 20 ml of *methanol (50 per cent)*, cool and filter. Combine all the filtrates and concentrate under vacuum to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with 0.2 per cent w/v solution of *ninhydrin* in mixture of 95 volumes of *butyl alcohol* and 5 volumes of *2 M acetic acid*. Heat the plate at 100° to 105° for about 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

D. Transfer about 10 g of garlic bulbs that have been cut into small pieces to a beaker, add 10 ml of *1 M sodium hydroxide* and 10 ml of *water*, heat the beaker in a boiling water for 10 minutes, cool and filter. To 2 ml of this filtrate add few drops of freshly prepared solution of *sodium nitroprusside*. Appearance of a red or orange red colour indicates the presence of sulphur containing compounds in the sample.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Total ash (2.3.19). Not more than 5.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 65.0 per cent for fresh bulbs, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh accurately 2 g of the freshly peeled substance under examination. Add 15 ml of hot *water* and grind in a porcelain mortar and filter. Grind the residue further with 2 × 15 ml of hot *water* and filter. Combine all the filtrates and make up to volume 50 ml with distilled *water*.

Reference solution. A 0.004 per cent w/v solution of *alliin IPRS* in *water*.

Chromatographic system
– a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
– mobile phase: 0.1 per cent v/v *phosphoric acid* prepared by diluting 1 ml of *orthophosphoric acid* to 1000 ml with *water*,
– flow rate: 0.5 ml per minute,
– spectrophotometer set at 210 nm,
– injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

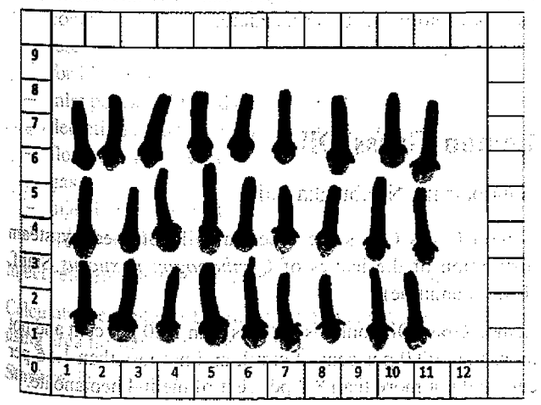
Inject the reference solution the the test solution.

Calculate the content of alliin.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Lavang

Syzigium aromaticum



Lavang consists of dried flower buds of *Syzigium aromaticum* (L.) Merr. & L.M. Perry. (Fam. Myrtaceae).

Lavang contains not less than 7.0 per cent w/w of eugenol, calculated on the dried basis.

Category. Digestant, carminative, stomachic, kasa.

Description. Aromatic odour and spicy taste.

Identification

A. *Macroscopic*—The flower bud is reddish brown in colour and consist of quadrangular stalked portion, the hypanthium, surrounded by four lobes of sepals.

B. *Microscopic*—Epidermis followed by thick cuticle, characteristic zone of bilateral vascular bundles, numerous schylogenous oil glands present beneath the epidermis. Central collumella and air spaces or lacuna in parenchyma region.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 93 volumes of *toluene* and 7 volumes of *ethylacetate*.

Test solution. Reflux 2.0 g of the coarsely powdered substance under examination with 50 ml *dichloromethane* for 15 minutes, cool and filter. Reflux the residue further for two times with 50 ml *dichloromethane*, cool and filter. Combine all the filtrates and concentrate under vacuum to 25 ml.

Reference solution. Reflux 1.0 g of the coarsely powdered *lavang IPBRS* with 25 ml *dichloromethane* for 15 minutes, cool and filter. Reflux the residue further for two times with 50 ml *dichloromethane*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. Spray the plate with a *vanillin sulphuric acid reagent*. Heat the plate at 105° for 5 minutes and examine the plate at 366 nm and in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol soluble extractive (2.6.2). Not less than 7.0 per cent.

Water soluble extractive (2.6.3). Not less than 20.0 per cent by Method I.

Total ash (2.3.19). Not more than 7.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

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Loss on drying (2.4.19). Not more than 12.0 per cent determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by gas chromatography (2.4.14).

Test solution. A 0.5 per cent w/v of substance under examination in *methanol*.

Reference solution. A 0.04 per cent w/v of *eugenol IPRS* in *methanol*.

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column 70° for 1 minute, then increased to 100° at a rate of 10° per minute, then increased to 200° at a rate of 20° per minute and maintained at this temperature for 2 minutes,
- inlet port at 220° and detector at 250°,
- detection by flame ionization detector,
- flow rate 1.5 ml per minute, using nitrogen as the carrier gas.

Inject the reference solution and the test solution.

Calculate the eugenol content in the substance under examination using the ratios of the area of peak corresponding to eugenol to the area of the peak obtained with test solution and the reference solution.

Storage. Store protected from light, moisture and against attack by rodents and insects.

Lavender Oil

Lavender Oil is the essential oil obtained by steam distillation of recently picked lavender flowers, *Lavendula angustifolia* P. Miller (Fam. Lamiaceae).

Lavender Oil (Indian variety) contains not less than 35 per cent and not more than 60 per cent of linalyl acetate, and not less than 25 per cent and not more than 35 per cent of linalool.

Description. A clear, pale yellow liquid, free from sediment, suspended matter; characteristic, rustic and recalling odour.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *lavender oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). Not less than 100°.

Tests

Relative density (2.4.29). 0.877 to 0.890.

Refractive index (2.4.27). 1.458 to 1.468.

Optical rotation (2.4.22). −11.0° to −7.0°.

Acid value (2.3.23). Not more than 1.0.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *linalyl acetate IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *linalool IPRS* in *ethanol (95 per cent)*.

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute, using nitrogen as the carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

Inject reference solutions (a), (b) and the test solution.

Calculate the contents of linalyl acetate, and linalool in the oil using area normalisation procedure.

Lemon Grass Oil

Gandhatrina, Nimbu ghas tail

Lemon Grass Oil is the essential oil obtained by steam distillation of the leaves of *Cymbopogon flexuosus* Stapf, (Fam. Gramineae).

Lemon Grass Oil contains not less than 70.0 per cent and not more than 90.0 per cent of citral, and not less than 1.56 per cent and not more than 8.0 per cent of methyl heptanoate.

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Description. A clear, pale yellow to amber liquid, free from sediment, suspended matter and characteristic lemon like odour.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *lemon grass oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described under Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). Not less than 100.0°.

Tests

Relative density (2.4.29). 0.852 to 0.905.

Refractive index (2.4.27). 1.483 to 1.4890.

Optical rotation (2.4.22). −4.0° to +1.0°.

Acid value (2.3.23). Not more than 1.0.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *citral IPRS* in *ethanol (95 per cent)*.

Reference solution (b) A 2.0 per cent w/v solution of *methyl heptanoate IPRS* in *ethanol (95 per cent)*.

Chromatographic system
- a capillary column 30 m x 0.25 mm, coated with methy 15 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute, using nitrogen as the carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

Inject reference solutions (a), (b) and the test solution.

Calculate the content of citral and methyl heptanoate in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Lemon Oil

Nimbu ka tail

Lemon Oil is the essential oil obtained by steam distillation from the epicarps of fresh fruits of *Citrus limon* (L.) Burm., (Fam. Rutaceae).

Lemon Oil contains not less than 10 per cent and not more than 16.5 per cent of β-pinene, not less than 60 per cent and not more than 69 per cent of limonene and not less than 8 per cent and not more than 12 per cent of γ-terpinene.

Description. A clear, pale yellow to dark green liquid, free from sediment, suspended matter and characteristic fresh lemon pericarp odour.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *lemon oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash Point (2.4.44). 47.0° to 51.0°.

Tests

Relative density (2.4.29). 0.849 to 0.858.

Refractive index (2.4.27). 1.473 to 1.476.

Optical rotation (2.4.22). +57.0° to +66.0°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *β-pinene IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *limonene IPRS* in *ethanol (95 per cent)*.

Chromatographic system
- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,

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- detection by flame ionization detector,
- flow rate 1 ml per minute, using nitrogen as the carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

Inject reference solutions (a), (b) and the test solution.

Calculate the β-pinene, and limonene contents in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Lime Oil

Nimbu ka tail

Lime Oil is obtained by centrifuging the emulsion of juice and oil obtained by crushing the whole fruits of *Citrus aurantiifolia* (Christman) Swingle, (Fam.Rutaceae).

Lime Oil contains not less than 45.0 per cent and not more than 60.0 per cent of D-limonene; and not less than 10.0 per cent and not more than 20.0 per cent of γ-terpinene.

Description. A clear, amber green to dark green liquid, free from sediment, suspended matter and characteristic odour.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

Reference solution. A 2.0 per cent w/v solution of *lime oil IPRS* in *ethanol (95 per cent).*

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 58.56° to 62.56°.

Tests

Relative density (2.4.29). 0.875 to 0.884.

Refractive index (2.4.27). 1.482 to 1.486.

Optical rotation (2.4.22). +35.0° to +41.0°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

Reference solution (a). A solution containing 2.0 per cent w/v solution of *D–limonene* in *ethanol (95 per cent).*

Reference solution (b) A solution containing 2.0 per cent w/v solution of γ-terpinene in *ethanol (95 per cent).*

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute, using nitrogen as the carrier gas,
- injection volume: 1.0 µl,
- split ratio : 1 : 25.

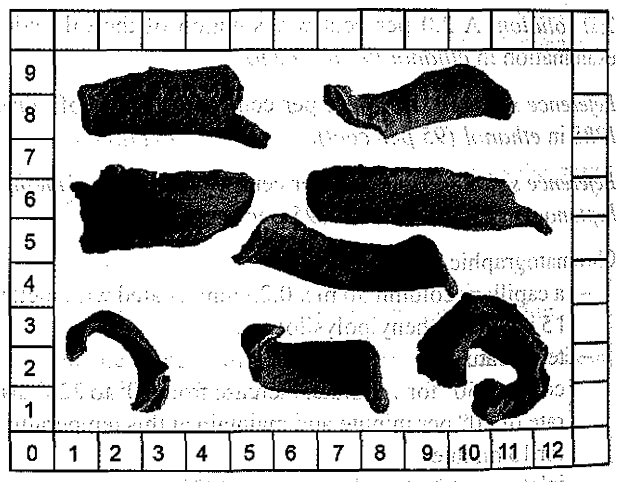
Inject reference solutions (a), (b) and the test solution.

Calculate the D-limonene, and γ-terpinene contents in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Lodhra

Lodh; *Symplocos racemosa*



Lodhra consists of dried stem bark of *Symplocos racemosa* Roxb. Syn. *S. racemosa* Roxb. (Fam. Symplocaceae)

Lodhra contains not less than 0.14 per cent *epiafzelechin,* calculated on the dried basis.

Category. Anti-leucorrhoea, antidiarrhoeal, antimicrobial and wound healing.

Description. A yellowish brown colored, flat or curved thick pieces of bark, astringent, tastes sweetish at beginning and bitter later on.

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Identification

A. *Macroscopic* — Mature stem bark occurs in channelled or curved pieces, with smooth uneven outer surface, exhibiting narrow longitudinally running fissures and occasional transverse cracks, yellowish brown colour externally, pale to whitish-brown internally, fracture short and granular in cortical region and somewhat fibrous in inner region.

B. *Microscopic* — Mature bark shows outer multilayered lignified cork, narrow cortex and wide phloem transverse with stone cells, fibers and medullary rays. Stratified cork consisting of many rows of squarish, radially arranged cells alternating with one to two rows of very narrow tangentially elongated suberized cells; underneath this lies eight to ten layers of parenchymatous cortex transversed with tangentially running, isolated or in groups of two to three, stone cells and fibrous sclereids of various sizes and shapes with narrow or wide lumen; phloem vary wide, transversed with isolated or large groups of stone cells and fibrous sclereids associated with idoblasts containing prismatic crystals of calcium oxalate. Medullary rays wavy, multiseriate, mostly uni- to triseriate, becoming very narrow in peripheral region and often embedded with isolated fibers. Few oval to circular secretary canals, cells containing brownish pigments and plenty of prismatic crystals of calcium oxalate and simple starch grains transverse throughout the section.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 80 volumes of *chloroform* and 20 volumes of *methanol*.

Test solution. Reflux about 2.5 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water bath for 30 minutes, cool and filter. Reflux the residue further for 2 more times with 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10.0 ml.

Reference solution. Reflux about 2.5 g of *lodhra IPBRS* with 50 ml of *methanol* on a water bath for 30 minutes, cool and filter. Reflux the residue further for 2 more times with 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10.0 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm, 366 nm. Spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 105° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 3.0 per cent.

Water-soluble extractive (2.6.3). Not less than 15.0 per cent.

Total ash (2.3.19). Not more than 12.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 4.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals. Method B (20 ppm).

Loss on drying (2.4.19). Not more than 8.0 per cent determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 1 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water bath for 30 minutes, cool and filter. Reflux the residue further for 2 more times with 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 100.0 ml. Dilute 1 ml of concentrate to 5 ml with *methanol*.

Reference solution. 0.001 per cent w/v solution of *epiafzelechin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. *acetonitrile*,
  B. *water*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	5	95
10	5	95
20	55	45
25	5	95

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injection is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of epiafzelechin.

Storage. Store protected from moisture, light, heat and against attack by insects and rodents.

Malt Extract

Malt Extract is a product obtained by extracting malted grains of cereals (barley, cholam or wheat) with water at a suitable temperature and evaporation of the strained liquid until a viscous product is obtained. It may be mixed with 10 per cent by weight of Glycerin.

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Malt Extract contains nitrogen equivalent to not less than 4.0 per cent w/w of protein.

Category. Pharmaceutical aid

Description. A sweet, viscous, light brown liquid; odour, pleasant and characteristic.

Tests

Refractive index (2.4.27). 1.489 to 1.498, determined at 20°.

Arsenic (2.3.10). Dissolve 10.0 g in 10 ml of *water*, add 10 ml of *brominated hydrochloric acid*, allow to stand for 5 minutes and remove the excess of bromine with a few drops of *stannous chloride solution AsT*. The resulting solution complies with the limit test for arsenic (1 ppm).

Lipase. To 95 ml of *water* add 6.5 ml of *triacetin* and 0.2 ml of a 0.1 per cent w/v solution of *bromocresol purple*, neutralise with *0.5 M sodium hydroxide* and add sufficient *water* to produce 110 ml. Place 50 ml of this solution in each of two large test-tubes (20 cm × 3 cm) A and B kept in a water-bath at 30° ± 1°. Insert in each tube a rubber stopper having two holes, one for the tip of a burette and the other for a short glass tube through which passes a thread operating a glass stirring coil. Stir the contents of the tube until they attain the temperature of the water-bath. Prepare a solution of 5.0 g of the substance under examination in 10 ml of *water*. To tube A add 1 ml of this solution; to tube B add 1 ml of this solution after previous boiling. Adjust and maintain the pH of the two tubes to between 6.2 and 6.4 by the dropwise addition of *0.05 M sodium hydroxide*, stirring frequently. After 6 hours, the difference between the amounts of *0.05 M sodium hydroxide* added to the tubes is not more than 1.0 ml.

Assay. Weigh accurately 5.0 g into a 200-ml long-necked flask and carry out the determination of nitrogen, Method A (2.3.30), using *0.05 M sulphuric acid* instead of *0.1 M sulphuric acid*.

1 ml of *0.05 M sulphuric acid* is equivalent to 0.001401 g of N_2 and multiply the result by 6.25 to obtain the protein content.

Storage. Store protected from moisture.

Mandarin Oil

Mandarin oil is cold pressed oil obtained by suitable mechanical treatment from the peel of the fresh fruit of *Citrus reticulate* Blanco (Fam. Rutaceae).

Mandarin oil contains not less than 1.6 per cent and not more than 3.0 per cent of α-pinene, not less than 1.2 per cent and not more than 2.0 per cent of β-pinene, not more than 0.3 per cent of sabinene, not less than 1.5 per cent and not more than 2.0 per cent of β-myrcene, not more than 1.0 per cent of p-cymene, not less than 65.0 per cent and not more than 75.0 per cent of limonene, not less than 16.0 per cent and not more than 22.0 per cent of γ-terpinene, not less than 0.30 per cent and not more than 0.60 per cent of methyl N-methylanthranilate.

Category. Antiseptic, antispasmodic, carminative.

Description. A greenish yellow or greenish orange liquid. Intensely sweet, citrusy, tangy, fresh odour.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *mandarin oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). Not more than 48.9°.

Tests

Relative density (2.4.29). 0.845 to 0.852.

Refractive index (2.4.27). 1.472 to 1.476.

Optical rotation (2.4.22). +63° to +78°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution(a). 2.0 per cent w/v solution of *α-pinene IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *β-pinene IPRS* in *ethanol (95 per cent)*.

Reference solution(c). A 2.0 per cent w/v solution of *sabinene IPRS* in *ethanol (95 per cent)*.

Reference solution (d). A 2.0 per cent w/v solution of *β-myrcene IPRS* in *ethanol (95 per cent)*.

Reference solution (e). A 2.0 per cent w/v solution of *β-cymene IPRS* in *ethanol (95 per cent)*.

Reference solution (f). A 2.0 per cent w/v solution of *limonene IPRS* in *ethanol (95 per cent)*.

Reference solution (g). A 2.0 per cent w/v solution of *γ-terpinene IPRS* in *ethanol (95 per cent)*.

Reference solution (h). A 2.0 per cent w/v solution of *methyl N-methylanthranilate IPRS* in *ethanol (95 per cent)*.

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Chromatographic system
- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  - column. 60° for 1 minute, increase from 60° to 230° at a rate of 4° per minute, and maintain at this temperature for 10 minutes,
  - inlet port at 230° and detector at 240°,
- detection by flame ionization detector,
- flow rate: 1 ml per minute using helium as carrier gas,
- injection volume: 0.5 µl,
- split ratio: 1: 200.

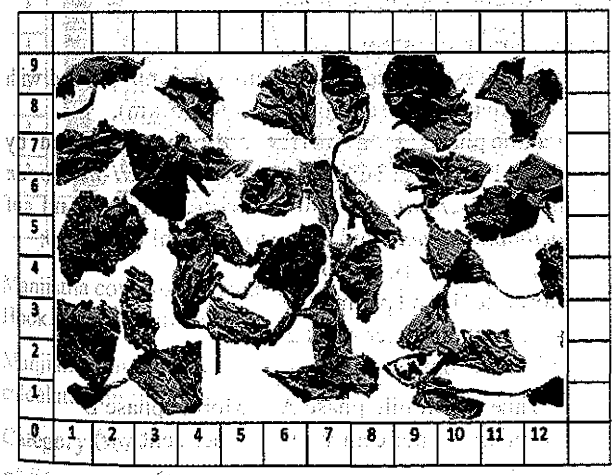
Inject reference solutions (a), (b), (c), (d), (e), (f), (g), (h) and the test solution.

Calculate the content of α-pinene, β-pinene, sabinene, β-myrcene, β-cymene, limonene, γ-terpinene, N-methylanthranilate in the oil using area normalization procedure.

Storage. Store in cool, dry place in tightly sealed containers, protected from heat and light.

Mandukaparni

Gotu Kola; *Centella asiatica*



Mandukaparni consists of the dried aerial parts of *Centella asiatica* (Linn.) Urban. (Fam. Umbelliferae).

Mandukaparni contains not less than 0.5 per cent w/w of asiaticoside, calculated on the dried basis.

Category. Brain tonic, anabolic, anxiolytic, alterative, raktapitta.

Description. A green to greenish yellow in colour, taste, slightly bitter and sweet.

Identification

A. *Macroscopic* — A slender trailing herb with rooted nodes and internodes. Leaves with elongated petioles and sheathing leaf bases; lamina reniform with crenate margin.

B. *Microscopic* — Animocytic stomata on both surfaces, more on lower surface; petiole epidermis has calcium oxalate prisms; vascular bundles seven arranged in 'U' shape, parenchyma cells contain simple and compound starch granules.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *chloroform*, 32 volumes of *glacial acetic acid*, 12 volumes of *methanol* and 8 volumes of *water*.

Test solution. Reflux 2 g of coarsely powdered substance under examination with 50 ml *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 × 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate to 10 ml under reduced pressure.

Reference solution. Reflux 1 g of *mandukaparni IPBRS* with 50 ml *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 × 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 100° for 5-10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 6.0 per cent.

Water-soluble extractive (2.6.3). Not less than 15.0 per cent by Method I.

Total ash (2.3.19). Not more than 24 per cent.

Acid-insoluble ash (2.3.19). Not more than 5.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 3 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a

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water bath for 15 minutes, cool and filter. Reflux the residue further with *methanol* till the last extract turns colorless, cool and filter. Combine all the filtrates and concentrate to 100.0 ml.

Reference solution. A 0.1 per cent w/v solution of *asiaticoside IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octylsilane bonded to porous silica (10 µm),
- mobile phase: 25 volumes of *acetonitrile* and 75 volumes of *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of asiaticoside.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Mandukaparni Dry Extract

Gotu Kola; *Centella asiatica*

Mandukaparni Dry Extract is obtained by extracting Mandukaparni aerial parts with *aqueous ethanol* or *ethanol* or any other suitable solvent and evaporation of solvents.

Mandukaparni Dry Extract contains not less than 90.0 per cent w/w and not more than 110.0 per cent w/w of the stated amount of total triterpenes (sum of asiaticoside, asiatic acid as asiaticoside and madecassoside (mixture of asiaticoside B and madecassoside), madecassic acid (mixture of terminolic acid and madecassic acid) as madecassoside) calculated on the dried basis. It may contain suitable added substances.

Category. Brain tonic, anabolic, anxiolytic, alterative, raktapitta.

Description. A greenish brown to green powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *chroroform*, 32 volumes of *glacial acetic acid* and 12 volumes of *methanol* and 8 volumes of *water*.

Test solution. Dissolve 500 mg of the extract under examination with 50 ml *methanol* and filter.

Reference solution (a). A 0.02 per cent w/v solution of *asiaticoside IPRS* in *methanol*.

Reference solution (b). A 0.02 per cent w/v solution of *madecassoside IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise to 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 100° for 5-10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of reference solutions (a) and (b).

Tests

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 5.0 g by drying in oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 250 mg of the extract under examination in *methanol* by gently heating, dilute to 100.0 ml with *methanol* and filter.

Reference solution (a). A 0.01 per cent w/v solution of *asiaticoside IPRS* in *methanol*.

Reference solution (b). A 0.01 per cent w/v solution of *madecassoside IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution prepared by dissolving 0.136 g of *potassium di-hydrogen orthophosphate* in 500 ml *water*, add 0.5 ml of *orthophoric acid* and dilute to 1000 ml with *water*,
  B. *acetonitrile*,
- flow rate. 1.8 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume. 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	85	15
5	75	25
15	55	45
20	40	60
25	40	60
30	50	50
35	55	45
40	85	15
45	85	15

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Inject reference solutions (a) and (b). The relative retention time is about 0.88 for madecassoside, 1.0 for asiaticoside, 1.6 for madecassic acid and 1.8 for asiatic acid. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject reference solutions (a), (b) and the test solution.

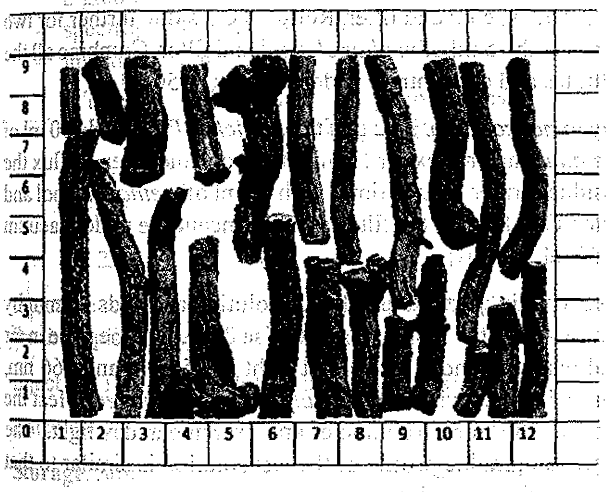
Calculate the content of asiaticoside, asiatic acid as asiaticoside and madecassoside (mixture of asiaticoside B and madecassoside), madecassic acid (mixture of terminolic acid and madecassic acid) as madecassoside.

Calculate the total triterpenes by summing asiaticoside, madecassoside, asiatic acid and madecassic acid.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Manjistha

Indian Madder; *Rubia cordifolia*



Manjistha consists of dried stem of *Rubia cordifolia* Linn. *sensu* Hook.f. (Fam. Rubiaceae).

Manjistha contains not less than 0.02 per cent w/w of rubiadin, calculated on the dried basis.

Category. Yoni roga.

Description. A cylindrical, slightly flattened, wiry pieces of brown to purple coloured root with mild bitter taste.

Identification

A. *Microscopic* — Exfoliating cork, consisting of 4-12 or more layered radially arranged, thin walled cells.

B. *Macroscopic* — Stem, slender, cylindrical, wiry and about 0.5 cm thick. It is brown to purple coloured and with longitudinal cracks.

C. Determine by thin-layer chromatography (2.4.17), the plate with *silica gel GF254*.

Mobile phase. A mixture of 7 volumes of *toluene*, 25 vo... of *ethyl acetate* and 0.5 volumes *glacial acetic acid*.

Test solution. To 4 g of coarsely powdered substance under examination, add 100 ml of *methanol*, reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 50 ml *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 100 ml.

Reference solution. To 1 g of coarsely powdered *manjistha IPBRS*, add 100 ml of *methanol*, reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 50 ml *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 25 ml.

Apply to the plate 20 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with *anisaldehyde sulphuric acid reagent*. Heat the plate at 100° for 5 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 4.0 per cent.

Water-soluble extractive (2.6.3). Not less than 20.0 per cent by Method I.

Total ash (2.3.19). Not more than 10.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.5 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh 4 g of coarsely powdered substance under examination, add 100 ml of *methanol*, reflux on a water-bath 15 minutes, cool and filter. Reflux the residue 2 × 50 ml *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 100.0 ml.

Reference solution. A 0.002 per cent w/v solution of *rubiadin IPRS* in *methanol*.

Chromatographic system

– a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),

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— mobile phase: A. a buffer solution pH 2.5 prepared by dissolving 0.136 g of *potassium di-hydrogen orthophosphate* in 900 ml of *water*, add 0.5 ml of *orthophosphoric acid* and dilute to 1000 ml with *water*, B. *acetonitrile*,
— a gradient programme using the conditions given below,
— flow rate: 1.5 ml per minute,
— spectrophotometer set at 278 nm,
— injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	65	35
5	50	50
15	20	80
30	20	80
35	50	50
40	65	35

Inject the reference solution. The relative standard deviation for the replicate injections is not more than 2.0 per cent.

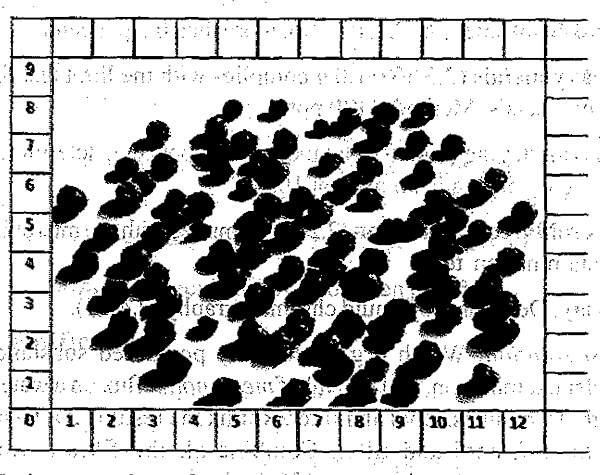
Inject the reference solution and the test solution.

Calculate the content of rubiadin.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Maricha

Black pepper; pepper; *Piper nigrum*



Maricha consists of the unripe fruits of *Piper nigrum* Linn. (Fam. Piperaceae).

Maricha contains not less than 2.5 per cent w/w of piperine, calculated on the dried basis.

Category. Anticonvulsant, bioavailability enhancer, appetizer, anthelmintic, swasa.

Description. Fruits are globular or oblong. They have a blackish brown cover, with raised reticulated wrinkles. The odour is aromatic and the taste is strong and pungent.

Identification

A. *Macroscopic* — The fruits are globular or oblong, 4-6 mm in diameter. The outer cover is blackish brown, with raised reticulated wrinkles. One seeded, seeds are white and hollow.

B. *Microscopic* — The fruit has a well differentiated pericarp, testa and perisperm. Isolated, tangentially elongated oil cells are in the outer region of the mesocarp. Endocarp has beaker shaped stone cells and numerous polyhedral masses of starch grains. Testa has a single layer of yellow colored cells.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *benzene*, 30 volumes of *ethyl acetate* and 10 volumes of *diethyl ether*.

Test solution. To 2 g of the coarsely powdered substance under examination, add 50 ml of *methanol* and reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 75 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 50 ml.

Reference solution. To 2 g of the *maricha IPRS*, add 50 ml of *methanol* and reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 75 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 50 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with *vanillin sulphuric acid reagent*. Heat the plate at 100° for 5-10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 6.0 per cent.

Water-soluble extractive (2.6.3). Not less than 6.0 per cent by Method I.

Total ash (2.3.19). Not more than 7.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

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Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh 2 g of coarsely powdered substance under examination, add 50 ml of *methanol*, sonicate for 3 minutes and heat on a boiling water bath for 15 minutes, cool and dilute to 100.0 ml with *methanol* and filter. Dilute further if necessary.

Reference solution. A 0.01 per cent w/v solution of *piperine IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution pH 2.5 prepared by dissolving 0.136 g of *potassium di-hydrogen orthophosphate* in 500 ml of *water*, add 0.5 ml of *orthophosphoric acid* and dilute to 1000 ml with *water*, B. *acetonitrile*,
- flow rate: 1.5 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 345 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per centv/v)
0	95	5
18	55	45
25	20	80

Inject the reference solution. The relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of piperine.

Storage. Store protected from moisture and against attack by insects and rodents.

Mentha Oil

Mentha

Mentha Oil is the volatile oil distilled with steam from various species of *Mentha* (Fam. Labiatae) and rectified if necessary.

Mentha Oil contains not less than 50.0 per cent w/w of total menthol, $C_{10}H_{20}O$.

Category. Pharmaceutical aid.

Description. A colourless or yellowish, clear liquid; odour, characteristic and pleasant.

Tests

Acidity or alkalinity. A solution of 1 ml of mentha oil in 3.5 ml of *ethanol (70 per cent)* is neutral to *litmus*.

Weight per ml (2.4.29). 0.892 g to 0.910 g.

Specific optical rotation (2.4.22). –18.0° to –33.0°.

Assay. Place about 10.0 g in an acetylation flask, add 10 ml of *acetic anhydride* and 1 g of *anhydrous sodium acetate*, attach a reflux condenser and boil for 2 hours. Cool, add 30 ml of *water* and warm on a water-bath for 15 minutes with occasional shaking. Transfer the contents of the flask to a separating funnel, reject the water layer and wash the remaining oil with *water* until the last washing no longer shows acid reaction. Dry the resulting oil by shaking with 2 g of *anhydrous sodium sulphate*, allow to stand for 30 minutes and filter through a dry filter paper. Weigh accurately about 1.5 g of the dry acetylated oil, add 3 ml of *ethanol (95 per cent)* and 0.1 ml of *phenolphthalein solution* and dropwise, *0.5 M ethanolic potassium hydroxide* until the solution acquires a faint pink colour. Add further 20.0 ml of the alkali, attach a reflux condenser and boil for 1 hour on a water-bath. Cool, add 1 ml of *phenolphthalein solution* and titrate the excess of alkali with *0.5 M hydrochloric acid*. Repeat the operation with the same quantities of the same reagents in the same manner without the oil and calculate the amount of total menthol from the following expression.

$$\text{Total menthol (per cent)} = \frac{(a-b) \times 7.813}{S - (a-b) \times 0.021}$$

where S is the amount, in g, of the acetylated sample taken, a is the amount, in ml, of *0.5 M hydrochloric acid* consumed in the blank test, and b is the amount, in ml, of *0.5 M hydrochloric acid* consumed in saponification of the acetylated oil.

Storage. Store protected from light and moisture.

Mentha Arvensis Oil

Mentha Arvensis Oil is the essential oil obtained by steam distillation of the flowering tops of *Mentha arvensis* Lin var. piperascens Malinvaud and var. glabrata (Fam. Lamiaceae).

Mentha Arvensis Oil contains not less than 33.0 per cent and not more than 45.0 per cent of menthol, and not less than 17.0 per cent and not more than 26.0 per cent w/w of menthone.

Category. Pharmaceutical aid.

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Description. A clear, almost colourless to amber yellow liquid, free from sediment, suspended matter and a characteristic mint and menthol like odor.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

Reference solution. A 2.0 per cent w/v solution of *mentha arvensis oil IPRS* in *ethanol (95 per cent).*

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash Point (2.4.44). 118.0° to 122.0°.

Tests

Relative density (2.4.29). 0.890 to 0.910.

Refractive index (2.4.27). 1.457 to 1.465.

Optical rotation (2.4.22). –13.0° to –22.0°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent).*

Reference solution (a). A 2.0 per cent w/v solution of *menthol IPRS* in *ethanol (95 per cent).*

Reference solution (b). A 2.0 per cent w/v solution of *menthone IPRS* in *ethanol (95 per cent).*

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate: 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

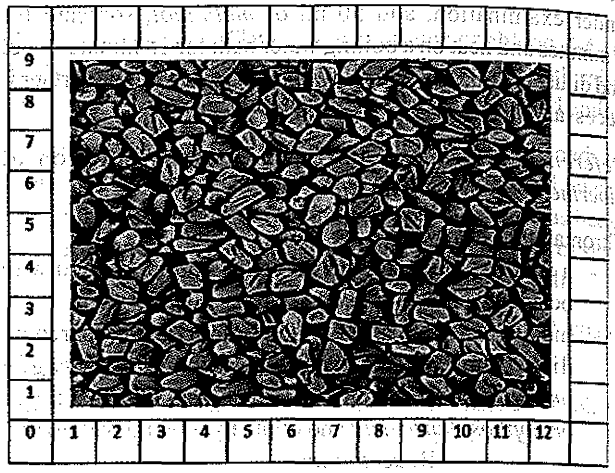
Inject reference solutions (a), (b) and the test solution.

Calculate the contents of menthol, and menthone in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Methi

Trigonella foenum-graecum



Methi is dried, ripe seeds of *Trigonella foenum-graecum* L. (Fam. Fabaceae).

Methi contains not less than 0.1 per cent w/w of trigonelline on dry basis.

Category. Antiinflammatory, analgesic, febrifuge, galactogogue.

Description. The seed is hard, flattened, brown or reddish-brown and more or less rhomboidal with rounded edges. The widest surfaces are marked by a groove that divides the seed into 2 unequal parts. The smaller part contains the radicle; the larger part contains the cotyledons. They have a strong aromatic characteristic odour.

Identification

A. *Macroscopic* — Seed is rhomboidal with rounded edges. It is 3 mm to 5 mm long, 2 mm to 3 mm wide and 1.5 mm to 2 mm thick. The widest surfaces are marked by a groove that divides the seed into two unequal parts. The smaller part contains the radicle; the larger part contains the cotyledons.

B. Reduce to a powder. The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution.* The powder shows fragments of the testa in sectional view with thick cuticle covering lageniform epidermal cells, with an underlying hypodermis of large cells, narrower at the upper end and constricted in the middle, with bar-like thickenings of the radial walls; yellowish-brown fragments of the epidermis in surface view, composed of small, polygonal cells with

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thickened and pitted walls, frequently associated with the hypodermal cells, circular in outline with thickened and closely beaded walls; fragments of the hypodermis viewed from below, composed of polygonal cells whose bar-like thickenings extend to the upper and lower walls; parenchyma of the testa with elongated, rectangular cells with slightly thickened and beaded walls; fragments of endosperm with irregularly thickened, sometimes elongated cells, containing mucilage.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 30 volumes of *water* and 70 volumes of *methanol*.

Test solution. To 1.0 g of the powdered substance under examination, add 5.0 ml of the *methanol*. Heat on a water-bath at 65° for 5 minutes, cool and filter.

Reference solution. Dissolve 3 mg of *trigonelline hydrochloride IPRS* in 1.0 ml of *methanol*.

Apply to the plate 20 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 10 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *potassium iodobismuthate solution*. Heat the plate at 105° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution. It also shows in its upper half, a broad light brownish-yellow zone (triglycerides).

Tests

Swelling Power. Not less than 6.0, determined on the powdered substance under examination, determined by the following method.

Transfer 1 g to a 100 ml stoppered cylinder containing 90 ml of *water*, shake well for 30 seconds and allow to stand 24 hours, shaking gently on three occasions during this period. Add sufficient water to produce 100 ml, mix gently for 30 seconds, avoiding the entrapment of air, allow to stand for 5 hours and measure the volume of mucilage. Repeat the determination three times. The average of four determinations is not less than 40 ml.

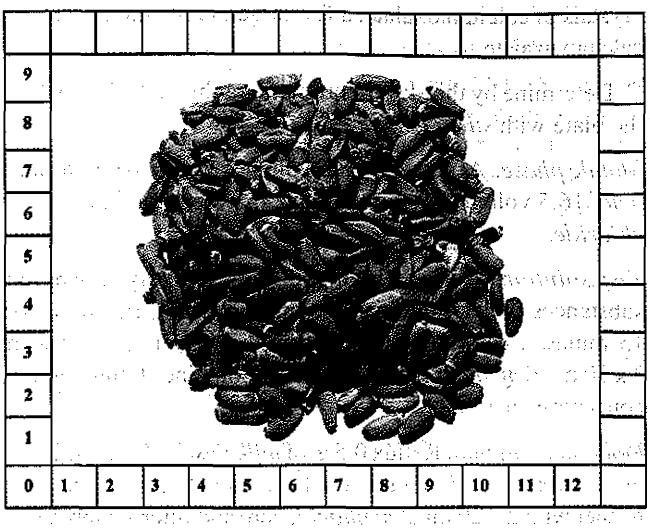
Total ash (2.3.19). Not more than 5.0 per cent.

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 1 g by drying in an oven at 105°.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Milk Thistle

Silybum marianum



Milk thistle consists of dried mature fruit, devoid of the pappus of *Silybum marianum* (Fam. Asteraceae).

Milk thistle contains not less than 1.5 per cent w/w of silymarin, expressed as silibinin calculated on the dried basis.

Category. Hepatoprotective, chemopreventive.

Description. Fruits are elongate-obovate with smooth and shiny outer surface. They have an overall pale greyish or brown colour with no rancid odour.

Identification

A. *Macroscopic* — The achene is strongly compressed, elongate-obovate, about 6-8 mm long, 3 mm broad and 1.5 mm thick, the outer surface is smooth and shiny with a grey or pale brown ground colour variably streaked dark brown longitudinally to give an overall pale greyish or brown colour; the fruit is tapering at the base and crowned at the apex with a glistening, pale yellow extension forming a collar about 1 mm high surrounding the remains of the style. Cut transversely, the fruit shows a narrow, brown outer area and 2 large, dense, white oily cotyledons.

B. *Microscopic* — The powder is brownish-yellow with dark specks. Examine under a microscope using *chloral hydrate solution*. The powder shows the following diagnostic characters: fragments of the epicarp composed of colourless cells, polygonal in surface view, the lumen appearing fairly large or as a small slit, depending on the orientation, groups of parenchymatous cells from the pigment layer, some of them containing colouring matter which appears bright red, very abundant groups of large sclereids from the testa with bright yellow pitted walls and a narrow lumen, occasionally

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fragments of small-celled parenchyma with pitted and beaded walls, abundant thin-walled parenchymatous cells from the cotyledons containing oil globules and scattered cluster crystals of calcium oxalate, a few larger, prismatic crystals of calcium oxalate.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 8.5 volumes of *anhydrous formic acid*, 16.5 volumes of *acetone* and 75 volumes of *methylene chloride*.

Test solution. Reflux about 1.0 g of coarsely powdered substances under examination with 50 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 x 50 ml of *methanol*, cool and filter. Combine all filtrates and concentrate to 10 ml.

Reference solution. Reflux 0.5 g of *milk thistle IPBRS* in 20 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 x 20 ml of *methanol*, cool and filter. Combine all filtrates and concentrate to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate at 100-105° for 5 minutes and examine under ultraviolet light at 254 nm. Spray the warm plate with a 1.0 per cent w/v solution of *diphenylboric acid aminoethyl ester* in *methanol* and subsequently spray with a 5.0 per cent w/v solution of *macrogol 400* in *methanol*. Heat the plate 105° for 5 minutes and examine in ultraviolet light at 254 nm and 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol soluble extractive (2.6.2.). Not less than 3.0 per cent.

Water soluble extractive (2.6.3). Not less than 12.0 per cent by Method I.

Total ash (2.3.19). Not more than 8.0 per cent.

Acid insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metal (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 8.0 per cent, determined on 1.0 g of the powdered drug by drying in an oven at 105° for 2 hours.

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 5 g of the powdered drug in a continuous extraction apparatus. Add 100 ml of *light petroleum* and heat on a water-bath for 8 hours. Allow the defatted drug to dry at room temperature. In a continuous extraction apparatus, extract the latter with 100 ml of *methanol* in a water-bath for 5 hours. Evaporate the methanolic extract in vacuum to a volume of 30 ml. Filter into a 50 ml volumetric flask, rinse the extraction flask and the filter, and dilute to 50 ml with *methanol*.

Reference solution. Dissolve a 10 mg of *silibinin IPRS* in *methanol* and dilute to 100 ml with the same solvent.

Chromatographic system

- a stainless steel column 12.5 cm x 4 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 0.5 volumes of *orthophosphoric acid*, 35 volumes of *methanol* and 65 volumes of *water*,

  B. a mixture of 0.5 volumes of *orthophosphoric acid*, 50 volumes of *methanol* and 50 volumes of *water*,
- a gradient programme using the conditions given below,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 288 nm,
- injection volume: 10 µl.

Time (in min.)	mobile phase A (per cent v/v)	mobile phase B (per cent v/v)
0	100	0
28	0	100
35	0	100
36	100	0
51	100	0

Inject the reference solution. The test is not valid unless the resolution between silibinin A and silibinin B is not less than 1.8.

Inject the reference solution and the test solution.

Calculate the percentage content of total silymarin, expressed as silibinin, using the following expression:

$$\frac{(A_1 + A_2) \times M_2 \times P}{(A_3 + A_4) \times M_1}$$

where, A_1 = area of the peak due to silibinin A in the chromatogram obtained with the test solution.

A_2 = area of the peak due to silibinin B in the chromatogram obtained with the test solution.

A_3 = area of the peak due to silibinin A in the chromatogram obtained with the reference solution.

A_4 = area of the peak due to silibinin B in the chromatogram obtained with the reference solution.

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M_1 = mass of the herbal drug used to prepare the test solution, in grams.

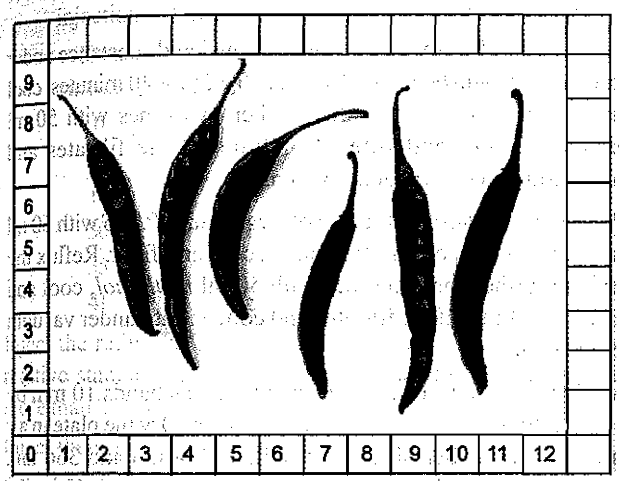
M_2 = mass of *silbinin IPRS* in the reference solution, in grams.

P = percentage content *of silibinin IPRS*.

Storage. Store in air tight amber colour containers protected from light, heat and moisture.

Mirch

Capsicum Fruit, *Capsicum annuum*



Mirch consists of dried ripe fruits of *Capsicum annuum* (Linn.) *var. minimum* (Miller) Heiser and small fruited varieties of *Capsicum frutescens* (Linn.), (Fam. Solanaceae).

Mirch contains not less than 0.25 per cent of capsaicin calculated on the dried basis.

Category. Analgesic, anti-inflammatory, antibacterial, anti-microbial and antioxidant.

Description. The fruit is yellowish-orange or reddish-brown, oblong conical with an obtuse apex, it has a characteristic odour and bitter taste.

Identification

A. *Macroscopic* — The fruit is yellowish-orange or reddish-brown, oblong conical with an obtuse apex, about 1-3 cm long and up to 1 cm in diameter at the widest part, occasionally attached to a 5-toothed inferior calyx and a straight peduncle. Pericarp somewhat shrivelled, glabrous, enclosing about 10-20 flat, reniform seeds 3-4 mm long, either loose or attached to a reddish dissepiments.

B. *Microscopic — Powder.* it shows the fragments of the epicarp, in surface view, with cells often arranged in rows of 5 to 7 thick-walled when close to the peduncle and with a cuticle uniformly striated; fragments of the pericarp, in transverse section, showing the epicarp covered by a thick cuticle and parenchymatous cells frequently containing droplets of red oil, occasionally containing microsphenoidal crystals of calcium oxalate; fragments of endocarp with characteristic island groups of sclerenchymatous cells, the groups being separated by thin-walled parenchymatous cells; fragments of the seeds having an episperm composed of large, greenish-yellow, sinuous- walled sclereids with thin outer walls and strongly and unevenly thickened radial and inner wall.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 7 volumes of *toluene* and 3 volumes of *ethyl acetate.*

Test solution. Reflux 2 g of coarsely powdered substance under examination with 25 ml of *methanol.*

Reference solution. Reflux 2 g of *mirch IPBRS* with 25 ml of *methanol.*

Apply to the plate 10 µl of each solution as band length 8 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in ultraviolet light at 254 nm and 366 nm. Spray the plate with *anisaldehyde sulphuric acid reagent.* Heat the plate at 105° for 5 minutes. Examine the plate in visible light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Total ash (2.3.19). Not more than 10.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.2 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 11.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Extract 2.5 g of the substance under examination with 100 ml *methanol* by maceration for 30 minutes and sonicate for 15 minutes. Filter in 100 ml volumetric flask and make up the volume with *methanol.*

Reference solution. A 0.02 per cent w/v solution of *capsaicin IPRS* in *methanol.*

Chromatographic system

– a stainless steel column 25 cm x 4.6 mm packed with *phenylsilyl silica gel* (5 µm),

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– mobile phase: a mixture of 40 volumes of *acetonitrile*, 60 volumes of buffer solution prepared by dissolving 1.0 ml of *orthophosphoric acid* in 1000 ml of *water*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 225 nm,
– injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

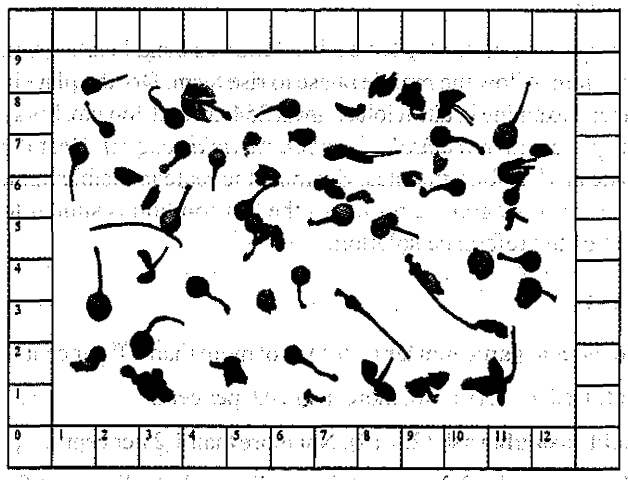
Inject the reference solution and the test solution.

Calculate the content of capsaicin.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Nagakesar

Cobra Saffron; *Mesua ferrea*



Nagakesar consists of dried stamen and flower buds of *Mesua ferrea* L. (Fam. Clusiaceae).

Nagakesar consists of not less than 0.3 per cent w/w of mesuol calculated on dried basis.

Category. Raktapitta, vatarakta, immunomodulator, wound healing.

Description. Odour fragrant and bitter in taste.

Identification

A. *Macroscopic* — Stamen consists of anther, connective and filament, coppery or golden brown, filament united at base, forming a fleshy ring, each stamen 0.9- 1.9 cm long, anther about 0.5 cm long, linear basifixed containing pollen grains, filament 0.8-1.0 cm long, slender, filiform, more or less twisted, soft to touch, quite brittle, connective not visible with naked eye, odour fragrant, taste astringent.

B. *Microscopic* — Androceium- Anther shows golden brown, longitudinally dehiscent, anther wall, consisting of thin walled, parenchymatous cells, pollen grains numerous in groups or in single, yellowish and thin walled, many pollen grains having 1-3 minute distinct protuberances on walls, thick walled, exine and intine distinct.

Powder - Brown, shows elongated cells of filament, connective and numerous golden yellow pollen grains having 1-3 protuberances.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 9 volumes of *toluene* and 1 volume of *ethyl acetate*.

Test solution. Reflux 2 g of coarsely powdered substance under examination with 60 ml *methanol* at 70 ± 5° for 40 minutes, cool and filter. Reflux the residue further two times with 50 ml *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 50 ml.

Reference solution. Reflux 2 g of *nagakesar IPBRS* with 60 ml *methanol* at 70 ± 5° for 40 minutes, cool and filter. Reflux the residue further for two times with 50 ml *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to get 50 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent.* Heat the plate at 100° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Forein organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol- soluble extractive (2.6.2). Not less than 15.0 per cent.

Water- soluble extractive (2.6.3). Not less than 14.0 per cent.

Total ash (2.3.19). Not more than 5.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.5 per cent

Heavy metals (2.3.13). 1 gm complies with limit test for heavy metals, Method B (20 ppm).

Loss on Drying (2.4.19). Not more than 9.0 per cent.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14)

Test solution. Reflux 1 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water bath for 15 minutes cool and filter. Reflux the residue further with 5 x 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate to 50.0 ml.

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Reference solution. A 0.2 per cent w/v solution of *mesuol IPRS* in *methanol.*

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with octadecysilane bonded to porous silica (5µm),
- mobile phase: A. a buffer solution prepared by dissolving 0.136 g of *potassium di-hydrogen orthophosphate* in 500 ml of *water,* add 0.5 ml of *orthophosphoric acid* and diluted to 1000 ml with water, B. *acetonitrile,*
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	15	85
12	45	55
18	85	15
30	85	15
35	45	55
41	15	85

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injection is not more than 2.0 per cent.

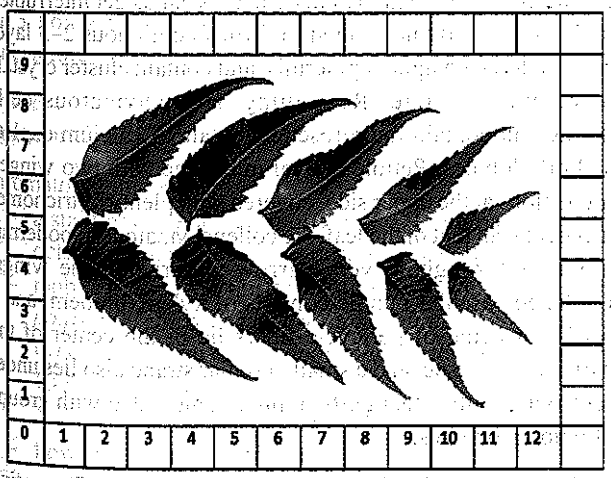
Inject the reference solution and the test solution.

Calculate the content of mesuol.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Neem

Azadirachta indica



Neem consist of dried leaves of *Azadirachta indica* A. Juss. (Fam. Meliaceae).

Neem leaves contain not less than 1.0 per cent w/w of the stated amount of rutin, calculated on the dry basis.

Category. Antileprotic, antipruritic, antifertility, jvara, in skin diseases.

Description. Characteristic odour and bitter taste.

Identification

A. *Macroscopic*—The leaves dark green, the petioles are short.The shape of mature leaflets is more assymetrical and their margins are dentate with the exception of the base of their basiscopal half, which is very strongly reduced and cuneate or wedge-shaped leaves.

B. *Microscopic*—Upper and lower epidermis, exhibiting two layers of palisade cells below the upper epidermis. The spongy parenchyma with intercellular spaces abundant on the border line of palisade cells. Midrib shows numerous collenchymatous cells below upper and lower epidermis. A characteristic zone of vascular bundles is present.

C. Determine by thin-layer chromatography (2.4.17) coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 25 volumes of *ethyl acetate,* 15 volumes of *n-butanol,* 5 volumes of *formic acid* and 5 volumes of *water.*

Test solution. Reflux 5.0 g of the coarsely powdered substance under examination with 100 ml of *hexane* for 2 hours, cool and filter. Reflux the residue further for 1hour with 100 ml of *methanol,* cool and filter. Concentrate under vacuum to 25 ml.

Reference solution. Reflux 2.5g of the coarsely powdered *neem IPBRS* with 100 ml *hexane* for 2 hours, cool and filter. Reflux the residue further for 1hour with 100 ml of *methanol,* cool and filter. Concentrate under vacuum to 10 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254. Spray the plate with *anisaldehyde sulphuric acid reagent.* Heat the plate at 105° for 5 minutes and examine the plate at 366 nm and in day light. The chromatographic profile of test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol soluble extractive (2.6.2). Not less than 7.0 per cent.

Water soluble extractive (2.6.3). Not less than 19.0 per cent by Method I.

Total ash (2.3.19). Not more than 12.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

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Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 5.0 g of the coarsely powdered substance under examination with 100 ml of *n-hexane* for 2 hours, cool and filter. Reflux the residue further for 1 hour with 100 ml of *methanol*, cool and filter. Concentrate under vacuum to 25 ml.

Reference solution. A 0.05 percent w/v of *rutin IPRS* in *methanol*.

Chromatographic system

- stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5µm),
- mobile phase: a mixture of 30 volumes of *acetonitrile* and 70 volumes of 0.5 per cent w/v of *formic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 340 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2 per cent.

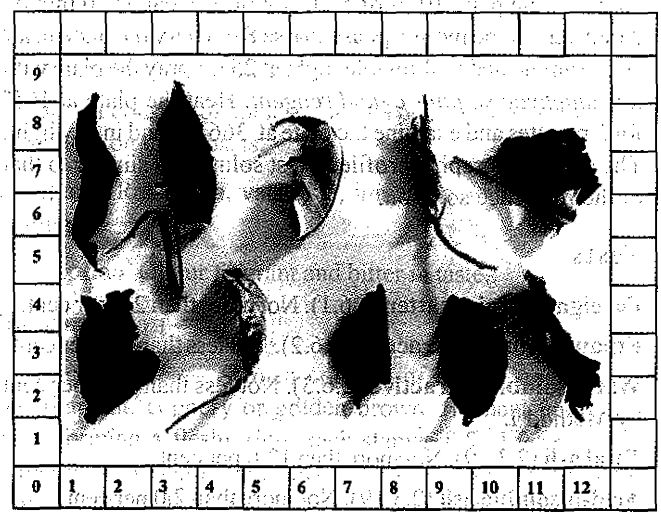
Inject the reference solution and the test solution.

Calculate the content of rutin.

Storage. Store protected from heat, moisture and against attack by rodents and insects.

Nirgundi

Sambhalu, Mewri, Nisinda, *Vitex negundo*



Nirgundi consists of dried leaves of *Vitex negundo* Linn (Fam. Lamiaceae).

Nirgundi contains not less than 0.3 per cent w/w of negundoside calculated on the dried basis.

Category. Analgesic, anti-inflammatory and antirheumatic.

Description. Green to brownish coloured leaves and having characteristic odour.

Identification

A. *Macroscopic* — Leaf is leathery, palmately trifoliate to pentafoliate, middle leaflet largest 6–12 cm long, 1.5–3 cm wide, 1–3 cm long petiole; two lowest lateral leaflets of the pentafoliates are small and almost sessile. Leaflets, lanceolate, acute, entire or rarely crenate, pubescent and dark green on upper surface, tomentose and whitish on lower surface; venation, reticulate, lateral vein arising at an angle of 45°, almost running parallel to each other. Rachis long, circular in outline, with somewhat irregular margin covered with plenty of trichomes.

B. *Microscopic* — Upper epidermis of the lamina, composed of polygonal tubular cells with straight thick anticlinal walls, covered with thin cuticle and bears few trichomes; mesophyll 3–5 compact rows of palisade cells discontinuous over the midrib region, slightly narrower, reaching up to two lateral edges of a centrally located crescent-shape meristele associated with a narrow arc of parenchymatous phloem at its lower side and small islets of them on its upper side; the cells of the lower epidermis much smaller, stomata and trichomes plenty, the latter almost concealing whole lower surface of leaf, both simple and glandular trichomes of various types present; simple, unicellular, on upper epidermis, a few multicellular, uniseriate 2–6 cells often collapsed or warty cell, rarely branched; grandular trichomes, sessile with 1–4 cell globular head and with unicellular stalk. Along meristeles of the mesophyll, vertical parenchymatous bands get interrupted in the palisade tissue, extend to form a continuous 2–3 layer zone underneath upper epidermis, and contain cluster crystals of calcium oxalate. Remaining parenchymatous cells throughout contain microrosette crystals of calcium oxalate and oil globules. Petiole is oval in outline with two winged projections at the upper side, covered with plenty of trichomes identical with that of the leaf, the collenchymatous hypodermis traverses throughout very narrow underneath the wings, U-shaped vascular bundle consists of xylem, phloem, and a continuous ring of pericycle fibers lie in the center of the parnechyma tissue, and a small vascular strand also lies under each wing; pith cells lignified, pitted embedded with groups of phloem bundles.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with silica gel *GF254*.

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Mobile phase. A mixture of 55 volumes of *ethyl acetate*, 45 volumes of water, 2 volumes of *acetic acid*.

Test solution. Reflux 2 g of coarsely powdered substance under examination with 25 ml of *methanol* for 15 minutes on a water bath, cool and filter.

Reference solution. Reflux 2 g of *nirgundi IPBRS* in *methanol* for 15 minutes on a water bath. Cool and filter.

Apply to the plate 10 µl of each solution band length 8 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in ultraviolet light at 254 and 366 nm, spray with the *anisaldehyde sulphuric acid reagent* and heat the plate at 105° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more that 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 10.0 per cent.

Water-soluble extractive (2.6.3). Not less than 20.0 per cent.

Total ash (2.3.19). Not more that 8.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 5 g coarsely powdered substance under examination with 200 ml *methanol* by soxhlet apparatus for 3 hours till extract turn colourless, cool and filter, concentrates to 100 ml.

Reference solution. A 0.005 per cent w/v solution of *negundoside IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm packed with octadecylsilane bonded to porous silica (10 µm)
- mobile phase: A. a buffer solution dissolving 0.136 g of *potassium dihydrogen orthophosphate* in 900 ml of *water* and 0.5 ml of *orthophosphoric acid* and dilute to 1000 ml with *water*.

B. *acetonitrile*

- flow rate. 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
10	85	15
20	80	20
23	80	20
25	85	15
30	90	10

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

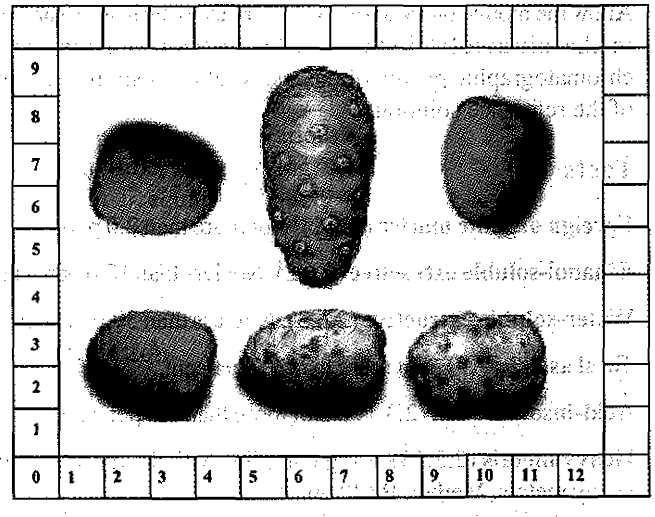
Inject the reference solution and the test solution.

Calculate the content of negundoside.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Noni

Bartundi, Indian mulberry, Morinda; *Morinda citrifolia*



Noni consists of dried fruits of *Morinda citrifolia* Linn. (Fam. Rubiaceae).

Noni contains not less than 0.33 per cent w/w of scopoletin, calculated on the dried basis.

Category. Antidiabetic, immunomodulator.

Description. Fruit is light green, odour characteristic, somewhat disagreeable.

Identification

A. *Macroscopic* — 3 to 10 cm long and about 3 to 6 cm in diameter, with an embossed appearance, slightly wrinkled,

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waxy, semi-translucent skin, green to yellow and to almost white on ripening, the fruit surface is faintly patterned with 4 to 6 sided outlines, each with a central eye, the pulp is fleshy and juicy, having numerous hard oblong-triangular reddish-brown pits, each containing 4 seeds about 3.5 mm long.

B. *Microscopic* — Thin-walled parenchyma cells, exocarp and sparsely distributed thin strands of vascular tissue containing tracheary elements with annular, spiral and rarely, pitted secondary walls, acicular calcium oxalate crystals, starch granules and oil globules.

C. Determine by thin layer chromatography (2.4.17) coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 9 volumes of *chlorform* and 1 volume of *methanol*.

Test solution. Reflux about 2.0 g of coarsely powdered substance under examination with 25 ml of *methanol* for 15 minutes. Cool and filter. Concentrate under vacuum to 5 ml.

Reference solution. Reflux about 2.0 g of *noni IPBRS* in 25 ml of *methanol* for 15 minutes. Cool and filter. Concentrate under vacuum to 5 ml.

Apply to the plate 10 µl of each solution as band length 8 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air, examine in the ultraviolet light at 254 nm and 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 17.0 per cent.

Water-soluble extractive (2.6.3). Not less than 39.0 per cent.

Total ash (2.3.19). Not more than 6.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 6.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh about 5.0 g of coarsely powdered substance under examination, add 20 ml of *methanol*. Sonicate for 60 minutes at 50°. Cool, centrifuge and filter supernatant through 0.45 µm filter.

Reference solution. A 0.04 per cent w/v solution of *scopoletin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 30 cm x 3.9 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. 0.1 per cent w/v solution of *formic acid* in *water*,

  B. *acetonitrile*,
- column temperature: 30°
- a gradient programme using the conditions given below,
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 350 nm,
- injection volume: 10 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
6	85	15
15	82	18
25	90	10
30	90	10

Inject the reference solution. The relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of scopoletin.

Storage. Store protected from light, heat, moisture and against attack by insects and rodents.

Nutmeg Oil

Jaiphal ka tail

Nutmeg Oil is the essential oil obtained by steam distillation of dried and crushed nuts of *Myristica fragans* Houttuyn (Fam. Myristicaceae).

Nutmeg oil contains not less than 15.0 per cent and not more than 28.0 per cent of α -pinene, not less than 13.0 per cent and not more than 18.0 per cent of β -pinene, not less than 14.0 per cent and not more than 29.0 per cent of sabinene, not less than 2.0 percent and not more than 7.0 percent of limonene, not less than 0.5 per cent and not more than 2.0 per cent Δ-3 carene, not less than 2.0 per cent and not more than 6.0 per ecnt of γ -terpinene, not less than 2.0 per cent and not more than 6.0 per cent of terpinen-4-ol, not less than 1.0 per cent and not more than 2.5 per cent safrole and not less than 5.0 per cent and not more than 12.0 percent of myristicine.

Description. A clear colourless to light yellow liquid, free from sediment and suspended matter, spicy, sweet, aromatic odour with characteristic nuances.

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Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *nutmeg oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 46.0° to 50.0°.

Tests

Relative density (2.4.29). 0.885 to 0.905.

Refractive index (2.4.27). 1.475 to 1.485.

Optical rotation (2.4.22). +8.0° to +18.0°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *α-pinene IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *β-pinene IPRS* in *ethanol (95 per cent)*.

Reference solution (c). A 2.0 per cent w/v solution of *sabinene IPRS* in *ethanol (95 per cent)*.

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  - column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
  - inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

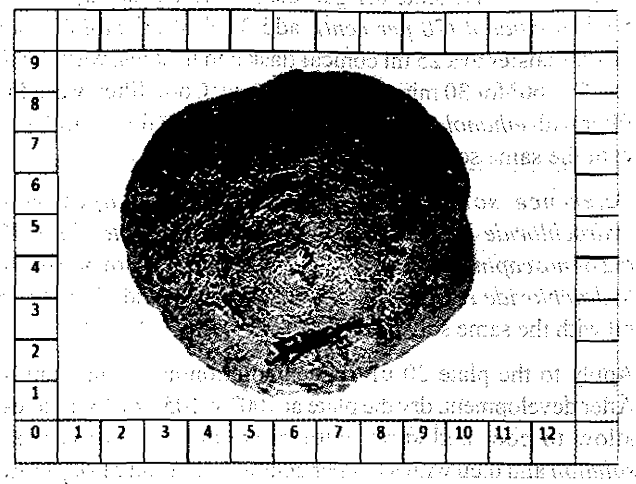
Inject reference solutions (a) (b), (c) and the test solution.

Calculate the contents of α-pinene, sabinene and β-pinene in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Opium

Raw Opium; *Papaver somniferum*



Opium is the air-dried latex obtained by incision from the unripe capsules of *Papaver somniferum* Linn (Fam. Papaveraceae).

Opium contains not less than 10.0 per cent w/w of morphine, $C_{17}H_{19}NO_3$, and not less than 2.0 per cent w/w of codeine, $C_{18}H_{21}NO_3$, both calculated on the dried basis.

Category. Narcotic analgesic, antitussive.

Description. Masses of various sizes which tend to be soft and shiny and, after drying, hard and brittle; usually in somewhat irregularly shaped masses (natural opium) or moulded into masses of more uniform size and shape (manipulated opium); colour, blackish brown; odour, strong and characteristic.

Identification

Strip off any covering, cut the substance under examination into thin slices, if necessary, dry at about 60° for 48 hours and reduce to a powder.

A. When examined under a microscope, a suspension in a 2 per cent w/v solution of *potassium hydroxide* appears as granules of latex agglomerated in irregular masses and light brown elongated filaments. Some fragments of vessels and rather elongated, refringent crystals are also visible, as well as a smaller number of round pollen grains and fragments of elongated fibres. Hairs of various lengths with sharp points and a few grains of starch introduced during the handling of the latex may be present. Fragments of epicarp consisting of polygonal cells with thick walls defining a stellate lumen may also be present.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel G*.

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Mobile phase. A freshly prepared mixture of 20 volumes of *acetone*, 20 volumes of *toluene*, 3 volumes of *ethanol (95 per cent)* and 1 volume of *strong ammonia solution*.

Test solution. Triturate 0.1 g of the powdered substance with 5 ml of *ethanol (70 per cent)*, add 3 ml of *ethanol (70 per cent)*, transfer to a 25 ml conical flask and heat in a water-bath at 50° to 60° for 30 minutes, with stirring. Cool, filter, wash the filter with *ethanol (70 per cent)* and dilute the filtrate to 10 ml with the same solvent.

Reference solution. Dissolve 2 mg of *papaverine hydrochloride IPRS*, 12 mg of *codeine phosphate IPRS*, 12 mg of *noscapine hydrochloride IPRS*, and 25 mg of *morphine hydrochloride IPRS* in *ethanol (70 per cent)* and dilute to 25 ml with the same solvent.

Apply to the plate 20 µl of each solution as 20 mm bands. After development, dry the plate at 100° to 105° for 15 minutes, allow to cool and spray with *potassium iodobismuthate solution* and then with a 0.4 per cent w/v solution of *sulphuric acid*. The chromatogram obtained with the reference solution shows in the lower part an orange-red or red band (morphine), above it a similarly coloured band (codeine) and in the upper part an orange-red or red band (papaverine) and above it a similarly coloured band (noscapine). The chromatogram obtained with the test solution shows orange-red or red bands corresponding to those in the chromatogram obtained with the reference solution. The chromatogram obtained with the test solution may also show a dark red band (thebaine) situated between those due to codeine and to papaverine.

C. To 1 g of the powdered substance add 5 ml of *water*, shake for 5 minutes, filter and add to the filtrate 0.25 ml of *ferric chloride solution*; a red colour develops which does not disappear on the addition of 0.5 ml of *2 M hydrochloric acid*.

Tests

Thebaine. Not more than 3 per cent, calculated on the dried basis.

Determine by liquid chromatography (2.4.14).

Test solution. Use the test solution prepared in the assay.

Reference solution. Dissolve 25 mg of *thebaine* in sufficient mobile phase to produce 25.0 ml and dilute 10.0 ml of this solution to 100.0 ml with the mobile phase.

Chromatographic system as described in the Assay.

The test is not valid unless the capacity factor for thebaine is not less than 3.0 and the number of theoretical plates is not less than 3000. Calculate the percentage content of thebaine from the expression given in the assay.

Total ash (2.3.19). Not more than 6.0 per cent, determined on 0.5 g.

Loss on drying (2.4.19). Not more than 15.0 per cent, determined on 0.25 g cut into thin slices, by drying in an oven at 105° for 4 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Suspend about 1.0 g, accurately weighed substance under examination, cut into thin slices, in 50 ml of *ethanol (50 per cent)*, mix with the aid of ultrasound for 1 hour, allow to cool, dilute to 100.0 ml with the same solvent and allow to stand. To 10.0 ml of the supernatant liquid add 5 ml of *ammonia buffer pH 9.5*, dilute to 25.0 ml with *water*, mix, transfer 20.0 ml of the solution to a column (about 15 cm × 30 mm) containing 15 g of kieselguhr for column chromatography and allow to stand for 15 minutes. Elute with two quantities, each of 40 ml, of a mixture of 85 volumes of *dichloromethane* and 15 volumes of *2-propanol*, evaporate the eluate to dryness at 40° at a pressure of 2 kPa, transfer the residue to a 25-ml volumetric flask with the aid of the mobile phase and dilute to volume with the same solvent.

Reference solution. Weigh accurately 0.1 g of *morphine hydrochloride IPRS* and 25 mg of *codeine phosphate IPRS*, dissolve in sufficient of the mobile phase to produce 25.0 ml and dilute 10.0 ml of this solution to 100.0 ml with the same solvent.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm), fitted with a guard column 4 cm x 4.6 mm packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: 1.0 g of *sodium heptanesulphonate* in 420 ml of *water*, adjusting to pH 3.2 with *phosphoric acid* that has been diluted to contain 0.49 per cent w/v solution of *orthophosphoric acid* (about 5 ml) and adding 180 ml of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm.

Inject suitable volumes of each solution. The assay is not valid unless the resolution between the peaks corresponding to morphine and codeine is at least 2.5. If necessary, adjust the volume of *acetonitrile* in the mobile phase. Inject the reference solution six times. The assay is not valid unless the relative standard deviation of the peak area for morphine is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the percentage content of each alkaloid from the expression

$$\frac{w_1 \times A_2 \times 625 \times 100}{w_2 \times A_1 \times 5(100-h)}$$

Where, w_1 = weight, in g, of the alkaloid used to prepare the reference solution,

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w_2 = weight, in g, of the substance under examination used to prepare the test solution,

A_1 = area of the peak corresponding to the alkaloid in the chromatogram obtained with the reference solution,

A_2 = area of the peak corresponding to the alkaloid in the chromatogram obtained with the test solution,

h = percentage loss on drying.

For calculation, 1 mg of morphine hydrochloride may be taken as equivalent to 0.759 mg of morphine and 1 mg of codeine phosphate may be taken as equivalent to 0.943 mg of codeine.

Storage. Store protected from light and moisture.

Opium Powder

Opium Powder is Opium dried at a temperature not exceeding 70°, reduced to a fine or moderately fine powder and adjusted by the addition of Lactose, suitably coloured with Caramel, or other suitable diluent to contain about 10 per cent of morphine and 2 per cent of codeine.

Opium Powder contains not less than 9.5 per cent w/w and not more than 10.5 per cent w/w of morphine, $C_{17}H_{19}NO_3$, and not less than 1.9 per cent w/w and not more than 2.1 per cent w/w of codeine, $C_{18}H_{21}NO_3$, both calculated on the dried basis.

Category. Narcotic analgesic, antitussive.

Description. A light brown powder consisting of yellowish brown or brownish red particles; odour, characteristic.

Identification

A. When examined under a microscope, the residue obtained after extraction with *water*, appears as granules of latex agglomerated in irregular masses and light brown elongated filaments. Some fragments of vessels and rather elongated, refringent crystals are also visible, as well as a smaller number of round pollen grains and fragments of elongated fibres. Hairs of various lengths with sharp points and a few grains of starch introduced during the handling of the latex may be present. Fragments of epicarp consisting of polygonal cells with thick walls defining a stellate lumen may also be present and if powdered cocoa husk is present, the following: brown colour of the fragments; narrow spiral vessels about 10 to 20 mm wide, in groups of from one to six, traversing a spongy parenchyma of thin-walled cells about 40 to 60 mm in either direction and united by arm-like projections enclosing almost circular intercellular spaces; fragments of the sclerenchymatous layer, consisting of thick-walled lignified brown, rectangular to polyhedral cells in a single layer, individual cells about 5 to 10 µm wide and 10 to 30 µm long; fragments of mucilage staining in *ruthenium red solution*.

B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 20 volumes of *acetone*, 20 volumes of *toluene*, 3 volumes of *ethanol (95 per cent)* and 1 volume of *strong ammonia solution*.

Test solution. Triturate 0.10 g of the powdered substance with 5 ml of *ethanol (70 per cent)*, add 3 ml of *ethanol (70 per cent)*, transfer to a 25 ml conical flask and heat in a water-bath at 50° to 60° for 30 minutes, with stirring. Cool, filter, wash the filter with *ethanol (70 per cent)* and dilute the filtrate to 10 ml with the same solvent.

Reference solution. Dissolve 2 mg of *papaverine hydrochloride IPRS*, 12 mg of *codeine phosphate IPRS*, 12 mg of *noscapine hydrochloride IPRS*, and 25 mg of *morphine hydrochloride IPRS* in *ethanol (70 per cent)* and dilute to 25 ml with the same solvent.

Apply to the plate 20 µl of each solution as 20 mm bands. After development, dry the plate at 100° to 105° for 15 minutes, allow to cool and spray with *potassium iodobismuthate solution* and then with a 0.4 per cent w/v solution of *sulphuric acid*. The chromatogram obtained with the reference solution shows in the lower part an orange-red or red band (morphine), above it a similarly coloured band (codeine) and in the upper part an orange-red or red band (papaverine) and above it a similarly coloured band (noscapine). The chromatogram obtained with the test solution shows orange-red or red bands corresponding to those in the chromatogram obtained with the reference solution. The chromatogram obtained with the test solution may also show a dark red band (thebaine) situated between those due to codeine and to papaverine.

C. To 1 g of the powdered substance add 5 ml of *water*, shake for 5 minutes, filter and add to the filtrate 0.25 ml of *ferric chloride solution*; a red colour develops which does not disappear on the addition of 0.5 ml of *2 M hydrochloric acid*.

Tests

Thebaine. Not more than 3.0 per cent, calculated on the dried basis.

Determine by liquid chromatography (2.4.14).

Test solution. Use the test solution prepared in the Assay.

Reference solution. Dissolve 25 mg of *thebaine* in sufficient mobile phase to produce 25.0 ml and dilute 10.0 ml of this solution to 100.0 ml with the mobile phase.

Chromatographic condition as described in the Assay.

The test is not valid unless the capacity factor for thebaine is at least 3.0 and the number of theoretical plates is at least

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3000. Calculate the percentage content of thebaine from the expression given in the Assay.

Total ash (2.3.19). Not more than 6.0 per cent, determined on 0.5 g.

Loss on drying (2.4.19). Not more than 15.0 per cent, determined on 0.25 g by drying in an oven at 105° for 4 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Suspend about 1.0 g of the substance under examination, cut into thin slices, in 50 ml of *ethanol (50 per cent)*, mix with the aid of ultrasound for 1 hour, allow to cool, dilute to 100.0 ml with the same solvent and allow to stand. To 10.0 ml of the supernatant liquid add 5 ml of *ammonia buffer pH 9.5*, dilute to 25.0 ml with *water*, mix, transfer 20.0 ml of the solution to a column (about 15 cm × 30 mm) containing 15 g of kieselguhr for column chromatography and allow to stand for 15 minutes. Elute with two quantities, each of 40 ml, of a mixture of 85 volumes of *dichloromethane* and 15 volumes of *2-propanol*, evaporate the eluate to dryness at 40° at a pressure of 2 kPa, transfer the residue to a 25-ml volumetric flask with the aid of the mobile phase and dilute to volume with the same solvent.

Reference solution. Weigh accurately 0.1 g of *morphine hydrochloride IPRS* and 25 mg of *codeine phosphate IPRS*, dissolve in sufficient of the mobile phase to produce 25.0 ml and dilute 10.0 ml of this solution to 100.0 ml with the same solvent.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octylsilane bonded to porous silica (5 µm), fitted with a guard column 4 cm x 4.6 mm packed with octylsilane bonded to porous silica (5 µm),
- mobile phase: 1.0 g of *sodium heptane sulphonate* in 420 ml of *water*, adjusting to pH 3.2 with *phosphoric acid* that has been diluted to contain 0.49 per cent w/v solution of *orthophosphoric acid* (about 5 ml) and adding 180 ml of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 280 nm.

Inject suitable volume of reference solution. The test is not valid unless the resolution between the peaks corresponding to *morphine* and *codeine* is at least 2.5. If necessary, adjust the volume of *acetonitrile* in the mobile phase. The relative standard deviation of the peak area for morphine is not more than 1.0 per cent.

Inject the reference solution and the test solution.

Calculate the percentage content of each alkaloid from the expression

$$\frac{w_1 \times A_2 \times 625 \times 100}{w_2 \times A_1 \times 5(100-h)}$$

Where, w_1 = weight, in g, of the alkaloid used to prepare the reference solution,

w_2 = weight, in g, of the substance under examination used to prepare the test solution,

A_1 = area of the peak corresponding to the alkaloid in the chromatogram obtained with the reference solution,

A_2 = area of the peak corresponding to the alkaloid in the chromatogram obtained with the test solution,

h = percentage loss on drying.

For calculation, 1 mg of morphine hydrochloride may be taken as equivalent to 0.759 mg of morphine and 1 mg of codeine phosphate may be taken as equivalent to 0.943 mg of codeine.

Storage. Store protected from light and moisture.

Papain

Carica papaya

Papain is an enzyme or a mixture of enzymes obtained from the juice of the unripe fruit of *Carica papaya* Linn. (Fam. Caricaceae). It may contain a suitable diluent such as Lactose.

Papain contains not less than the minimum protease activity determined under the conditions of the Assay.

Category. Proteolytic enzyme.

Description. A white to light brown, amorphous or slightly granular powder; odour, characteristic.

Tests

Microbial contamination (2.2.9). 1.0 g is free from *Escherichia coli* and 10.0 g is free from *Salmonella*.

Loss on drying (2.4.19). Not more than 7.0 per cent, determined on 1 g by drying in an oven at 60° at a pressure not exceeding 0.7 kPa for 4 hours.

Assay. Weigh accurately about 0.5 g, triturate with 10 ml of *cysteine hydrochloride solution* and dilute to 100.0 ml with *water*. To 30 ml of *water* in each of two flasks add 15.0 ml of *casein solution* and maintain at 60° by heating on a *water-bath*. To the first flask add 5.0 ml of the solution of the substance under examination, and to the second flask add 5.0 ml of the same solution, previously boiled for 2 minutes and cooled. Maintain the solutions at 60° for 30 minutes, cool rapidly to room temperature and add to each flask 0.75 ml of *phenolphthalein solution* and 10 ml of *formaldehyde solution*, previously neutralised to *phenolphthalein solution*. Titrate both solutions with *0.1 M sodium hydroxide* to the same

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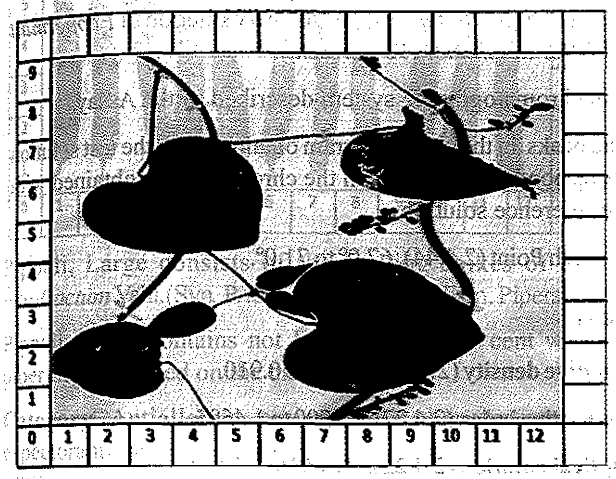
definite pink colour; the difference between the two titrations is not less than 4.5 ml.

Storage. Store protected from light and moisture.

Labelling. The label states the name of any added substance.

Patha

Cissampelos pareira



Patha consists of aerial parts of *Cissampelos pareira* Linn. (Fam. Menispermaceae).

Patha contains not less than 0.06 per cent w/w of methyl seconeolitsine, calculated on the dried basis.

Category. Antidiarrheal, antiemetic, antipyretic, anti-dermis.

Description. It is a perennial climbing shrub with small greenish-yellow flowers. Stems are long cylindrical; leaves are triangular with long petioles.

Identification

A. *Macroscopic* — It is a perennial climbing shrub with small greenish – yellow flowers. Stems are long cylindrical; leaves are triangular with long petioles. Leaves are 3-12 cm long.

B. *Microscopic* — *Stem.* It shows a single layer of epidermis, many cells of which are elongated to form bi-cellular four sepals which are hairy and obovate to oblong. Petals combined into a cyathiform corolla, which is half the length of the sepals. The filaments are longer than the corolla. Female flowers are elongate, solitary or twin, axillary racemes with very short pedicles; bracts foliaceous or nearly sessile and orbicular or reniform. It has one sepal, which is ovate to oblong. It has one petal, which is covering trichomes. Only 3-5 layers of cells, which are slightly collenchymatous in the outer two layers, represent the cortex. Vascular bundles are 5-7 in number and arranged in ring, each capped by a pericycle and separated by 4-7 cells wide medullary rays, which are parenchymatous and radially elongated, but with age, the outer ray cells and adjoining cortical cells become sclerosed, forming moderately thick walled stone cells. The cells of pericycle gradually become thick walled and lignified in outer 5-7 layers and form sclerenchymatous fibers. The vessels are drum shaped with simple or bordered pits.

Leaf. It is dorsi-ventral. Stomata are of anomocytic type, present on lower surface. Upper surface is practically free from stomata. Epidermal cells have wavy walls. A single layer of palisade is present below upper epidermis. Mesophyll consists of parenchymatous cells. Leaf bears bicellular covering trichomes. The midrib region shows collenchymatous cells beneath both epidermal layers. The central region is occupied by vascular bundle. The phloem is present on dorsal side and radiating medullary traverses these.

Trichomes. Trichomes are bicellular with the length upto 360 micron.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 25 volumes of *toluene*, 7.5 volumes of *ethyl acetate* and 2.5 volumes of *formic acid*.

Test solution. Weigh approximately 5.0 g of the coarsely powdered substance under examination, add 50 ml of *methanol*, reflux for 15 minutes, cool and filter. Reflux the residue further with *methanol* till the extract turns colourless, cool and filter. Combine all the filtrates and concentrate to 10 ml.

Reference solution. Boil 5 g of dried *patha IPBRS* powder with 25 ml of *methanol* under reflux for 10 minutes, cool and filter. Boil under reflux with 10 ml of *methanol*, cool and filter.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, Spray with the *anisaldehyde sulphuric acid reagent.* Heat the plate at 105° for 5 minutes. Examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 2.0 per cent.

Water-soluble extractive (2.6.3). Not less than 5.0 per cent by Method I.

Total ash (2.3.19). Not more than 12.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

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Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Extract 2 g of the substance under examination with 50 ml of *methanol* by sonication for 15 minutes followed by warming on water-bath for another 15 minutes, cool and filter. Further extract the marc, twice 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 50.0 ml.

Reference solution. A 0.01 per cent w/v solution of *methyl seconeolitsine IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. 0.5 per cent v/v solution of *ortho phosphoric acid*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 315 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	50	50
15	100	0
20	100	0

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of methyl seconeolitsine.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Peppermint Oil

Pudina ka tail

Peppermint Oil is the essential oil obtained by steam distillation of the flowering herb *Mentha piperita* L.var.(Fam. Lamiaceae).

Peppermint Oil contains not less than 13.0 per cent w/w and not more than 28.0 per cent w/w of menthone, and not less than 32.0 per cent w/w and not more than 45.0 per cent w/w of menthol.

Category. Pharmaceutical aid.

Description. A clear, almost colourless to amber yellow liquid, free from sediment, suspended matter and characteristic of mint and menthol-like odour.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *Peppermint oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash Point (2.4.44). 67.0° to 71.0°.

Tests

Relative density (2.4.29). 0.890 to 0.910.

Refractive index (2.4.27). 1.4500 to 1.4595.

Optical rotation (2.4.22). – 14.0° to – 38.5°.

Assay. Determine by gas chromatography (2.4.14).

Test solution. A 2.0 per cent w/v solution of the oil under examination in ethanol (95 per cent).

Reference solution(a). A solution containing 2.0 per cent w/v solution of *menthone IPRS* in *ethanol (95 per cent)*.

Reference solution(b) A solution containing 2.0 per cent w/v solution of *menthol IPRS* in *ethanol (95 per cent)*.

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute using nitrogen as carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

Inject reference solutions (a), (b) and the test solution

Calculate the menthone, and menthol contents in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

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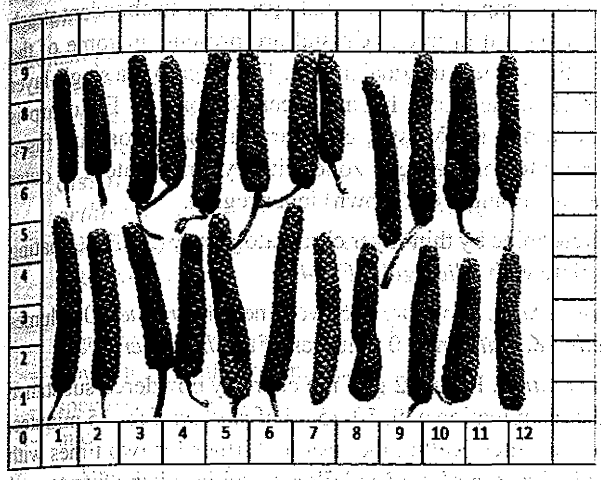
Pippali, Large

Long Pepper; Catkins (Big); *Piper retrofractum*



Pippali, Large consists of the fruiting spikes of *Piper retrofractum* Vahl.(Syn. P. *latifolium* Hunter, (Fam. Piperaceae).

Pippali, Large contains not less than 1.0 per cent w/w of piperine, calculated on the dried basis.

Category. Antiallergic, antiasthmatic, hepatoprotective, expectorant, carminative, arsa.

Description. The spikes are blacking green to green in colour, cylindrical, erect and blunt. It has pungent taste and the odour is aromatic and characteristic. The spikes are 2 to 4 cm long and 0.4-0.7 cm in diameter.

Identification

A. *Macroscopic* — The spikes are blackish green to green in colour, surface rough. The spikes bear bracts and numerous small fruits sunk in solid spike.

B. *Microscopic* — The penduncle is circular in outline, trichomes are absent, epidermis is a single layer of tangentially elongated cells. Vascular traces are in five to six bundles. Pith is star shaped. Endosperm cells are larger, cells are packed with abundant starch grains and oil droplets. Crystals are present in some of the cells, they appear unequal in size. The epicarp is a single layer of thick walled cells having greenish content. Endocarp is wavy in outline. Mostly, endocarp and seed coat are fused together to form a deep zone with hyaline content in outer layer and orange red (brown) inner region.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *benzene*, 30 volumes of *ethyl acetate* and 10 volumes of *diethyl ether*.

Test solution. Reflux 2 g of the coarsely powdered substance under examination with 50-75 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further for two times with 75 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 50 ml.

Reference solution. Reflux 0.4 g of the coarsely powdered *pippali, large IPBRS* with 50-75 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further for two times with 75 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with *vanillin sulphuric acid reagent*. Heat the plate at 100° for 5-10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 8.0 per cent.

Water-soluble extractive (2.6.3). Not less than 10.0 per cent by Method I.

Total ash (2.3.19). Not more than 8.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh 2 g of coarsely powdered substance under examination, add 50 ml of *methanol*, sonicate for 3 minutes and heat on a boiling water bath for 15 minutes, cool and dilute to 100.0 ml with *methanol* and filter. Dilute further if necessary.

Reference solution. A 0.01 per cent w/v solution of *piperine IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution prepared by dissolving 0.136 g of *potassium di-hydrogen orthophosphate* in 900 ml of *water*, adjusted to pH 2.5 with *orthophosphoric acid* and dilute to 1000 ml with *water*,

  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

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Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
18	55	45
25	20	80
30	95	5

Inject the reference solution. The relative standard deviation for the replicate injections is not more than 2.0 per cent.

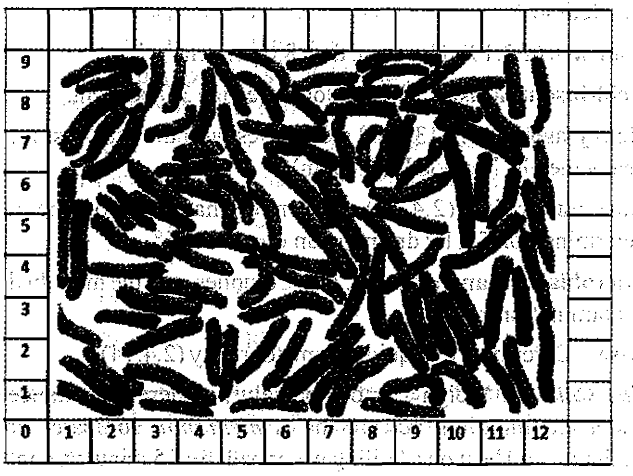
Inject the reference solution and the test solution

Calculate the content of piperine.

Storage. Store protected from heat, moisture and against attach by insects and rodents.

Pippali, Small

Small Pepper; Catkins (small); *Piper longum*



Pippali, small consists of the fruiting spike of *Piper longum* Linn. (Fam. Piperaceae).

Pippali, small contains not less than 0.4 per cent w/w of piperine, calculated on the dried basis.

Category. Antiallergic, antiasthmatic, hepatoprotective, expectorant, carminative, arsa.

Description. The spikes are greenish black to black in colour, cylindrical, erect and blunt. It has pungent taste and the odour is aromatic and characteristic. The spikes are 1.0 to 1.9 cm long and 0.2 to 0.3 cm in diameter.

Identification

A. *Macroscopic* — The spikes are greenish black to black in colour, surface rough. The spikes bear bracts and numerous small fruits sunk in solid spike.

B. *Microscopic* — The peduncle has uniseriate septate trichomes, epidermis is a single layer of tangentially elongated cells. The penduncle vascular traces are in 2-3 bundles, pith is dumbbell shaped. Cells are small, packed with abundant strach grains and oil droplets. Crystal are present in some of the cells, they appear unequal in size. The epicarp is a single layer of thick walled cells having greenish content. Endocarp is wavy in outline. Mostly, endocarp and seed coat are fused together to form a deep zone with hyaline content in outer layer and orange red (brown) inner region.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 60 volumes of *benzene*, 30 volumes of *ethyl acetate* and 10 volumes of *diethyl ether*.

Test solution. Reflux 2 g of the coarsely powdered substance under examination with 50-75 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further for two times with 75 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 50 ml.

Reference solution. Reflux 0.4 g of the coarsely powdered *pippali, small IPBRS* with 50-75 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further for two times with 75 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with *vanillin sulphuric acid reagent*. Heat the plate at 100° for 5-10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 8.0 per cent.

Water-soluble extractive (2.6.3). Not less than 10.0 per cent by Method I.

Total ash (2.3.19). Not more than 8.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh 2 g of coarsely powdered substance under examination, add 50 ml of *methanol*, sonicate for 3 minutes and heat on a boiling water bath for 15 minutes, cool

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and dilute to 100.0 ml with *methanol* and filter. Dilute further if necessary.

Reference solution. A 0.01 per cent w/v solution of *piperine IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a buffer solution prepared by dissolving 0.136 g of *potassium di-hydrogen orthophosphate* in 900 ml of *water*, adjusted to pH 2.5 with *orthophosphoric acid* and dilute to 1000 ml with *water*,

B. *acetonitrile*,

- a gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 270 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
18	55	45
25	20	80
30	95	5

Inject the reference solution. The relative standard deviation for the replicate injections is not more than 2.0 per cent.

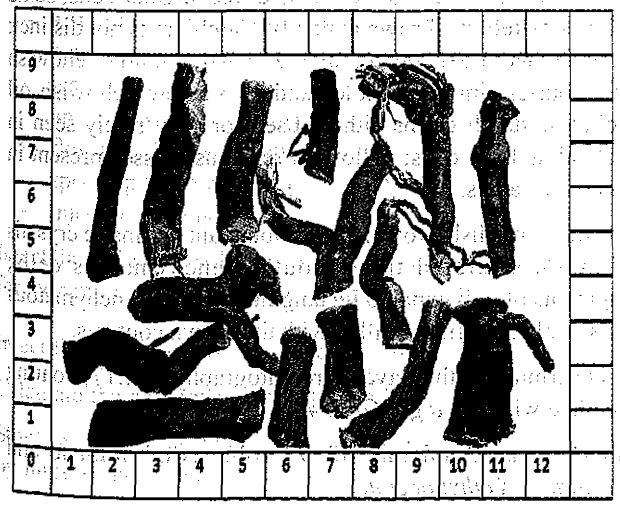
Inject the reference solution and the test solution. The relative retention time of piperine is 1.

Calculate the content of piperine.

Storage. Store protected from heat, moisture and against attach by insects and rodents.

Punarnava

Hogweed; *Boerhaavia diffusa*



Punarnava consists of the dried root of *Boerhaavia diffusa* Linn. (syn. *B. reperis* Linn) (Fam. Nyctaginaceae).

Punarnava contains not less than 0.005 per cent w/w of boeravinone B, calculated on the dried basis.

Category. Hepatoprotective, diuretic, antinephritic, pandu.

Description. A greyish-brown in colour, short and fibrous fracture and no distinct odour with bitter taste.

Identification

A. *Macroscopic* — Stout, tapering, somewhat knotty and twisted roots upto 30 cm or more long and 0.5-1.5 cm thick often crowned with stem bases, greyish-brown, surface is rough due to minute, irregular, longitudinal striations and root scars.

B. *Microscopic* — Transverse section of root shows outermost layer of cork which consists of thin walled tangentially elongated cells with brownish walls followed by thin walled 1-2 layered cork cambium. Cortex many layered and composed of thin walled cells. Secondary cortex 2-4 layered and parenchymtous. Concentric bands of xylem tissues alternating with parenchymatous tissues. Vessels are radial with reticulate thickening. Simple and compound starch grains with centric hilum and raphide crystals of calcium oxalate in cortex region. Fibres are aseptate and spindle shaped with pointed ends.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 35 volumes of *chloroform*, 6 volumes of *methanol and* 1 volume of *glacial acetic acid*.

Test solution. Reflux 2 g of the coarsely powdered substance under examination with 25 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 × 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 5 ml.

Reference solution. Reflux 2 g of the *punarnava IPBRS* with 25 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 × 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with *anisaldehyde sulphuric acid reagent*. Heat the plate at 110° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

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Ethanol-soluble extractive (2.6.2). Not less than 0.5 per cent.

Water-soluble extractive (2.6.3). Not less than 9.0 per cent by Method I.

Total ash (2.3.19). Not more than 10.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 2 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water-bath for 15 minutes, cool and filter. Reflux the residue further with *methanol* till the extract turns colourless, cool and filter. Combine all the filtrates and concentrate to a volume slightly less than 25 ml. Dilute to 25.0 ml with *methanol*.

Reference solution. A 0.002 per cent w/v solution of *boeravinone B IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. *water*,
  B. *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 272 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
25	10	90
35	90	10

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

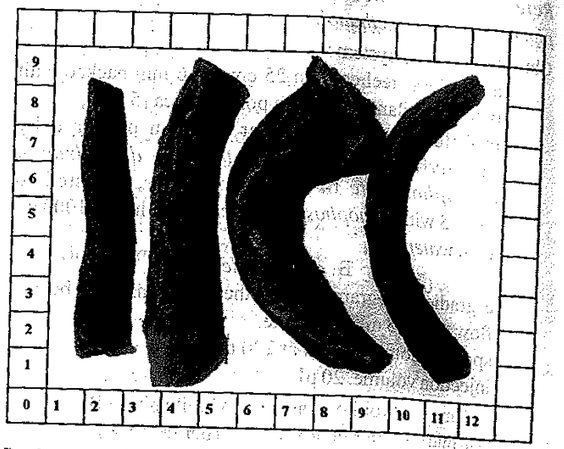
Inject the reference solution and the test solution.

Calculate the content of boeravinone B.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Puskara

Pushkarmula, Orris root; *Inula racemosa*



Puskara consists of dried roots of *Inula racemosa* Hook. F. (Fam. Asteraceae).

Puskara contains not less than 0.05 per cent of alantolactone, calculated on the dried basis.

Category. Anti-inflammatory, antibacterial and aphrodisiac.

Description. Brownish-grey externally, cylindrical root, camphoraceous and taste is bitter.

Identification

A. *Macroscopic* — Root cylindrical, conical to fusiform in shape, unbranched, 5-12 cm long and 0.5 to 2.0 cm in diameter; longitudinally irregularly wrinkled and furrowed surface. Lateral rootlets very thin, wiry and brittle. Fracture, short and smooth.

B. *Microscopic* — Mature roots show a wavy outline due to development of rhytidoma, cork composed of 8 to 12 layers of thick walled, tangentially elongated, rectangular cells, some filled with reddish-brown contents. Cambium is not distinct; parenchyma in general contains granular, slightly yellowish or colourless inulin granules and also a few yellowish oil globules; starch grains either absent or very rarely seen in cortical and ray cells; yellowish resinous masses present in secretary canals.

Powder is reddish-brown; shows abundant prismatic crystals of inulin scattered throughout parenchymatous cells; fragments of polygonal to rectangular cells. Parenchymatous cells of phelloderm are filled with the brown contents.

C. Determine by thin layer chromatography (2.4.17) coating the plate with *silica gel GF254*.

Mobile phase A mixture of 93 volumes of *toluene* and 7 volumes of *ethyl acetate*.

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Test solution. Reflux 2.0 g of powdered substance under examination with 100 ml of *n-hexane* on water-bath for 30 minutes, cool and filter. Concentrate under vacuum to 5 ml.

Reference solution. Reflux 2.0 g of *puskara IPBRS* with 100 ml of *n-hexane* on water-bath for 30 minutes, cool and filter. Concentrate under vacuum to 5 ml.

Apply to the plate 10 µl of each solution as band length 8 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in the ultraviolet light at 254 nm and 366 nm. Spray the plate with *anisaldehyde sulphuric acid reagent* and heat the plate at 105° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 25.0 per cent.

Water-soluble extractive (2.6.3). Not less than 15.0 per cent.

Total ash (2.3.19). Not more than 5.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.6 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 9.0 per cent, determined on 5.0 g by drying in an oven at 105°.

Assay. Determine by liquid chromatography (2.4.14)

Test solution. Reflux about 2.0 g of the coarsely powdered substance under examination with 200 ml of *methanol* by soxhlet apparatus for 4 hours till extract turn colourless. Cool and filter. Concentrate under vacuum to 10 ml.

Reference solution. A 0.01 per cent w/v solution of *alanatolactone IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 30 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (10 µm),
- mobile phase: a mixture of 70 volumes of *acetonitrile* and 30 volumes of *water*,
- flow rate: 1ml per minute,
- spectrophotometer set as 210 nm,
- injection volume: 20 µl.

Inject the reference solution. The relative standard deviation for the replicate injection is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of alantolactone.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Rosemary Oil

Rosemary Oil is the essential oil obtained by steam distillation of the twigs and blossoming tips of *Rosmarinus officinalis* Linn. (Fam. Lamiaceae).

Rosemary Oil (Indian variety) contains not less than 2.0 per cent and not more than 10.0 per cent of borneol, and not less than 15.0 per cent and not more than 40.0 per cent of α-pinene.

Description. A clear, pale yellow to amber yellow liquid, free from sediment and suspended matter, spicy, characteristic and anise-like odour.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *rosemary oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 118.0° to 122.0°.

Tests

Relative density (2.4.29). 0.905 to 0.962.

Refractive index (2.4.27). 1.458 to 1.548.

Optical rotation (2.4.22). –6.0° to +7.5°.

Acid value (2.3.23). Not more than 1.0 per cent.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *borneol IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *α-pinene IPRS* in *ethanol (95 per cent)*.

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,

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– detection by flame ionization detector,
– flow rate 1 ml per minute using nitrogen as carrier gas,
– injection volume: 1.0 µl,
– split ratio: 1 : 25.

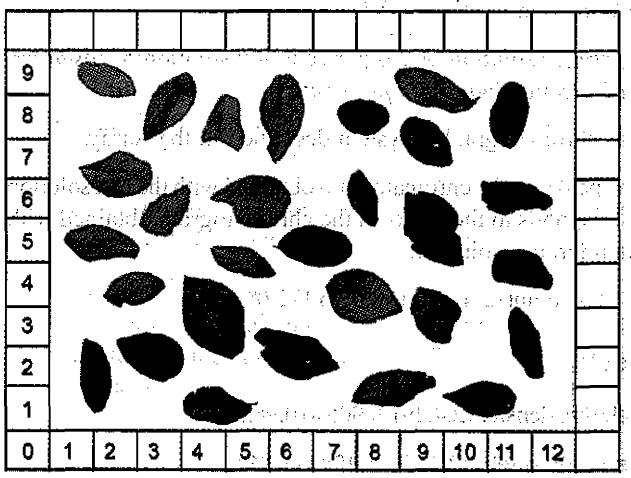
Inject reference solutions (a), (b) and the test solution.

Calculate the contents of borneol, and α-pinene in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Sahajana Leaf

Saijna leaf; *Moringa oleifera*



Sahajana consist of dried leaves of *Moringa oleifera* Lamk. (Fam. Moringaceae)

Category. Antidiarrhoeal, antibacterial, antiinflammatory, anti microbial and anticancer.

Description. Leaves are small, dark green in colour with smooth surface.

Identification

A. *Macroscopic* — The leaves are alternate, usually thrice pinnate, and 25-50 cm long. There are 2 to 9 leaflets on the ultimate pinnules. Leaflets are thin, ovate to elliptic and 1to 2 cm long.

B. *Microscopic* — Leaflet shows dorsiventral structure; epidermis and unicellular hair present on both the surfaces; palisade single layered; spongy parenchyma 2-3 layers; central region occupied by crescent shaped, collateral vascular bundle surrounded by 2-4 layers of collenchymatous cells; crystals of calcium oxalate present in mesophyll and collenchymatous cells.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 65 volumes of *chloroform*, 30 volumes of *methanol* and 5 volumes of *water*.

Test solution. Reflux about 2.5 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water bath for 30 minutes, cool and filter. Reflux the residue further for 2 more times with 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 25.0 ml.

Reference solution. Reflux about 2.5 g of *sahajana IPBRS* with 50 ml of *methanol* on a water bath for 30 minutes, cool and filter. Reflux the residue further for 2 more times with 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 25.0 ml.

Apply to plate 15 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm, 366 nm, spray separately with 5 per cent *sulphuric acid solution* and 0.5 per cent *ninhydrin solution* in *ethanol*. Heat the plate at 105° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 2.0 per cent.

Water-soluble extractive (2.6.3). Not less than 10.0 per cent.

Total ash (2.3.19). Not more than 16.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 4.0 per cent.

Heavy metals (2.3.13). 1.0 g comply with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 8.0 per cent determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Vitamin A (2.3.41). Not less than 0.20 per cent, Method B.

Protein content (2.3.30). Not less than 14.0 per cent, Method B.

Storage. Store protected from light and moisture and against attack by insects and rodents.

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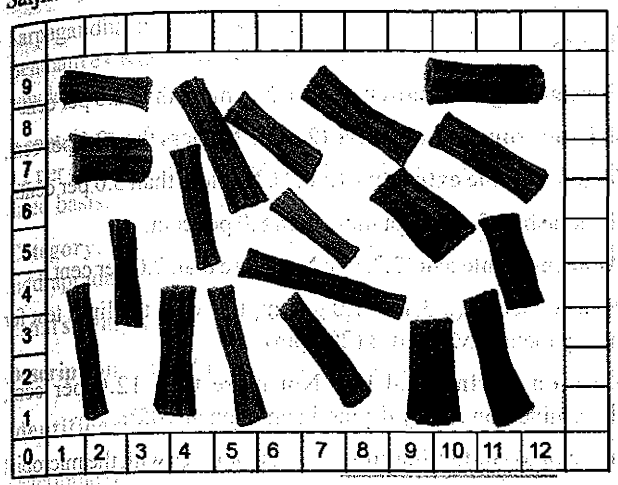
Sahajana Stick

Saijna drumstick; *Moringa oleifera*



Sahajana consist of dried fruit of *Moringa oleifera* Lamk. (Fam. Moringaceae)

Category. Antidiarrhoeal, antibacterial , antiinflammatory, antimicrobial and anticancer.

Description. Fruit is long green stick shaped with diameter of 1.0 to 1.5 cm.

Identification

A. *Macroscopic* — The fruit is a hanging, three-sided brown capsule of 20–45 cm size which holds dark brown, globular 26 seeds on an average with diameter of approx. 1 cm.

B. *Microscopic* — Fruit is divided into epicarp, mesocarp and endocarp. Epicarp consists of thin rectangular cells with a thick cuticle whereas mesocarp is comprised of many thin layers of compactly arranged parenchymatous cells with phloem and xylem fibers, vascular tissue. A large polygonal parenchymatous cell makes the endocarp part of fruit.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 40 volumes of *n-butanol*, 10 volumes of *acetic acid* and 20 volumes of *water*.

Test solution. Reflux about 2.5 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water-bath for 30 minutes, cool and filter. Reflux the residue further for 2 more times with 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 25.0 ml.

Reference solution. Reflux about 2.5 g of *sahajana IPBRS* with 50 ml of *methanol* on a water bath for 30 minutes, cool and filter. Reflux the residue further for 2 more times with 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 25.0 ml.

Apply to plate 15 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray separately with 5 per cent *sulphuric acid solution* and 0.5 per cent *ninhydrin solution* in *ethanol*. Heat the plate at 105° for 10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 1.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 1.5 per cent.

Water-soluble extractive (2.6.3). Not less than 20.0 per cent by Method I.

Total ash (2.3.19). Not more than 8.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.25 per cent.

Heavy metals (2.3.13). 1.0 g comply with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

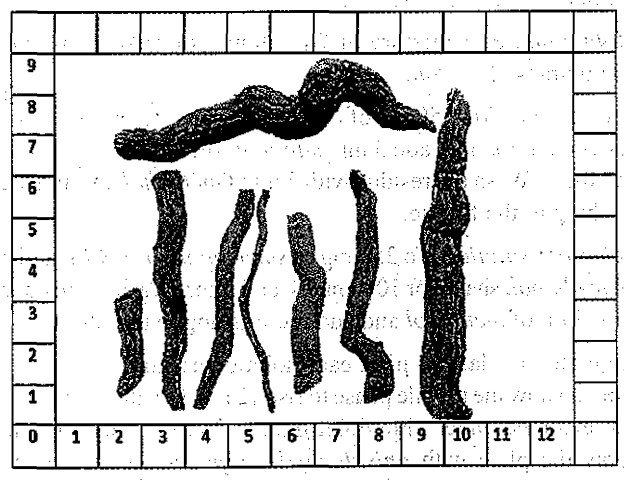
Vitamin A (2.3.41). Not less than 0.003 per cent, Method B.

Protein content (2.3.30). Not less than 9.0 per cent, Method B.

Storage. Store protected from light and moisture and against attack by insects and rodents.

Sarpagandha

Rauwolfia serpentina



Sarpagandha consists of the dried roots of *Rauwolfia serpentina Bentham* ex Kurz (Fam. Apocynaceae).

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Sarpagandha contains not less than 0.15 per cent of reserpine and ajmalicine, calculated on the dried basis.

Category. Antihypertensive, antipsychotic, neurosedative, niddrapradaa.

Description. Taste bitter, odour indistinct.

Identification

A. *Macroscopic* — Roots are sub cylindrical to tapering, tortuous or curved, rarely branched. Occurs as segments usually from 5 to 15 cm in length and 3 to 20 mm in diameter. Externally grayish yellow to brown, wood pale yellow. Roots tough with longitudinal marking and slightly wrinkled surface. When scraped, bark separates readily from the wood. Fracture is short and irregular.

B. *Microscopic* — In transverse section, cork cells in 2 to 8 alternating bands of radically narrow and broader cells, thin, lignified up to 75 µm in tangential width, broader cells up to about 90 µm in radial length; phelloderm, tangentially elongated to isodimetric parenchyma cells containing starch and short latex cells with brown resinous matter; secondary cortex consists of parenchyma cells, heavily packed with starch grains secondary phloem contains phloem parenchyma and sieve elements, parenchyma contains starch and angular crystals of calcium oxalate 3 to 20 µm in length. Xylem is about 4/5 of the diameter of the root, wood is transversed by medullay rays 1 to 5 cells in width. Xylem consists of vessels, tracheids, wood parenchyma and wood fibers. Xylem vessels are elongated up to 350 µm in length and 50 µm in width and contains simple or bordered pits; tracheids lignified, pitted; wood parenchyma with moderately thick, lignified and pitted walls containing starch; wood fibres highly thickened with pointed ends, stone cells absent.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *chloroform* and 30 volumes of *acetone*.

Test solution. To 250 mg of the coarsely powdered substance under examination, add 5 ml *methanol*, shake for 10 minutes, and filter. Wash the residue with 5 ml of *methanol* and add the washing to the filtrate.

Reference solution. To 250 mg of *sarpagandha IPBRS*, add 5 ml *methanol*, shake for 10 minutes, and filter. Wash the residue with 5 ml of *methanol* and add the washing to the filtrate.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 12 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with *anisaldehyde sulphuric acid reagent*. Heat the plate at 100° for 5 minutes and examine the plate in day light. The chromatogram obtained with test solution shows two pinkish-violet bands corresponding to the bands in the chromatogram obtained with reference solution. A dark brown band may also appear at the line of application in the chromatogram obtained with test solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 2.0 per cent.

Water-soluble extractive (2.6.3). Not less than 5.0 per cent.

Total ash (2.3.19). Not more than 8.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 2 g of the coarsely powdered substance under examination with 50 ml of *ethanol (95 per cent)*, on a water bath for 15 minutes, cool and filter. Reflux the residue further with *ethanol (95 per cent)*, till the last extract turns colorless, cool and filter. Combine all the filterates and concentrate to 100.0 ml.

Reference solution (a). A solution containing a 0.002 per cent w/v *reserpine IPRS* and 0.002 per cent w/v *ajmalicine IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 0.002 per cent w/v solution of *reserpine IPRS* in *ethanol (95 per cent)* for peak identification.

Chromatographic system :

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of *acetonitrile* and 65 volumes of a buffer solution prepared by dissolving 6.80 g of *potassium dihydrogen phosphate* in 1000 ml *water*, adjust the pH to 3.0 with *dilute orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 268 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent. Inject reference solution (b) for peak identification.

Inject reference solution (a) and the test solution.

Calculate the content of reserpine and ajmalicine.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

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Sarpagandha Powder

Rauwolfia serpentina Powder

Sarpagandha powder is obtained by *Rauwolfia serpentina* Bentham ex Kurz roots (Fam. Apocynaceae) reduced to a fine powder.

It contains not less than 0.15 per cent w/w and not more than 0.2 per cent w/w of reserpine-ajmalicine alkaloid calculated on dried basis.

Category. Antihypertensive, antipsychotic, neurosedative, niddrapradaa.

Usual strength. 0.175 per cent w/w.

Description. Taste bitter, odour indistinct.

Identification

Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *chloroform* and 30 volumes of *acetone*.

Test solution. To 0.25 g of the substance under examination, add 5 ml *methanol*, shake for 10 minutes, and filter. Wash the residue with 5 ml of *methanol* and add the washing to the filtrate.

Reference solution. To 0.25 g of *sarpagandha IPBRS*, add 5 ml *methanol*, shake for 10 minutes, and filter. Wash the residue with 5 ml of *methanol* and add the washing to the filtrate.

Apply to the plate 20 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 12 cm. Dry the plate in air and examine under ultraviolet light at 366 nm. Spray the plate with *anisaldehyde sulphuric acid reagent*. Heat the plate at 100° for 5 minutes and examine the plate at 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Total ash (2.3.19). Not more than 5.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 gm complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 5.0 per cent determined on 1 gm by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 2.0 g of the substance under examination with 50 ml of *ethanol (95 per cent)*, on a water bath for 15 minutes, cool and filter. Reflux the residue further with *ethanol (95 per cent)*, till the last extract turns colourless, cool and filter. Combine all the filterates and concentrate to 100.0 ml.

Reference solution (a). A solution containing 0.002 per cent w/v *reserpine IPRS* and 0.002 per cent w/v *ajmalicine IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 0.002 per cent w/v solution of *reserpine IPRS* in *ethanol (95 per cent)*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 35 volumes of *acetonitrile* and 65 volumes of a buffer solution prepared by dissolving 6.80 g of *potassium dihydrogen phosphate* in 1000 ml *water*, adjusted to pH 3.0 with *dilute orthophosphoric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 268 nm,
- injection volume: 10 µl.

Inject reference solution (a). The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent. Inject reference solution (b) for peak identification.

Inject reference solution (a) and the test solution.

Calculate the content of reserpine and ajmalicine.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Sarpagandha Tablets

Rauwolfia serpentina Tablets

Sarpagandha Tablets contain not less than 85.0 per cent w/w and not more than 115.0 per cent w/w of stated amount of the alkaloids contents mainly of reserpine together with ajmalicine.

Category. Antihypertensive, antipsychotic, neurosedative, niddrapradaa.

Usual strengths. 0.1 mg; 0.25 mg.

Identification

In the assay the principal peak in chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution.

Tests

Other tests. Comply with the tests stated under tablets.

Loss on drying (2.4.19). Not more than 8.0 per cent, determined in 1.0 gm by drying in an oven at 105°.

Assay. Determine by liquid chromatography (2.4.14).

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Test solution. Weigh and powder 20 tablets. Weigh accurately a quantity of the powder containing about 1 g of *Rauwolfia serpentina* into a 200 ml flat bottom flask. Add 50 ml *of ethanol (95 per cent)*, reflux on a water bath for 30 minutes, cool and filter. Reflux the residue further with *ethanol (95 per cent)* till the last extract turns colorless, cool and filter. Combine all the filtrates and concentrate to 100.0 ml.

Reference solution. A 0.001 per cent w/v *Reserpine IPRS* and 0.001 per cent w/v *Ajmalicin IPRS* in *ethanol* (95 per cent).

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecyl silane chemically bonded to porous silica (5µ),
- mobile phase: a mixture of 35 volumes of *acetonitrile* and 65 volumes of a buffer (pH 3.0), prepared by dissolving 6.80 g of *potassium dihydrogen* phosphate in 1000 ml water, adjusted to pH 3.0 with *dilute* orthophosphoric *acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 268 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the refernce solution and the test solution.

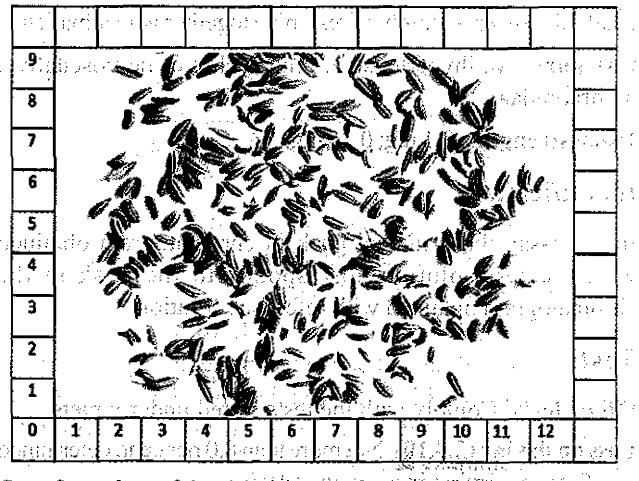
Calculate the content of ajmalicin and reserpine.

Storage. Store protected from light at a temperature not exceeding 30°.

Labelling. The quantity of active ingredient is stated in terms of reserpine-ajmalicine content in labelled amount.

Saunf

Fennel; *Foeniculum vulgare*



Saunf consists of the dried fruits of *Foeniculum vulgare* Mill. (Fam. Apiaceae).

Saunf contains not less than 0.60 per cent of anethole, calculated on the dried basis.

Category. Carminative, spasmolytic.

Description. The fruits are oval-oblong, greenish brown to yellowish brown in colour. They have an aromatic characteristic odour and the taste is sweet aromatic.

Identification

A. *Macroscopic* — Fruit cylindrical to oval with pedicel attached, consists of mericarps. Each mericap is 10 mm long and 4 mm broad, five sided with a wider commissural surface, tapered at apex and base, crowned with a conical stylepod, glabrous, greenish or yellowish brown with five ridges.

B. *Microscopic* — Transverse section of fruit shows pericarp with outer epidermis of quadrangular to polygonal cells with smooth cuticle. Vittae 4 dorsal and 2 commissural extending with length of each mericarp, having brown cells and volatile oil in cavity. Mesocarp with reticulate lignified parenchyma. Endocarp cells thin walled arranged parallel to one another in groups of 5-7. Endosperm consists of thick walled, cellulosic parenchyma containing fixed oil cells and rosette crystals of calcium oxalate.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 93 volumes of *toluene* and 7 volumes of *ethyl acetate.*

Test solution. To 1 g of the coarsely powdered substance under examination, add 25 ml of *dichloromethane,* reflux for 15 minutes, cool and filter. Reflux the residue further for two times with 25 ml of *dichloromethane*, cool and filter. Combine all the filtrates and evaporate under vaccum to 25 ml.

Reference solution. To 2.0 g of the *saunf IPBRS*, add 40 ml of *methanol,* reflux for 15 minutes, cool and filter. Reflux the residue further with 25 ml of *methanol* twice, cool and filter. Combine all the filtrates and concentrate under vacuum to 25 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with a *anisaldehyde sulphuric acid reagent.* Heat at 110° for 10 minutes and examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 5.0 per cent.

Water-soluble extractive (2.6.3). Not less than 14.0 per cent by Method I.

Total ash (2.3.19). Not more than 10.0 per cent.

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Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 10 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by gas chromatography (2.4.13).

Test solution. Weigh 2.0 g of the coarsely powdered substance under examination, add 50 ml of *methanol,* reflux on a water-bath for 15 minutes, cool and filter. Reflux the residue further with *methanol* till the extract turns colourless, cool and filter. Combine all the filtrates and concentrate to a volume slightly less than 100 ml. Dilute to 100.0 ml with *methanol.*

Reference solution. A 0.01 per cent w/v solution of *anethole IPRS* in *methanol.*

Chromatographic system

- a capillary column 30 m x 0.25 m, packed with DB1,
- temperature:
  - oven 90° to 260° at a rate of 10° per minute (for 5 minutes),
  - injector 240° and detector 280°,
- flow rate: 0.8 ml per minute,
- split flow: 20 ml per minute.

Inject 1 µl of the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 10.0 per cent.

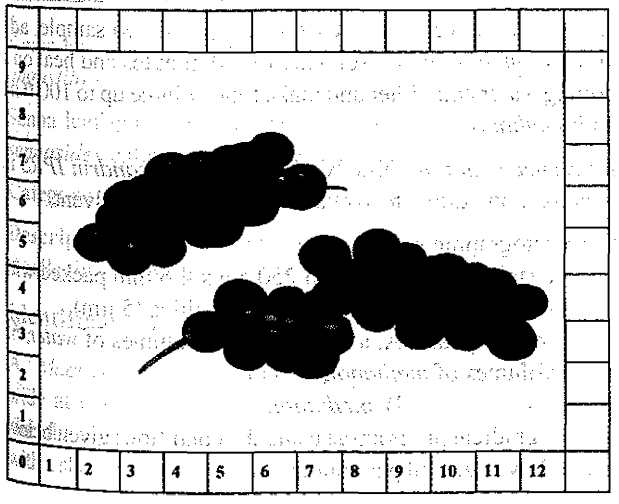
Inject 1 µl of the reference solution and the test solution.

Calculate the content of anethole.

Storage. Store protected from light in well-filled containers, at a temperature not exceeding 30°.

Schisandra Fruit

Chinese Magnolia Vine; five-flavor Berry; *Schisandra chinensis*



It consists of whole, dried or steamed and dried, ripe fruit of *Schisandra chinensis* (Fam. Schisandraceae)

Schisandra fruit contains not less than 0.4 per cent w/w of schisandrin calculated on the dried basis.

Category. Energizer, hepatoprotective

Description. Fruits are irregularly spheroid and have a diameter of 5 to 8 mm. The fruits surface is oily and wrinkled and color is red to black brown depending on its degree of ripeness.

Identification

A. *Macroscopic* — The berry is more or less spherical, up to 8 mm in diameter; red, reddish- brown or blackish outer surface, sometimes covered in a whitish frost, strongly shrivelled pericarp, presence of 1-2 reniform, yellowish-brown, lustrous seeds, with thin seed-coat.

B. *Microscopic* — Examine under a microscope using chloral hydrate solution. The powder shows the following diagnostic characters: reddish-brown fragments of pericarp, consisting of 1 layer of thin-walled epicarp cells, accompanied by sparse oil cells and several layers of ovoid, more-or-less flattened mesocarp cells, fragments of the outer testa of the seed consisting of thick-walled, finely channelled sclereids, polygonal in surface view (15-50 µm in diameter) and in palisade arrangement in side view; fragments of the inner testa with sclereids, isolated or in small groups, about 80 µm in diameter, with slightly thickened and markedly channelled walls, fragments of endosperm consisting of polyhedral cells containing oil droplets and aleurone grains.

C. Determine by thin-layer chromatography (2.4.17) coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 2 volumes of *acetic acid,* 22 volumes of *ethyl acetate* and 46 volumes of *toluene.*

Test solution. Reflux about 2 g of coarsely powdered substances under examination with 20 ml of *methanol* for 10 minutes, cool and filter. Reflux the residue further with 2 x 20 ml of *methanol,* cool and filter, combine all the filtrates and concentrate under vacuum to 10 ml.

Reference solution. Reflux about 0.5 g of *schisandra IPBRS* with 10 ml of *methanol* for 10 minutes, cool and filter. Reflux the residue further with 2 x 10 ml of *methanol,* cool and filter, combine all the filtrates and concentrate under vacuum to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 10 cm. Dry the plate at 100-105°. Spray the warm plate with a 10 per cent w/v solution of *sulphuric acid* in *methanol.* Heat at 120° for 10 minutes examine in ultraviolet light at 254 nm and 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

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Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Total ash (2.3.19). Not more than 6.0 per cent.

Heavy metal (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 1 g powder of herbal drug by drying in an oven at 105° for 3 hours.

Microbial Contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh about 1 g of the powdered sample, add 90 ml of *methanol,* sonicate for 30 minutes and heat on a boiling water bath, filter and make final volume up to 100.0 ml with *methanol*.

Reference solution. Dissolve 5.0 mg of *schisandrin IPRS* in *methanol* and dilute to 100.0 ml with the same solvent.

Chromatographic system

- a stainless steel column 250 cm x 4.6 mm packed with octadecylsilane bound to porous silica (5 µm),
- mobile phase: A. a mixture of 35 volumes of *water* and 65 volumes of *methanol,*
  B. *methanol,*
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 250 nm,
- injection volume: 10 µl.

Time (in min.)	mobile phase A (per cent v/v)	mobile phase B (per cent v/v)
0	100	0
10	100	0
16	58	42
26	58	42

Inject the reference solution. The test is not valid unless the column efficiency determined from the schisandrin peak is not less than 5000 theoretical plates.

Inject the reference solution and the test solution.

Calculate the content of schisandrin.

Storage. Store protected from light, heat, moisture and against attack of insects and rodents.

Schisandra Dry Extract

Schisandra chinensis

Schisandra Dry Extract is obtained by extracting *Schisandra chinensis* (Fam. Schisandraceae) fruit with *methanol* or any other suitable solvent.

Schisandra Dry Extract contains not less than 2.0 per cent w/w of schisandrin, calculated on the dried basis.

Category. Energizer, Hepatoprotective

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 2 volumes of *acetic acid*, 22 volumes of *ethyl acetate* and 46 volumes of *toluene*.

Test solution. Dissolve about 1 g of the dry extract under examination with 10 ml of *methanol* then sonicate for 10 minutes, and filter.

Reference solution. Reflux about 0.5 g of *schisandra IPBRS* with 10 ml of *methanol* for 10 minutes, cool and filter. Reflux the residue further with 2 x 10 ml of *methanol*, cool and filter, combine all the filtrates and concentrate under vacuum to 5 ml.

Apply to the plate 5 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 10 cm. Dry the plate at 100 -105°. Spray the warm plate with a 10 per cent w/v solution of *sulphuric acid* in *methanol*. Heat at 120° for 7 minutes examine in ultraviolet light at 254 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 1 g powder of herbal drug by drying in an oven at 105° for 2 hours.

Heavy metal (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Microbial Contamination (2.2.9.). Complies with the microbial contamination test.

Total ash (2.3.19). Not more than 6.0 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh about 1 g of the powdered sample, add 90 ml of *methanol,* utrasonicate for 30 minutes and heat on a boiling water bath, filter and make final volume up to 100.0 ml with *methanol*.

Reference solution. Dissolve 5.0 mg of *schisandrin IPRS* in *methanol* and dilute to 100.0 ml with the same solvent.

Chromatographic system

- a stainless steel column 250 cm x 4.6 mm packed with octadecylsilane bound to porous silica (5 µm),
- mobile phase. A. a mixture of 35 volumes of *water*, 65 volumes of *methanol,*
  B. *methanol,*
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,

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– spectrophotometer set at 250 nm,
– injection volume: 10 µl.

Time (in min.)	mobile phase A (per cent v/v)	mobile phase B (per cent v/v)
0	100	0
10	100	0
16	58	42
26	58	42

Inject the reference solutions. The test is not valid unless the column efficiency determined from the schisandrin peak is not less than 5000 theoretical plates.

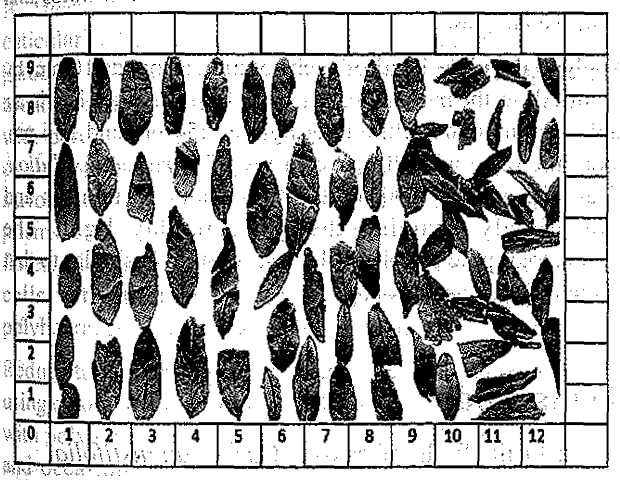
Inject the reference solution and the test solution.

Calculate the content of schisandrin in extract.

Storage. Store protected from light, heat, moisture and against attack of insects and rodents.

Senna Leaf

Cassia leaf; *Cassia angustifolia*



Senna leaf consists of the dried compound leaves of *Cassia angustifolia* or *Cassia senna* Vahal. (Fam. Leguminosae).

Senna leaf contains not less than 1.0 per cent w/w of total *sennosides* calculated as *sennoside B*, on the dried basis.

Category. Purgative, anthelmintic, febrifuge, vibandha.

Description. Pale yellowish green coloured leaflets with mucilaginous and faint odour.

Identification

A. *Macroscopic* — Leaflets, 2.5 to 8 cm long and 5-15 mm wide at centre, pale yellowish green, elongated lanceolate, slightly asymmetric at base; margins entire, flat, apex acute with a sharp spine; both surface smooth with sparce trichomes; odour, faint but distinctive; taste, mucilaginous and disagreeable but not distinctly bitter.

B. *Microscopic* — Transverse section shows outer single layered mucilaginous epidermal cells. Unicellular hairs presents. Stomata paracytic, numerous on both surface. Mesophyll consists of upper and lower palisade layers with spongy layer in between , primatic crystals of calcium oxalate present on larger veins.

C. In the Assay, the chromatogram obtained with test solution corresponds to the chromatogram obtained with reference solution.

D. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 40 volumes of *n- propyl alcohol*, 40 volumes of *ethyl acetate*, 29 volumes of *water* and 1 volume of *glacial acetic acid*.

Test solution. Take about 1 g of the dried leaves powder substance under examination. Add 25 ml of *methanol*, reflux for 10 minutes, cool and filter. Reflux the residue with another 20 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate to 10 ml.

Reference solution. Boil 0.5 g of dried *senna IPBRS* powder with 25 ml of *methanol* under reflux for 10 minutes, cool and filter. Boil under reflux the residue with another 20 ml of *methanol*, cool and filter. Combine all the filtrates and evaporate to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with 20 per cent v/v of *nitric acid solution*. Heat the plate at 100° to 105° for about 10 minutes and immediately examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 1.0 per cent.

Total ash (2.3.19). Not more than 14.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.5 per cent.

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh accurately 1.0 g of the coarsely powdered substance in a round bottom flask, add about 10 ml of 1.0 per cent v/v *glacial acetic acid* and 25 ml of *methanol* and reflux on a water bath for about 30 minutes. Cool to room temperature; make up the volume up to 50.0 ml with *methanol* and filter.

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Reference solution. Dissolve an accurately weighed quantity of *calcium sennosides IPRS* containing about 10 mg of *sennosides* in 50 ml of the mixture of 10 ml of 1.0 per cent v/v *glacial acetic acid* and 40 ml of *methanol.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 82 volumes of 1 per cent v/v *acetic acid* in *water* and 18 volumes of *acetonitrile,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 350 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the total sennosides from the peak area of sennoside A and B using the content of total sennosides in calcium sennoside.

1 mg of calcium sennoside contain 0.9579 mg sennoside.

Storage. Store protected from light and moisture.

Powdered Senna Leaf

Cassia leaf; *Cassia angustifolia*

Powdered Senna is Senna dried compound leaves of *Cassia angustifolia* L. Vahal or *Cassia senna* L. (Fam. Leguminosae) reduced to a fine or very fine powder.

Category. Purgative, anthelmintic, febrifuge, vibandha

Description. A light brown to dark brown powder with faint and distinctive odour; taste, mucilaginous and disagreeable but not distinctly bitter.

Identification

Powder study

Examine under a microscope (2.6.7). The powder shows the following diagnostic characters: Fragments of upper and lower epidermis, composed of cells with smooth and straight anticlinal walls, polygonal in outline, embedded with numerous paracytic and few anomocytic stomata; numerous, thick walled, unicellular, warty, straight or bent trichomes scattered and/or attached to the cells of epidermis, both epidermises have cicatrices where the trichomes were attached; abundant *calcium oxalate crystals,* as prisms in cells of parenchymatous sheath surrounding the group of fibres (crystal sheath), as cluster crystals in cells of spongy mesophyll,both crystals are also found scattered; fibres in groups, thick-walled, lignified with few pits; few simple spherical starchgrains scattered or in parenchymatous cells.

Quantitative microscopy. Stomatal index (upper surface) for *Cassia angustifolia* is 17.1 to 19.0 to 20.7, (lower surface) is 17.0 to 18.3 to 19.3; for *Cassia senna* (upper surface) 11.4 to 12.4 to 13.3, (lower surface) is 10.8 to 11.8 to 12.6.

Vein-islet numbers for *C. angustifolia* range. 19.5 – 22.5; average 21; *C. senna is* 15 – 29.5; average 26

Vein termination numbers: ***C. angustifolia*** is 25.9 – 32.8; *C. senna* 32.7 – 40.2.

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel* GF_{254}.

Mobile phase. A mixture of 40 volumes of *n- propyl alcohol,* 40 volumes of *ethyl acetate,* 29 volumes of *water* and 1 volume of *glacial acetic acid.*

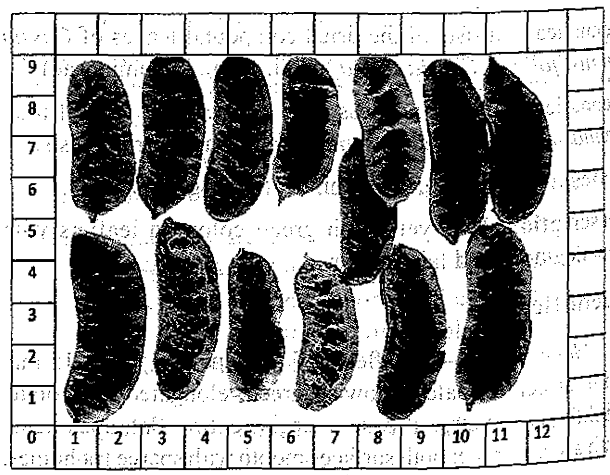
Test solution. Take about 1 g of the dried leaves powder substance under examination. Add 25 ml of *methanol,* reflux for 10 minutes, cool and filter. Reflux the residue with another 20 ml of *methanol,* cool and filter. Combine all the filtrates and concentrate to 10 ml.

Reference solution. Boil 0.5 g of dried *senna leaves IPRS* powder with 25 ml of *methanol* under reflux for 10 minutes, cool and filter. Boil under reflux the residue with another 20 ml of *methanol,* cool and filter. Combine all the filtrates and evaporate to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with 20 per cent v/v of *nitric acid solution.* Heat the plate at 100° to 105° for about 10 minutes and immediately examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Senna Pods

Senna Fruit; Pods of cassia; *Cassia angustifolia*



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Senna pods consist of the dried compound Pods of *Cassia angustifolia* or *Cassia senna* Vahal. (Fam. Leguminosae).

Senna Pods contains not less than 1.0 per cent w/w of total *sennosides* calculated as *sennoside B,* on the dried basis.

Category. Purgative, anthelmintic, febrifuge, vibandha.

Description. Pale yellowish green coloured pods with slight odour. Leaflets with mucilaginous and faint odour.

Identification

A. *Macroscopic* — Flattened reniform pods, brownish yellow at the edges, dark brown in the central area about 40 to 50 mm long and about at least 20 mm wide. At one end is a stylar point and at the other a short stalk. The pods contains 5 to 7 flattened and obovate seeds, green to pale brown, with a continuous network of prominent ridges on the tests and in complate wavy transverse ridges on the testa. Leaflets, 2.5 to 8 cm long and 5-15 mm wide at centre, pale yellowish green, elongated lanceolate, slightly asymmetric at base; margins entire, flat, apex acute with a sharp spine; both surface smooth with sparce trichomes; odour, faint but distinctive; taste, mucilaginous and disagreeable but not distinctly bitter.

B. *Microscopic* — The pods present an epicarp with strongly cuticularised isodiametric cells, occasional anomocytic or paracytic stomata, and very few conical, unicellular and warty trichomes. Hypodermis with collenchymatous cells, mesocarp with parenchymatous tissue, a layer of prism s of calcium oxalate and containing vascular bundles incompletely surrounded by fibres with a crystals sheth of calcium oxalate prisms, endocarp consisting of thick-walled and inter lacing fibres. The seeds present a sub epidermal layers of palisade cells with thick outer walls, endosperm composed of polyhedral cells with mucilaginous wall.

Reduce to moderately fine powder examine microscopically using chloral hydrate solution. The powder consists of epicarp with polygonal cells and a small number of warty trichomes and occasional anomocytic stomata, fibres in two crossed layers, accomparied by a crystal sheath of calcium oxalate prism, characteristic palisade cells in the seeds and stratified cells in the endosperm, cluster and prisms of calcium oxalate

C. In the Assay, the chromatogram obtained with test solution corresponds to the chromatogram obtained with reference solution.

D. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 40 volumes of *n- propyl alcohol,* 40 volumes of *ethyl acetate,* 29 volumes of *water* and 1 volume of *glacial acetic acid.*

Test solution. Take 1 g of the dried pods powder substance under examination. Add 25 ml of *methanol,* reflux for 10 minutes, cool and filter. Reflux the residue with another 20 ml of *methanol,* cool and filter. Combine all the filtrates and concentrate to 10 ml.

Reference solution. Boil 0.5 g of dried *senna IPBRS* powder with 25 ml of *methanol* under reflux for 10 minutes, cool and filter. Boil under reflux the residue with another 20 ml of *methanol,* cool and filter. Combine all the filtrates and evaporate to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with 20 per cent v/v of *nitric acid solution.* Heat the plate at 100° to 105° for about 10 minutes and immediately examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 1.0 per cent.

Total ash (2.3.19). Not more than 14.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.5 per cent.

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh accurately 1.0 g of the coarsely powdered substance in a round bottom flask, add about 10 ml of 1.0 per cent v/v *glacial acetic acid* and 25 ml of *methanol* and reflux on a water bath for about 30 minutes. Cool to room temperature; make up the volume up to 50 ml with *methanol* and filter.

Reference solution. Dissolve an accurately weighed quantity of *calcium sennosides IPRS* containing about 10 mg of *sennosides* in 50 ml of the mixture of 10 ml of 1.0 per cent v/v *glacial acetic acid* and 40 ml of *methanol.*

Chromatographic system:

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase. a mixtures of 82 volumes of 1 per cent v/v *acetic acid* in *water* and 18 volumes of *acetonitrile.*
- flow rate: 1 ml per minute,
- spectrophotometer set at 350 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

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Calculate the total sennosides from the peak area of sennoside A and B using content of total sennosides in calcium sennoside.

1 mg calcium sennoside contains 0.9579 mg sennosides.

Storage. Store protected from light and moisture.

Senna Dry Extract

Senna Dry Extract is produced from Senna leaves or pods of *Cassia angustifolia* (Tinnevelly Senna) or *Cassia acutifolia* (Cassia Senna) as calcium salts.

Senna dry extract contains not less than 85.0 per cent w/w and not more than 115.0 per cent w/w of the stated amount of total sennosides, calculated as sennoside B, as calcium salt on dried basis.

Category. Purgative, anthelmintic, febrifuge, vibandha.

Description. A light brown to dark brown powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 40 volumes of *n-propyl alcohol*, 40 volumes of *ethylacetate*, 30 volumes of *water* and 1 volume of *glacial acetic acid*.

Test solution. Shake well 0.1 g of the extract under examination with 5 ml of a mixture of equal volumes of *methanol* and *water* for 5 minutes, heat to 60°, cool and allow to settle. Use the supernatant liquid.

Reference solution. Dissolve 10 mg of *calcium sennosides IPRS* in 1 ml of a mixture of equal volumes of *methanol* and *water*.

Apply to the plate, 10 µl of each solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 10 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with 20 per cent v/v of *nitric acid solution*. Heat the plate at 110° for 10 minutes and examine the plate in day light. Allow to cool and spray with a 5 per cent w/v solution of *potassium hydroxide* in *ethanol (50 per cent v/v)* until the zones appear. The chromatographic profile of the test solution is similar to that of the reference solution.

B. To about 25 mg of the extract add 50 ml of *water* and 2 ml of *hydrochloric acid*. Heat in a water-bath for 15 minutes. Cool and shake with 40 ml of *ether*. Separate ether layer, dry over *anhydrous sodium sulphate* and evaporate 5 ml to dryness. To the cooled residue add 5 ml of *dilute ammonia*. A yellow or orange colour develops. Heat on a water-bath for 2 minutes. A reddish-violet colour develops.

Tests

pH (2.4.24). 5.5 to 7.5, determined in a 10 per cent solution in *water*.

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 gm by drying in an oven at 105°.

Microbial contamination (2.2.9). Total aerobic viable count is not more than 10^4 CFU per g and total fungal count is not more than 10^2 CFU per g. 1 g is free from *Escherichia coli* and 10 g is free from *Salmonella*.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. A 0.3 per cent v/v solution of *acetic acid* with the pH adjusted to 5.9 with *1 M sodium hydroxide*.

Test solution. Dissolve an accurately weighed quantity of substance under examination containing about 10 mg of *calcium sennosides* in 50.0 ml of the solvent mixture.

Reference solution. *Reference solution*: Dissolve an accurately weighed quantity of *calcium sennoside IPRS* containing about 10 mg of *calcium sennosides* in 50.0 ml of the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane chemically bonded to porous silica (5 µm),
- mobile phase: a mixture of 83 volumes of a 1 per cent v/v solution of *glacial acetic acid* and 17 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 350 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the total sennosides from the peak area of sennoside A and B using the declared content of total sennosides in calcium sennosides.

1 mg of sennoside is equivalent to 1.044 mg of calcium sennoside.

Storage. Store protected from light, in air-tight containers.

Senna Tablets

Senna Tablets contains not less than 85.0 per cent w/w and not more than 115.0 per cent w/w of the stated amount of total *sennosides*, calculated as *sennoside B*, as calcium salt.

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1 mg of *sennoside* is equivalent to 1.044 mg of *calcium sennoside*.

Category. Purgative, anthelmintic, febrifuge, vibandha.

Identification

In the assay, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

Tests

Other tests. Comply with the tests stated under tablets.

Loss on drying (2.4.19). Not more than 8.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Assay. Determine by liquid chromatography (2.4.14).

Solvent mixture. A 0.3 per cent v/v solution of *acetic acid*, with the pH adjusted to 5.9 with *1 M sodium hydroxide*.

Test solution. Weigh and powder 20 tablets. Weigh accurately a quantity of the powder containing about 10 mg of *calcium sennosides*, dissolve about 40 ml of solvent mixture and mix with the aid of ultrasound for 15 minutes. Dilute to 50 ml with the solvent mixture and filter.

Reference solution. A 0.02 per cent w/v solution of *calcium sennoside IPRS* in the solvent mixture.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane chemically bonded to porous silica (5 µm),
- mobile phase: a mixture of 83 volumes of a 1 per cent v/v solution of *glacial acetic acid* and 17 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 350 nm,
- injection volume: 10 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

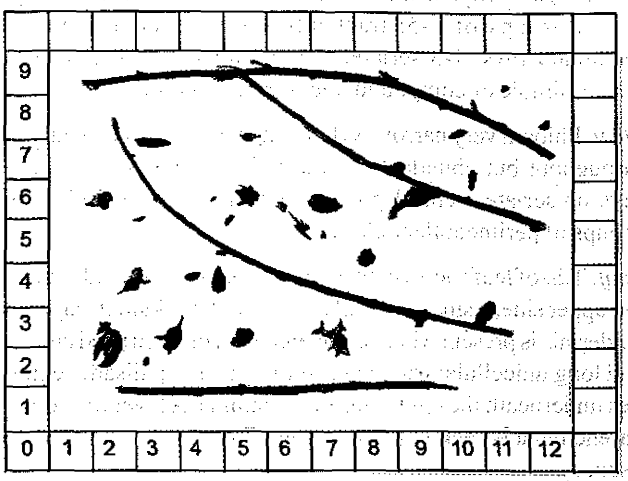
Calculate the total sennosides from the peak area of sennoside A and B using the declared content of total sennosides in calcium sennosides.

Storage. Store protected from light, at a temperature not exceeding 30°.

Labelling. Labelling. The quantity of active ingredient is stated in terms of total sennosides as calcium salt.

Shankhpushpi

Convolvulus pluricaulis



Shankhpushpi consists of whole plant of *Convolvulus pluricaulis* Choisy (Fam. Convolvulaceae).

Shankhpushpi contains not less than 0.1 per cent to 0.3 per cent of Scopoletin, calculated on the dried basis.

Category. Antiulcerogenic, antidepressant, antiepileptic, hepatoprotective and anti-inflammatory.

Description. A light green yellowish in colour and slightly bitter in taste with characteristic odour.

Identification

A. *Macroscopic — Root*. Cylindrical, straight and branched, 7-12 cm long, 0.1-0.4 cm thick, the root is faintly longitudinally striated and exhibits small circular rootlet scars, fractured surface and is yellowish brown to light brown in colour.

Stem. Cylindrical and aerial stems densely covered with hairs. The stem is 2 to 6 mm in diameter and 20 to 50 cm in length, light green in colour, taste is slightly bitter.

Leaf. Shortly petiolate, linear-lanceolate, acute, hairy on both surfaces; 0.5-2 cm long and 10-0.5 cm broad; light green.

Flower. White or pinkish; solitary or in pairs sessile or sub sessile in the leaf axis; sepals narrow, linear lanceolate, sparsely hairy; corolla shortly discoid; stamen 5, free, epipetalous, alternate with the petals, inserted deep in the corolla tube; ovary superior and bicarpellary

Fruit. Capsule, oblong globose with coriaceous, pale brown pericarp.

Seed. Brown, minutely puberulous.

B. *Microscopic — Root*. T.S. of root shows outer cork composed of 10-15 years of tangentially elongated, thick-

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walled suberized cells, cortex many layered, parenchymatous, traversed by yellowish-brown tanniniferous secretory cells and starch grains, phloem composed of sieve elements, phloem parenchyma and phloem rays. Xylem vessel solitary or in groups of 2-5; tracheids and parenchyma pitted, medullary rays 1-3 seriate; starch grains, round to oval in shape, simple or compound, measuring 3-8 µ in diameter.

Stem. Phloem very narrow, xylem ring wider, vessels scattered throughout but abundantly towards inner region, medullary rays uniseriate, central pith parenchymatous, encircled by groups of perimedullary phloem.

Leaf. T.S. of leaf shows convex midrib on the lower side and at the upper side, a small centrally placed notch. A single layered epidermis is present which is covered with thick striated cuticle and long unicellular trichomes. A two layered palisade region lies underneath the epidermis of the lamina. A layer of spongy parenchyma is seen in between the upper and lower palisade tissue.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 4 volumes of *toluene,* 2.5 volumes of *chloroform*, and 3.5 volumes of *acetone.*

Test solution. Reflux 2 g of coarsely powdered substance under examination with 50 ml of *methanol* for 30 minutes, cool and filter. Reflux the residue further with 50 ml of *methanol*, cool and filter. Combine the filtrates and concentrate under vacuum to 25.0 ml.

Reference solution. Reflux 2 g of *shankhpushpi IPBRS* with 50 ml of *methanol* for 30 minutes, cool and filter. Reflux the residue further with 50 ml of *methanol*, cool and filter. Combine the filtrates and concentrate under vacuum to 25.0 ml.

Apply to the plate 10 µl of both test and reference solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in ultraviolet light at 254 nm and 366 nm. Spray with *anisaldehyde sulphuric acid reagent* and heat the plate at 100° for 10 minutes. Examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 1.0 per cent.

Ethanol- soluble extractive (2.6.2). Not less than 6.0 per cent

Water soluble extractive (2.6.3). Not less than 13.0 per cent.

Total ash (2.3.19). Not more than 20.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 6.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 8.0 per cent determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 20 g of the coarsely powdered substances under examination with 100 ml of *methanol* on water-bath for 2 hours, cool and filter. Evaporate to dryness and dissolve in 5 ml of *methanol.*

Reference solution. A 0.2 per cent w/v solution of *scopoletin IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 30 volumes of *methanol* and 70 volumes of a buffer solution of 0.1 per cent v/v of *formic acid,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 365 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injection is not more than 2.0 per cent.

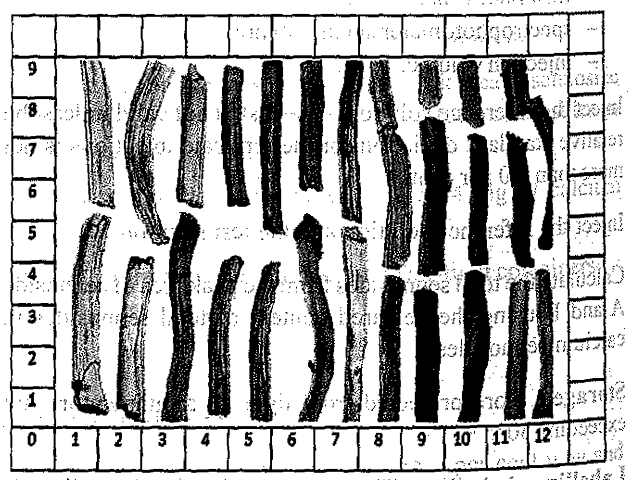
Inject the reference solution and the test solution.

Calculate the content of scopoletin.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Shatavari

Asparagus racemosus root



Shatavari consists of the tuberous roots of *Asparagus racemosus* Willd. (Fam. Liliaceae).

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Shatavari contains not less than 0.1 per cent w/w of shatavarin IV, calculated on the dried basis.

Category. Antiinflammatory, immunomodulatory, neuroprotective and antidysentery.

Description. The tuberous root bits are dirty white in color, longitudinally wrinkled with yellow hard central core. It is starchy and slightly bitter followed by sweet taste.

Identification

A. *Macroscopic* — The tuberous roots are borne in a compact bunch and are fleshy, and spindle shaped. They are marketed in pieces 5-15 cm in length and 2 cm in thickness. They are silvery white or ash-colored externally and white internally, more or less smooth when fresh, developing longitudinal wrinkles when dry. They are adventitious that measure about one meter in length tapering at both ends with roughly a hundred on each plant.

B. *Microscopic* — The inner parenchymatous zone of cortex is composed of 18-24 layers in upper and 42-47 layers in the middle tuberous portion of the roots. The cells are thin - walled cellulosic, with circular to oral outlines and distinct inter cellular spaces. In some roots 3-4 layers of cortex immediately adjacent to the endodermis are modified into a sheath of stone cells round the endodermis. The number of vascular bundles is 30-35 in the upper levels and 35-45 in the middle tuberous portions of the roots.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 13 volumes of *chloroform*, 10 volumes of *methanol* and 2 volumes of *water*.

Test solution. Reflux 1 g of the coarsely powdered substance under examination with 30 ml of *methanol* for 30 minutes, cool and filter. Reflux the residue further with 2 x 30 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Reference solution. Reflux 1 g of *shatavari IPBRS* with 30 ml of *methanol* for 30 minutes, cool and filter. Reflux the residue further with 2 x 30 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Apply to the plate 5 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with *vanillin sulphuric acid reagent*. Heat the plate at 120° for 10 minutes and examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

D. DNA-based Identification (2.2.17). Determine botanical identity by DNA-based testing, in samples requiring such testing due to suspected adulteration/subs disputes this test shall be mandatory.

Genomic DNA extraction

Reference solution

Rinse thoroughly the dried roots of a sample of *shatavari IPRS* in sterile distilled water and cut into pieces of approximately 1 mm using sterile blade. Grind 1 g of the sample in the presence of liquid nitrogen and 2 per cent w/w *polyvinyl pyrrolidone* using pre-chilled mortar and pestle. Transfer the frozen powder in 12 ml of extraction *cetyltrimethylammonium bromide buffer* containing 2 per cent *mercaptoethanol* to a 50 ml centrifuge tube. Mix well and incubate at 60° for 12 h. Cool the mixture to room temperature and add equal volume of 24:1 *chloroform-isoamylalcohol*. Mix by inversion (20-30 times) to form an emulsion. Centrifuge the mixture at 10,000 rpm for 10 minutes at 4°. Transfer the supernatant to a fresh tube and precipitate the DNA by adding 0.6 volumes of chilled *isopropanol* and 1/10th volume of *3M sodium acetate* and store at –20° for 5 hours. Centrifuge the precipitated DNA at 10,000 rpm for10 minutes at 4°. Carefully decant the supernatant and wash the pellet with 70 per cent *alcohol*. Decant the supernatant and dry the pellet at 37°. Dissolve the pellet in *tris buffer* and store at –20°. Treat the DNA with 3 µl of 1 per cent *RNase* for 1 hour at 37°. Add 3 µl of 1 per cent *proteinase K* and incubate at 37° for 15-20 minutes. Add 150 µl of *phenol* and 150 µl of 24:1 *chloroform-isoamylalcohol* to each microfuge tube. Vortex briefly and centrifuge at 14,000 rpm for 15 minutes. Transfer the supernatant to a fresh tube and re-precipitate the DNA by adding double the volume of *absolute alcohol* and 1/10th volume of *3M sodium acetate* and store at –80° for 3 hours. Precipitate DNA at 14,000 rpm for 12 minutes at 4° by centrifugation and wash the pellet with 70 per cent *ethanol*. Dry the pellet at 37° and dissolve in 100 µl - 200 µl of *tris buffer*.

Test solution

Perform DNA extraction adopting the same procedure as for reference solution but using dried roots of shatavari sample under examination in place of *shatavari IPRS*.

PCR amplification of ITS2 (2.8.1). Perform PCR with universal primers and conditions using the isolated genomic DNA to examine efficiency of amplification for the candidate DNA barcode. The universal primers (Forward primer: 5'-ATGCGATACTTGGTGTGAAT-3'; Reverse primer: 5'-GACGCTTCTCCAGACTACAAT-3') for candidate DNA barcode (ITS2) are designed. Carry out PCR for amplifying the DNA preparations in a 50 µl volume of reaction mixture consisting of following components:

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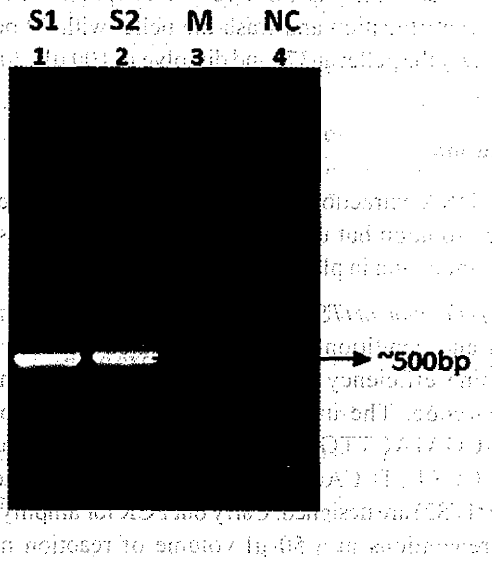
Components	Volume
50 ng Template genomic DNA	2 µl
100 ng Primer (Forward and Reverse) in equal proportions	1 µl
50 mM of Magnesium chloride solution PCR buffer (5 mM KCl; 0.15 mM $MgCl_2$; 1 mM Tris HCl, pH 8.8; 0.1 per cent Triton X-100)	1 µl
4 mM Deoxynucleotide triphosphate (dNTPs) in equal proportions	5 µl
0.5 U Taq DNA polymerase	1 µl
50 mM Tetramethylammonium chloride	1 µl
Double distilled water	34 µl
Total	50 µl

Carry out amplifications by using a DNA thermal cycler with the following cycling program: 94° for 5 minutes; 40 cycles at 94° for 1 minute, 56° for 30 seconds, and 72° for 45 seconds; and final extension at 72° for 10 minutes.

Visualization of PCR product: 5 µl of the PCR product is mixed with 1µl of 30 per cent glycerol and loaded on 1.5 per cent *agarose gel* in *Tris-borate-EDTA buffer* along with 100bp DNA ladder as size marker and 1 µl tracking dye (2.5 per cent Ficoll 400, 11 mM EDTA, 3.3 mM Tris-HCl, 0.017 per cent SDS, 0.015 per cent Bromophenol blue, pH 8.0 at 25°). Electrophoresis is carried out at 100V for 45 minutes and photograph is taken under UV light. Shatavari *IPRS* served as positive control in the PCR reaction.

The amplified DNA band of sample under examination corresponds to amplified DNA band of shatavari *IPRS*.



PCR for ~500 bp ITS2 region of root DNA samples of Shatavari

Gel elution. Load 50 µl of PCR product mixed with 7 µl of 30 per cent *glycerol* and 1µl of tracking dye (2.5 per cent Ficoll 400, 11 mM EDTA, 3.3 mM Tris-HCl, 0.017 per cent SDS, 0.015 per cent Bromophenol blue, pH 8.0 at 25°) on a 1.5 per cent *agarose gel*. Run the gel for 45 minutes with power pack set to 100 volts. Take a glass tray and wash it first with *detergent* and then rinse with hot *water* and then finally wipe thoroughly with cotton soaked in spirit. Keep the gel on the UV transilluminator and then cut the bands from gel carefully using a sterile surgical blade for each band and slice the gel bands with same sterile blades. Transfer the gel pieces into labeled microfuge tubes. To each microfuge tube add 500 µl of *buffer* saturated *phenol* (pH 7.4) and vortex properly. Store the tubes at –80° overnight. The next day thaw the tubes properly and add 100 µl of double distilled *water*. Centrifuge the tubes at 14,000 rpm for 15 minutes at 4° and transfer the supernatant into a fresh labeled tube. Add 500 µl of 24:1 *chloroform-isoamylalcohol* to each microfuge tube. Vortex briefly and centrifuge at 14,000 rpm for 15 minutes. Transfer the supernatant to a fresh tube and precipitate the DNA by adding double the volume of absolute *alcohol* and 1/10th volume of *3M sodium acetate* and store at –80° for 3h. Centrifuge the tubes at 14,000 rpm for 15 minutes at 4° and wash the pellet with 70 per cent *ethanol*. Dry the pellet at 37° and dissolve the DNA in 10 µl sterile double distilled *water*.

DNA sequencing. Pipette 1µl (20 ng) of reverse or forward primers (Forward primer: 5'-ATGCGATACTTGGTGTGAAT-3'; Reverse primer: 5'- GACGCTTCTCCAGACTACAAT-3') into appropriately labeled PCR tubes. Pipette 2 µl of big dye ready reaction termination mix (2.25µl of Bigdye having 6FAM ddC, VIC ddA, NED ddG, TED ddT) into each tube. Pipette 1 µl of the sequencing buffer containing 200 mM Tris-HCl pH 9.0, 5 mM $MgCl_2$ and 4 µl of double distilled water. Add 1 µl of PCR product to the appropriate tube and mix with the pipette up and down several times and spin briefly. Run the following cycling programme: 94° for 5 minutes; 25 cycles at 96° for 10 seconds, 50° for 5 seconds and 60° for 4 minutes and 4° for forever.(gel eluted PCR product was used for big dye ready reaction and hence final extension is not required in the reaction) After the big dye reaction termination, remove the tubes from the thermocycler. Add 10 µl of sterile *water* to each tube to make up the reaction volume to 20 µl. Add 2 µl of *3M sodium acetate*, pH 4.6 and 50 µl *ethanol* to labeled 1.5 ml microfuge tubes. Add each 20 µl sequencing reaction to the appropriate tube containing the *salt-ethanol* mixture. Vortex briefly to mix. Incubate at room temperature for 15 minutes. Spin the tubes for 15-30 minutes at 200 G speed. Immediately after spinning, remove the supernatant using a pipette and re-dissolve the pellet. Sequence, purified PCR products in an Applied Biosystems genetic analyzers using the amplification primers.

During DNA sequencing, the extension products of the cycle sequencing reaction enter the capillary as a result of

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electrokinetic injection. A high voltage charge applied to the buffered sequencing reaction forces the negatively charged fragments into the capillaries. The extension products are separated by size based on their total charge. The electrophoretic mobility of the sample can be affected by the run conditions: the buffer type, concentration, and pH; the run temperature; the amount of voltage applied and the type of polymer used. Shortly before reaching the positive electrode, the fluorescently labeled DNA fragments, separated by size, move across the path of a laser beam. The laser beam causes the dyes on the fragments to fluoresce. An optical detection device on Applied Biosystems genetic analyzers detects the fluorescence. The data collection software converts the fluorescence signal to digital data and then records the data in a *.abl file because each dye labeled ddNTP's (6FAM ddC, VIC ddA, NED ddG, TED ddT) emits light at a different wavelength when excited by the laser, all four colors and therefore all four bases can be detected and distinguished in one capillary injection.

Evaluation and interpretation of results. Assemble original forward and reverse sequences of PCR products using sequence alignment tools like Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI) to estimate the quality of the generated DNA sequence traced. Analyze the data for generated DNA sequence by amplified PCR product from Shatavari plant by comparing it to available sequence of ITS2 region of same family plants in the GenBank to find the intra and inter-species specific divergence. The homology between the Shatavari *IPRS* and its substitute plant Shatavari test should be not less than 95.0 per cent.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 15.0 per cent.

Water-soluble extractive (2.6.3). Not less than 20.0 per cent by Method I.

Total ash (2.3.19). Not more than 15.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 15.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Perform the Assay by following Method I or by Method II. The result of the Method II will be official in case of dispute.

Method I

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 6.5 volumes of *chloroform* and 3.5 volumes of *methanol*.

Test solution. Reflux 4 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water-bath for 30 minutes, cool and filter. Reflux the residue further with *methanol* till the last extract turns colourless, cool and filter. Combine all the filtrates and concentrate to 50 ml.

Reference solution. A 0.008 per cent w/v solution of *shatavarin IV IPRS* in *methanol*.

Apply to the plate 5 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and spray with a 10 per cent v/v solution of *sulphuric acid* in *methanol*. Heat the plate at 100° for 5 minutes, scan the plate in absorbance mode at 500 nm. Record the chromatograms and measure the responses for the analyte peak.

Calculate the content of shatavarin IV.

Method II

Determine by liquid chromatography (2.4.14).

Test solution. Reflux 5 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water-bath for 30 minutes, cool and filter. Reflux the residue further with *methanol* till the last extract turns colourless, cool and filter. Combine all the filtrates and concentrate to 50.0 ml.

Reference solution. A 0.1 per cent w/v solution of *shatavarin IV IPRS* in *methanol*. Dilute suitably to prepare 0.0075- 0.075 per cent w/v solution.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 60 volumes of *acetonitrile* and 40 volumes of *water*,
- flow rate: 1 ml per minute,
- use evaporative light scattering detector,
- temperature
  evaporator 110°,
  nebulizer 90°,
  nebulizer gas nitrogen and gas flow 1 SLM,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the regression coefficient is not more than 0.9.

Inject the reference solution and the test solution.

Calculate the content of shatavarin IV.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

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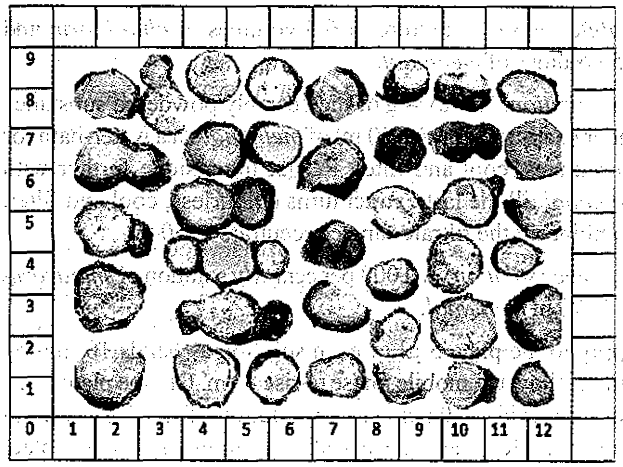
Shati

Hedychium spicatum



Shati consists of the dried rhizomes of *Hedychium spicatum* Buch.-Ham.ex Smith (Fam. Zingiberaceae).

Shati contains not less than 0.80 per cent w/w of p-methoxy cinnamic acid ethyl ester, calculated on the dried basis.

Category. Antiasthmatic, antiseptic, indicated in skin diseases, analgesic, kasa.

Description. A reddish-brown outer surface and white in side, short and uneven fracture and camphoraceous odour with aromatic and pungent taste.

Identification

A. *Macroscopic* — Tuberous rhizome having reddish brown, rough outer surface with round root scars or rootlets which remain attached at some places. Transversely sliced pieces of dried rhizome are spherical, flat, 1 cm in thickness and 2-3 cm in diameter having white and starchy surface.

B. *Microscopic* — Transverse section of rhizome shows outermost layer of cork having 2-6 layers of isodiametic, nonlignified and suberised cells which are radially arranged. In old rhizome, the cork cells are exfoliated or crushed. Cortex is a broad zone with 20-25 layers of thin walled parenchymatous cells. Cortex region is filled with abundant starch grains and numerous oleo-resin cells. Vascular bundles are closed and collateral and scattered throughout the ground tissues. Cambium has 4-5 rows of tangentially elongated cells. It forms a complete ring between the xylem and phloem groups. Starch grains tissue are simple, circular or oval in shape, found in ground cells.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 80 volumes of *n-hexane* and 20 volumes of *acetone.*

Test solution. Reflux 1 g of the coarsely powdered substance under examination with 25 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 × 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 25 ml.

Reference solution. Reflux 0.5 g of the *shati IPBRS* with 25 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 × 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 12.5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with *anisaldehyde sulphuric acid reagent.* Heat the plate at 110° for 10 minutes and examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 0.7 per cent.

Water-soluble extractive (2.6.3). Not less than 12.0 per cent by Method I.

Total ash (2.3.19). Not more than 8.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 14.0 per cent, determined on 5 g by drying in an oven at 105°

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux 2 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water-bath for 15 minutes, cool and filter. Reflux the residue further with *methanol* till the extract turns colourless, cool and filter. Combine all the filtrates and concentrate to a volume slightly less than 100 ml. Dilute to 100.0 ml with *methanol.*

Reference solution. A 0.01 per cent w/v solution of *p-methoxy cinnamic acid ethyl ester IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: 40 volumes of *water* and 60 volumes of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 310 nm,
- injection volume: 20 µl.

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Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of p-methoxy cinnamic acid ethyl ester.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Shellac

Lac

Shellac consists of a purified substance prepared from the resinous secretion of the female insect, *Laccifer lacca* Kerr (Fam. Coccoidae). Shellac is a polyester resin consisting of inter-and intra-esters of polyhydroxyl carboxylic acids formed from certain hydroxyl acids and sesquiterpinic acids, and also contains variable amounts of wax.

There are four types of shellac depending on the nature of the treatment of crude secretion.

Orange shellac is produced by a process of filtration in the molten state and/or by a process of solvent extraction. Orange shellac contains not more than 5.5 per cent w/w of wax.

Refined (dewaxed) orange shellac is produced by filtration of the wax in a solvent process. It may also be declorized by activated carbon. Refined orange shellac contains not more than 0.2 per cent w/w of wax.

Regular bleached (white) shellac is prepared by dissolving the lac in an alkaline solution and bleaching the solution with sodium hypochlorite. It is precipitated by dilute acid and dried. Regular bleached shellac contains not more than 5.5 per cent w/w of wax.

Refined bleached shellac is prepared by dissolving the lac in an alkaline solution and bleaching the solution with sodium hypochlorite. During the process, wax is removed by filtration. It is precipitated by dilute acid and dried. Refined bleached shellac contains not less than 0.2 per cent w/w of wax.

Category. Pharmaceutical aid.

Description. Brownish-orange or yellow, shining, translucent, hard or brittle, more or less thin flakes (wax-containing shellac and bleached shellac) or a creamy white or brownish-yellow powder (bleached shellac and bleached, dewaxed shellac).

Identification

A. To 50 mg add a few drops of a mixture of 1 g of *ammonium molybdate* and 3 ml of *sulphuric acid;* a green colour is produced and it becomes lilac on standing for 5 minutes.

B. Thin-layer chromatography (2.4.17), using the plate coated with *silica gel GF254.*

Mobile phase. A mixture of 1 volume of *acetic acid,* 8 volumes of *methanol,* 32 volumes of *methylene chloride* and 60 volumes of *ethyl acetate.*

Test solution. Heat 0.25 g of the coarsely powdered substance under examination with 2 ml of *dilute sodium hydroxide solution* for 5 minutes. Cool and add 5 ml of *dilute acetic acid.* Shake and filter the upper layer through *anhydrous sodium sulphate.*

Reference solution. Dissolve 6.0 mg of *aleuritic acid IPRS* in 1.0 ml of *methanol,* heat gently if required.

Apply to the plate 10 µl of each solution as band length 8 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine in ultraviolet light at 254 nm and 366 nm. Spray the plate with *anisaldehyde sulphuric acid reagent.* Heat the plate at 105° for 5 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Acid value (2.3.23). 65 to 95, determined by the following method. Weigh accurately 2.0 g and dissolve with the aid of gentle heat, in 50 ml of *ethanol (95 per cent)* previously neutralised to *ethanolic thymol blue solution.* Titrate with *0.1 M ethanolic potassium hydroxide* using *ethanolic thymol blue solution* as an external indicator. Calculate the acid value from the expression

$$5.61 \times a/w$$

where, a = number of ml of *0.1 M ethanolic potassium hydroxide* and

w = weight, in g, of the sample.

Ethanol-insoluble matter. Not more than 2.0 per cent, determined by the following method. Weigh accurately 5.0 g in an extraction thimble. Cover with *ethanol (95 per cent)* and allow to stand for 16 hours. Place in an apparatus for the continuous extraction of drugs, extract with *ethanol (95 per cent)* for 4 hours, dry the residue at 100° for 3 hours and weigh.

Colophony. Dissolve 2.0 g by shaking with 10 ml of *ethanol,* add slowly with shaking 50 ml of *light petroleum (40° to 60°),* wash with two successive portions, each of 50 ml of *water,* filter the washed *light petroleum* solution, and evaporate to dryness. To the residue, add 2 ml of a mixture of 1 volume of *bromine,* 4 volumes of *liquified phenol* and 2 volumes of *carbon tetrachloride,* and cover both cavities with an inverted watch-glass; no purple or deep indigo-blue colour is produced in the liquid containing the residue.

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Arsenic (2.3.10). Heat gently 5.0 g with 2 ml of *nitric acid* and 0.5 ml of *sulphuric acid* in a long-necked flask, until the first reaction has subsided, cool, add carefully and in small portions, 15 ml of *nitric acid* and 6 ml of *sulphuric acid* taking care to avoid excessive foaming, and continue heating, adding further small portion of *nitric acid*, if necessary, until white fumes are evolved and the solution becomes colourless or almost colourless. Cool, add carefully 10 ml of *water*, evaporate until white fumes are evolved, and repeat the addition of *water* and evaporation until all the *nitric acid* has been removed, cool, dilute to 50 ml with *water*, and 10 ml of *stannated hydrochloric acid AsT*. The resulting solution complies with the limit test for arsenic (2 ppm), Use 0.5 ml of *arsenic standard solution (10 ppm As)* for the standard stain.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

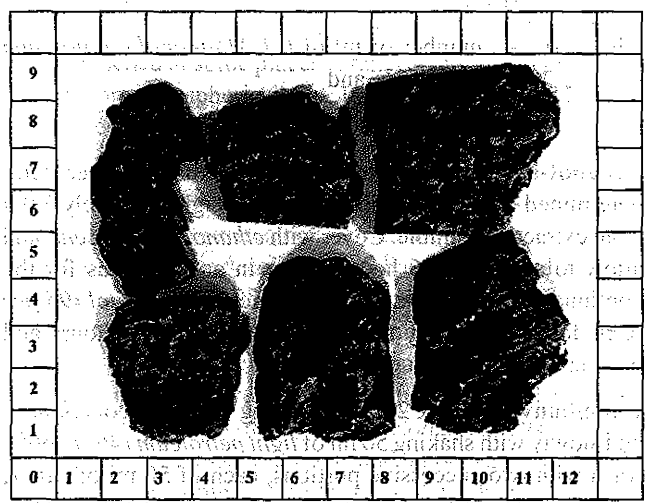
Sulphated ash (2.3.18). Not more than 1.0 per cent, determined on 0.5 g.

Loss on drying. Not more than 2.0 per cent for unbleached shellac and not more than 6.0 per cent for bleached shellac, determined on 1.0 g by drying in an oven at 40°- 45° for 24 hours.

Storage. Store protected from light and moisture. Store bleached shellac and bleached, dewaxed shellac at a temperature not exceeding 15°.

Siris

Albizia lebbeck



Siris consists of dried stem bark of *Albizia lebbeck* (Linn.) Benth. (Fam. Fabaceae). Siris contains not less than 0.03 per cent w/w of catechin hydrate, calculated on the dried basis.

Category. Anti-inflammatory, Anti-histaminic, Antioxidant, Antitussive.

Description. It is a medium to large sized tree growing throughout India and up to 900 m above mean sea level also in lower Himalayas and in the Andaman and Nicobar Islands.

Identification

A. *Macroscopic* — Bark 1.5-2.5 cm thick, dark brown, young bark with transversely elongated lenticels, older one rough longitudinally fissured and transversely cracked, exhibit well developed, brown to blackish brown rhytidoma, which often peels off in the flakes exposing a buff coloured inner surface, showing impressions of lenticels. The inner surface is smooth, yellowish to grayish brown in color, longitudinally finely striated. Fracture is fibrous. Perceptible acrid and astringent taste and no characteristic odour.

B. *Microscopic* — Transverse section of the mature bark externally shows about 4-10 mm thick rhytidoma occupying almost one third the area of the entire bark, remaining tissue being mostly of secondary phloem. Rhytidoma is composed of alternate zones of various tissues like cork, cortex, pericycle and phloem, deeply coloured with brown pigment and tannin. Isolated prisms of calcium oxalate, groups of sclerids and stone cells of various sizes and thickness also traversed throughout this region. The living tissue of the bark is mainly composed of wide secondary phloem consisting of groups of tangentially running sieve tissues frequently getting obliterated. Companion cells and phloem parenchyma alternating with phloem fibers are associated with idioblast containing prisms of calcium oxalate crystals; groups of stone cells occasionally associated with fibers. Medullary rays uni- to tri-seriate, mostly multi-seriate and wavy in outer phloem, and running almost parallel in the inner phloem region. Simple starch grains present in the paranchymatous cells of the phloem.

Powder highly fibrous reddish brown. The diagnostic microscopical characters include isolated or groups of thick-walled, pitted, lignified stone cells of different sizes and shapes; fragments of thick-walled fibers; paranchymatous cells associated with idioblast containing prisms of calcium oxalate crystals; cork cells in surface view; paranchymatous cells with starch grains; tangentially and radially cut medullary rays with fibers and prismatic crystals of calcium oxalate.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 5 volumes of *toluene*, 5 volumes of *ethyl acetate* and 1 volume of *formic acid*.

Test solution. Defat 10.0 g of the powdered substance under examination with 50 ml of *petroleum ether (60-80°)*. Reflux the residue under examination with 50 ml of *methanol* for 30 minutes, cool and filter. Reflux the residue further for 2 x 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 5.0 ml.

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Reference solution. A 0.01 per cent w/v solution of *catechin hydrate IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm, 366 nm and day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign Organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 12.0 per cent.

Water-soluble extractive (2.6.3). Not less than 15.0 per cent by method I.

Total ash (2.3.19). Not more than 5.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.4 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Sonicate 10.0 g of the coarsely powdered substance under examination with 50 ml of *methanol* for 15 minutes followed by reflux on water bath for another 15 minutes, cool and filter. Extract the residue further for 2 x 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum up to 5.0 ml.

Reference solution. A 0.01 per cent w/v solution of *catechin hydrate IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm, packed with octadecylsilane bonded to porus silica (5 µm).
- mobile phase: a mixture of 80 volumes of *water* and 20 volumes of *methanol* containing 0.1 per cent v/v *trifluoroacetic acid*,
- spectrophotometer set at 205 nm,
- flow rate: 0.75 ml per minute,
- injection volume: 2 µl,
- column temperature: 35°

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference and the test solution.

Calculate the content of catechin hydrate calculated on the dried basis.

Storage: Store protected from heat, moisture and against attack by insects and rodents.

Starch

Starch consists of polysaccharide granules obtained from the caryopsis of maize or corn, *Zea mays* Linn.(Fam. Poceae), or of rice, *Oryza sativa* Linn., or of wheat, *Triticum aestivum* Linn.(Fam. Graminae), or from the tuber of potato, *Solanum tuberosum* Linn (Fam. Solanaceae), or from the rhizomes of tapioca, *Manihot utilissima* Pohl.(Fam. Euphorbiaceae).

Category. Pharmaceutical aid.

Description. A very fine, white or slightly yellowish powder or irregular white masses which are readily reducible to powder, creaks when pressed between the fingers; odourless and tasteless. The presence of granules showing cracks or edge irregularities is exceptional in starches other than wheat starch; wheat starch may contain granules with cracks on the edges.

Identification

A. *Corn or maize starch* — Polyhedral granules, 2 to 23 µm in size, or rounded granules, 25 to 32 µm in diameter. The central hilum consists of a distinct cavity or two- to five-rayed cleft; no concentric striations. Viewed between crossed nicol prisms, a distinct black cross is seen intersecting at the hilum.

Potato starch — Single granules, either irregular, ovoid or pear-shaped, 30 to 100 µm in size, or rounded, 10 to 35 µm in size; compound granules consisting of groups of two to four elements are rare. Eccentric hilum; clearly visible concentric striations. Viewed between crossed nicol prisms, a distinct black cross is seen intersecting at the hilum.

Rice starch — Polyhedral granules, 2 to 5 µm in size, either isolated or aggregated in ovoid masses, 10 to 20 µm in size. Central hilum poorly visible; no concentric striations. Viewed between crossed nicol prisms, a distinct black cross is seen intersecting at the hilum.

Tapioca starch — Principally simple granules, sub-spherical, muller-shaped or rounded polyhedral; smaller granules 5 to 10 µm, larger granules 20 to 35 µm in diameter; hilum, central, punctate, linear or triradiate; striations, faint, concentric; compound granules, few, of two to three unequal components.

Wheat starch — Large discoid or, more rarely, reniform granules, 10 to 45 µm in size; profile, elliptical and fusiform, slit along the main axis. Small rounded or polyhedral granules, 2 to 10 µm in size. Granules of intermediate size very rarely occur. Hilum and striations invisible or barely visible. Viewed between crossed nicol prisms, a distinct black cross is seen intersecting at the hilum.

B. Heat to boiling for 1 minute a suspension of 1 g in 50 ml of *water* and cool; a thin and cloudy mucilage is produced with all starches except potato starch which gives a thick and more transparent mucilage.

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C. To 10 ml of the mucilage obtained in test B add 0.05 ml of *0.01 M iodine*; a dark blue colour is produced, which disappears on heating and reappears on cooling.

Tests

Acidity. Add 10.0 g to 100 ml of *ethanol (70 per cent)* previously neutralised to *phenolphthalein solution*, shake for 1 hour, filter and titrate 50 ml of the filtrate with *0.1 M sodium hydroxide*. Not more than 2.0 ml is required to change the colour of the solution.

Iron (2.3.14). Dissolve the residue obtained in the test for sulphated ash in 4 ml of *hydrochloric acid* with the aid of gentle heat, dilute with *water* to 50 ml and mix; 25 ml of the resulting solution complies with the limit test for iron (40 ppm).

Fluorescence. No fluorescence should be visible under screened ultra-violet light.

Oxidising substances. To 5.0 g add 10 ml of *water* and 1 ml of *acetic acid* and stir until a homogeneous suspension is obtained. Add 0.5 ml of a freshly prepared saturated solution of *potassium iodide*, mix and allow to stand for 5 minutes; no distinct brown or blue colour is observed.

Microbial contamination (2.2.9). 1.0 g is free from *Escherichia coli* and 10.0 g is free from *Salmonella* and *Shigella*.

Sulphated ash (2.3.18). Not more than 0.6 per cent (for all starches except rice starch) and not more than 0.8 per cent (for rice starch), determined on 2.0 g.

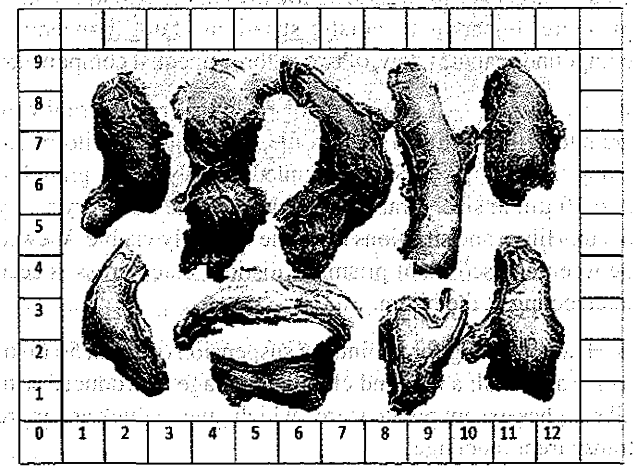
Loss on drying (2.4.19). Not more than 15.0 per cent (for all starches except potato starch) and not more than 20.0 per cent (for potato starch), determined on 0.2 g by drying in an oven at 105°.

Storage. Store protected from light and moisture.

Labelling. The label states the type of starch.

Sunthi

Saunth; Ginger; *Zingiber officinale*



Sunthi is the whole or cut scraped or unscraped, dried rhizomes of *Zingiber officinale* Roscoe. (Fam. Zingiberaceae).

Sunthi contains not less than 0.8 per cent w/w of total gingerols, calculated on the dried basis.

Category. Carminative, antiemetic, antiinflammatory, hepatoprotective, agnimandya.

Description. Odour, agreeable and aromatic; taste, agreeable and pungent.

Identification

A. *Macroscopic* — Rhizome laterally compressed, bearing short, flattened, oblique branches; outer surface buff-coloured, longitudinally striate; inner surface pale yellow, starchy and fibrous. Fracture short with projecting fibers.

B. *Microscopic* — Fibro-vascular bundles and oleoresin cells with yellow pigment scattered in ground tissue. Starch grains abundant in parenchyma cells, mostly simple, sack shaped, spherical; hilum eccentric.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 30 volumes of *hexane* and 70 volumes of *diethyl ether*.

Test solution. Reflux 1 g of the coarsely powdered substance under examination with 25 ml of *methanol* for 15 minutes, cool and filter. Wash the residue with 10 ml of *methanol*. Combine all the filtrates and concentrate to 10 ml.

Reference solution. Reflux 0.5 g of coarsely powdered *sunthi IPBRS* with 5 ml of *methanol* for 15 minutes, cool and filter.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray the plate with *vanillin sulphuric acid reagent*. Heat the plate at 100° for 5-10 minutes and examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 2.0 per cent.

Water-soluble extractive (2.6.3). Not less than 10.0 per cent by Method I.

Total ash (2.3.19). Not more than 8.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.5 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not more than 12.0 per cent, determined on 0.2 g.

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Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 3 g of the coarsely powdered substance under examination with 100 ml of *methanol* on a water–bath for 15 minutes cool and filter. Reflux the residue further with *methanol* till the last extract turns colorless, cool and filter. Combine all the filtrates and concentrate to 50.0 ml

Reference solution. A 0.1 per cent w/v solution of *6-gingerol IPRS* in *methanol.*

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: 55 volumes of *acetonitrile* and 45 volumes of *water,*
- flow rate: 1.3 ml per minute,
- spectrophotometer set at 278 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the contents of total gingerols by summing the peak areas of 6-gingerol with all other peaks, which elute after 6-gingerol and have a peak area of at least 5 per cent of the peak area of 6-gingerol.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Sunthi Extract

Sunthi extract is obtained by extracting Sunthi (*Zingiber officinale* Roscoe, Fam. Zingiberaceae) dried rhizome of with ethanol or any other suitable solvent. The powdered extract may contain suitable excipients.

Sunthi extract contains not less than 90.0 per cent w/w and not more than 120.0 percent w/w of the stated amount of total gingerols and shogaols (sum of 6 gingerol, 8 gingerol, 10 gingerol and 6 shogaol). The content of 6 shogaol shall not be more than the content of 6 gingerol.

Category. Carminative, antiemetic, antiinflammatory, hepatoprotective, agnimandya.

Usual strengths. 5 per cent w/w; 20 per cent w/w.

Description. Very light yellow to light brown powder or thick liquid with characteristic odour and pungent taste.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 30 volumes of *hexane* and 70 volumes of *diethyl ether.*

Test solution. Dissolve 0.5 g of extract under examination with 50 ml of *methanol* and filter.

Reference solution. 0.1 per cent w/v solution of *6 gingerol IPRS* in *methanol.*

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm. Spray the plate with *vanillin sulphuric acid.* Heat the plate at 100° for 10 minutes and examine the plate at 366 nm and in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Loss on drying (2.4.19). Not more than 5.0 per cent, determined on 2.0 g when dried at 105° for 3 hours.

Total ash (2.3.19). Not more than 3.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, (Method B) 20 ppm.

Microbial contamination (2.2.9). Complies with the microbial contamination test

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 1 g of the extract containing 100 mg of gingerols in *methanol* by heating, make up to 100 ml and filter.

Reference solution. A 0.1 per cent w/v solution of 6 *gingerol IPRS* in *methanol.*

Chromatographic system

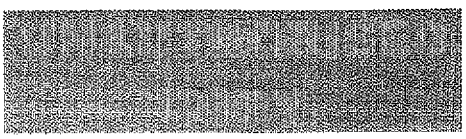
- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A mixture of 55 volumes of *acetonitrile* and 45 volumes of *water*
- flow rate 1.3 ml per minute,
- spectrophotometer set at 278 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of total gingerols by summing the peak areas of 6 gingerol, 8 gingerol, 10 gingerol and 6 shogaol. The relative retention time of 6 gingerol is 1.0, 8 gingerol is about 2.1, 10 gingerol is about 5.0 and 6 shogaol is about 2.6. The area of the peak of 6 gingerol in the sample chromatogram is more than the area of 6 shogaol indicating the content of 6 gingerol is more than the content of 6 shogaol.

Storage. Store protected from heat and moisture.



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Sweet Orange Oil

Narangi ka tail

Sweet orange oil is cold pressed oil obtained by mechanical treatment of the fresh peel of the fruit of *Citrus sinensis* Linn. Osbeck; *Citrus aurantium* Linn. var. dulcis (Fam. Rutaceae).

Sweet Orange oil contains not less than 0.4 per cent and not more than 0.6 per cent of α-pinene, not less than 0.02 per cent and not more than 0.3 per cent of β-pinene, not less 0.2 per cent and not more than 1.1 per cent of sabinene, not less 1.7 per cent and not more than 2.5 per cent of β-myrcene, not less than 92.0 per cent and not more than 97.0 per cent of limonene, not less than 0.1 per cent and not more than 0.4 per cent of octanal, not less than 0.1 per cent and not more than 0.4 per cent of decanal, not less than 0.02 per cent and not more than 0.1 per cent of neral, not less than 0.03 per cent and not more than 0.2 per cent of geranial, not less than 0.2 per cent and not more than 0.7 per cent of linalool, not less than 0.02 per cent and not more than 0.5 per cent of valencene,

Category. Antiseptic, antispasmodic, carminative.

Description. A clear, pale yellow or orange, mobile liquid, which may become cloudy when chilled. It has a characteristic odour of fresh orange peel.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *Sweet orange oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). 46° to 50°.

Tests

Relative density (2.4.29). 0.839 to 0.847.

Refractive index (2.4.27). 1.468 to 1.474.

Optical rotation (2.4.22). +94° to + 99°.

Peroxide Value (2.3.35). Maximum 20.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution(a). A 2.0 per cent w/v solution of *α-pinene IPRS* in *ethanol (95 per cent)*.

Reference solution (b). A 2.0 per cent w/v solution of *β- pinene IPRS* in *ethanol (95 per cent)*.

Reference solution (c). A 2.0 per cent w/v solution of *sabinene IPRS* in *ethanol (95 per cent)*.

Reference solution (d). A 2.0 per cent w/v solution of *limonene IPRS* in *ethanol (95 per cent)*.

Reference solution (e). A 2.0 per cent w/v solution of *octanal IPRS* in *ethanol (95 per cent)*.

Reference solution (f). A 2.0 per cent w/v solution of *decanal IPRS* in *ethanol (95 per cent)*.

Reference solution (g). A 2.0 per cent w/v solution of *neral IPRS* in *ethanol (95 per cent)*.

Reference solution (h). A 2.0 per cent w/v solution of *geranial IPRS* in *ethanol (95 per cent)*.

Reference solution (i). A 2.0 per cent w/v solution of *linalool IPRS* in *ethanol (95 per cent)*.

Reference solution (j). A 2.0 per cent w/v solution of *valencene IPRS* in *ethanol (95 per cent)*.

Reference solution (k). A 2.0 per cent w/v solution of *β-myrcene IPRS* in *ethanol (95 per cent)*.

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 60° for 1 minute, increase from 60° to 230° at a rate of 4° per minute, and maintain at this temperature for 10 minutes,
- inlet port at 230° and detector at 240°,
- detection by flame ionization detector,
- flow rate: 1 ml per minute, using helium or nitrogen as the carrier gas,
- injection volume: 0.5 µl,
- split ratio: 1: 200.

Inject reference solutions (a), (b), (c), (d), (e), (f), (g), (h), (i), (j), (k) and the test solution.

Calculate the content of α-pinene, β-pinene, limonene, sabinene, β-myrcene, octanal, decanal, neral, geranial, linalool and valencene in the oil using area normalization procedure.

Storage. Store protect from light and moisture.

Tea Tree Oil

Tea Tree oil is obtained by steam distillation of foliage and terminal branch of *Melaleuca alternifolia* (Maiden and Betch) Cheel, *M. linariifolia Smith, M dissitiflora* F. Mueller (Fam. Myrtaceae).

Tea Tree oil contains not less than 1.0 per cent and not more than 6.0 per cent of α-pinene, not less than 3.5 per cent of sabinene, not less than 5.0 per cent and not more than 13.0 per cent of α- terpinene, not less than 0.5 per cent and not more than 4.0 per cent of limonene, not less than 15.0 per cent of cineole, not less than 10.0 per cent and not more than 28.0 per cent of γ-terpinene, not less than 0.5 per cent and not more than 12.0 per cent of p-cymene, not less than 1.5 per cent and not more than 5.0 per cent of terpinolene, not less than 30.0 per cent of terpene-4-ol, not more than 7.0 per cent of aromadendrene, not less than 1.5 per cent and not more than 8.0 per cent of α-terpeniol.

Category. Antiacne, antiseptic and anti-fungal.

Description. A clear, colourless or pale yellow liquid with fresh, spicy-earthy odour.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *hexane*.

Reference solution. A 2.0 per cent w/v solution of *tea tree oil IPRS* in *hexane*.

Use chromatographic system described in the Assay.

The peaks in the chromatogram obtained with the test solution correspond to the peaks in the chromatogram obtained with the reference solution.

B. Flash point (2.4.44). Not less than 60°.

Tests

Relative density (2.4.29). 0.885 to 0.906 at 25°.

Refractive index (2.4.27). 1.475 to 1.482.

Optical rotation (2.4.22). +5° to +15°.

Assay. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *hexane*.

Reference solution. Dissolve 5 µl of *α-pinene IPRS*, 5 µl of *sabinene IPRS*, 15 µl of *α-terpinene IPRS*, 5 µl of *limonene IPRS*, 5 µl of *cineole IPRS*, 30 µl of *γ-terpinene IPRS*, 5 µl of *p-cymene IPRS*, 5 µl of *terpinolene IPRS*, 60 µl of *terpinen-4-ol IPRS*, 5 µl of *aromadendrene IPRS* and 5 mg of *α-terpineol IPRS* in 10 ml of *hexane*.

Chromatographic system

- a capillary column 30 m x 0.25 mm, coated with *macrogol 20000*,
- temperature:
  column. 50° for 1 minute, increase from 50° to 230° at a rate of 5° per minute and maintain at this temperature for 8 minutes,
- inlet port at 240° and defector at 240°,
- detection by flame ionization detector,
- flow rate: 1.3 ml per minute, using helium or nitrogen as the carrier gas,
- injection volume: 1.0 µl.
- split ratio: 1: 50

Inject the reference solution and the test solution.

Calculate the content of α-pinene, sabinene, α-terpinene, limonene, cineole, γ-terpinene, p-cymene, terpinolene, terpinen-4-ol, aromadendrene and α-terpineol in the oil using area normalization procedure.

Storage. Store protected from light and moisture.

Thyme Oil

Ajwain ka tail

Thyme Oil is the essential oil obtained by steam distillation of the flowering stems of *Thymus zygis* (Loefl.) Linn. (Fam. Lamiaceae).

Thyme Oil (Indian variety) contains not less than 2 per cent and not more than 10 per cent of borneol; and not less than 15 per cent and not more than 40 per cent of α-pinene.

Description. A clear, pale yellow to amber yellow liquid, free from sediment, suspended matter and characteristic spicy, herbal and anise-like odor.

Identification

A. Determine by gas chromatography (2.4.13).

Test solution. A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution. A 2.0 per cent w/v solution of *thyme Oil IPRS* in *ethanol (95 per cent)*.

Use chromatographic system described under Assay.

The peaks in the chromatogram obtained with the test solution corresponds to the peaks in the chromatogram obtained with the reference solution.

B. Flash Point (2.4.44). 61.0° to 65.0°.

Tests

Relative density (2.4.29). 0.912 to 0.935.

Refractive index (2.4.27). 1.495 to 1.505.

Assay. Determine by gas chromatography (2.4.13).

Test solution (a). A 2.0 per cent w/v solution of the oil under examination in *ethanol (95 per cent)*.

Reference solution (a). A 2.0 per cent w/v solution of *Borneol IPRS* in *ethanol (95 per cent)*.

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Reference solution (b). A 2.0 per cent w/v solution of *α-pinene IPRS* in *ethanol (95 per cent)*.

Chromatographic system
- a capillary column 30 m x 0.25 mm, coated with methyl 5 per cent phenylpolysiloxane,
- temperature:
  column. 50° for 1 minute, increase from 50° to 220° at a rate of 10° per minute and maintain at this temperature for 13 minutes,
- inlet port at 250° and detector at 280°,
- detection by flame ionization detector,
- flow rate 1 ml per minute, using nitrogen as the carrier gas,
- injection volume: 1.0 µl,
- split ratio: 1 : 25.

Inject reference solutions (a), (b) and the test solution.

Calculate the contents of borneol, and α-pinene in the oil using area normalisation procedure.

Storage. Store protected from light and moisture.

Tolu Balsam

Balsam of Tolu

Tolu Balsam is a solid or semi-solid, balsam obtained by incision from the trunk of *Myroxylon balsamum* (Linn.) Harms (Fam. Leguminosae).

Tolu Balsam contains not less than 35.0 per cent and not more than 50.0 per cent of total balsamic acids, calculated as cinnamic acid, $C_9H_8O_2$, on the dry, ethanol-soluble matter.

Category. Expectorant, antiseptic.

Description. A soft, tenacious, brownish yellow or brown mass, when first collected; subsequently, becoming harder and finally brittle. Transparent in thin films; odour, aromatic and vanilla-like. Warmed and pressed between pieces of glass and examined with a lens, it exhibits crystals of cinnamic acid.

Identification

A. To a solution in *ethanol (90 per cent)* add *ferric chloride test solution*; a green colour is produced.

B. To 1 g add to 5 ml of water, heat to boiling, filter, add 30 mg of *potassium permanganate* and continue heating; the odour of benzaldehyde is produced.

Tests

Acidity. A solution in *ethanol (90 per cent)* is acidic to *litmus solution*.

Acid value (2.3.23). 97 to 160, calculated on the dry, ethanol-soluble matter, determined by the following method. Dissolve 5.0 g in 50 ml of boiling *ethanol (90 per cent)*, add 3 ml of *phenolphthalein solution* and titrate the hot solution with *1 M ethanolic potassium hydroxide*. When the colour becomes dark brown, attach to a reflux condenser, boil for a few minutes to break up the precipitate and complete the titration.

Ethanol-insoluble matter. Not more than 5 per cent, determined by the following method. Digest 2.5 g with 50 ml of *ethanol (90 per cent)*, filter through a sintered glass crucible, transfer the residue to the filter crucible with the aid of more *ethanol (90 per cent)*, wash with hot *ethanol (90 per cent)* until all soluble matter is removed and dry to constant weight at 100°.

Colophony. Add 5 g to 25 ml of *carbon disulphide*, warm gently on a water-bath under a reflux condenser, filter, evaporate the solution to dryness, dissolve the residue in 6 ml of *light petroleum (40° to 60°)* and shake with 10 ml of a 0.5 per cent w/v solution of *cupric acetate*; the light petroleum layer is not coloured green.

Ester value (2.3.26). 47 to 95, calculated on the dry, ethanol-soluble basis.

Saponification value (2.3.37). 170 to 230, calculated on the dry, ethanol-soluble basis.

Loss on drying (2.4.19). Not more than 4.0 per cent, determined on 2.0 g by drying in a thin layer in an oven at 60° over *phosphorus pentoxide* at a pressure not exceeding 2.7 kPa.

Assay. Weigh accurately 2.0 g and boil with 25 ml of *dilute ethanolic potassium hydroxide solution* under a reflux condenser for 1 hour. Remove the ethanol and digest the residue with 50 ml of hot *water* until diffused. Cool the liquid, add 150 ml of *water* and 1.5 g of *magnesium sulphate* dissolved in 50 ml of *water*. Mix thoroughly and set aside for 10 minutes. Filter, wash the residue on the filter with 20 ml of water, acidify the combined filtrate and washings with *hydrochloric acid* and extract with successive quantities of 50, 40, 30, 30 and 30 ml of *ether*. Combine the ether extracts and discard the aqueous portion. Extract with successive quantities of 20, 20, 10, 10 and 10 ml of *sodium bicarbonate solution*, washing each aqueous extract with the same 20 ml of *ether*. Discard the ether layers, acidify the combined aqueous extracts with *hydrochloric acid* and extract with successive quantities of 30, 20, 20 and 10 ml of *chloroform*, filtering each chloroform extract through a plug of cotton wool on which a layer of *anhydrous sodium sulphate* is placed. Evaporate the chloroform on a water-bath until about 10 ml remains and remove the remainder in a current of air stopping immediately when the last trace of solvent is removed. Dissolve the residue by warming with 10 ml of *ethanol (95 per cent)*, previously neutralised to *phenol red solution*, cool and titrate

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with *0.1 M sodium hydroxide* using *phenol red solution* as indicator.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.01482 g of total balsamic acids, calculated as cinnamic acid, $C_9H_8O_2$.

Storage. Store protected from light and moisture. Avoid exposure to excessive heat.

Tragacanth

Tragacanth is the air-hardened gummy exudate, flowing naturally or obtained by incision, from the trunk and branches of *Astragalus gummifer* Labill. and certain other species of *Astragalus* (Fam. Fabaceae).

Category. Pharmaceutical aid.

Description. Pale yellow, thin, flattened ribbons or brittle pieces; odourless and almost tasteless. On the addition of about 10 times its weight of *water,* it forms a mucilaginous gel.

It has the macroscopic and microscopic characteristics described under Identification tests A and B.

Identification

A. *Macroscopic* — Occurs as thin, flattened pieces, 30 mm long, 10 mm wide and up to 1 mm in thickness, more or less curved; marked on the surface by fine longitudinal striae and concentric transverse ridges; white, translucent, horny; fracture, short. May also be in the form of thicker, less brittle pieces, white to pale yellow and more opaque.

B. *Microscopic* — Reduce to powder. Examine under a microscope; the powder shows in the gummy mass numerous stratified cellular membranes which turn violet on the addition of *iodinated zinc chloride solution*. The mass includes starch granules, isolated or in small clusters, rounded or occasionally deformed, diameter 4 to 10 µm, and up to 20 µm, with a central hilum, visible in polarised light.

C. Moisten 0.5 g of the powdered material with 1 ml of *ethanol (95 per cent)* and add gradually, while shaking, 50 ml of *water* until a homogeneous mucilage is obtained. To 5 ml of the mucilage add 5 ml of *water* and 2 ml of *barium hydroxide solution*. A slightly flocculent precipitate is formed which, when heated for 10 minutes on a water-bath, gives an intense yellow colour.

D. Add 4 ml of a 0.5 per cent w/v dispersion in *water* to 0.5 ml of *hydrochloric acid* and heat on a water-bath for 30 minutes. To one half of the resulting liquid add 1.5 ml of *sodium hydroxide solution* and 3 ml of *alkaline cupric-tartrate solution* and heat on a water-bath; a reddish brown precipitate is formed. To the other half of the liquid, add a few drops of *barium chloride solution*; no precipitate is formed (freedom from agar).

Tests

Acacia and other soluble gums. To 20 ml of a 2.5 per cent w/v suspension of the powdered material prepared with freshly boiled *water* add 10 ml of *lead acetate solution*; a flocculent precipitate is formed. Filter and add to the filtrate 10 ml of *lead subacetate solution*; a slight cloudiness may appear, but there is no precipitate.

Karaya gum. Boil 1 g with 20 ml of *water* until a mucilage is formed, add 5 ml of *hydrochloric acid* and again boil for 5 minutes; no pink or red colour develops.

Sterculia. A. Shake 0.2 g of the powdered material with 10 ml of *ethanol (60 per cent)* in a 10-ml stoppered cylinder; any gel formed occupies not more than 1.5 ml.

B. Shake 1 g of the powdered material with 100 ml of *water* and titrate with *0.01 M sodium hydroxide*, using *methyl red solution* as indicator. Not more than 5.0 ml is required to change the colour of the solution.

Foreign matter. Not more than 1.0 per cent, determined by the following method. To 2.0 g of the powdered material in a 250.0 ml round-bottomed flask add 95 ml of *methanol,* swirl to moisten the powder and add 60 ml of *7 M hydrochloric acid*. Add a few glass beads and heat under a reflux condenser in a water-bath for 3 hours, shaking occasionally. Remove the glass beads and filter the hot suspension under reduced pressure through a sintered-glass crucible (porosity No. 1). Rinse the flask with a small quantity of *water,* passing the rinsings through the filter. Wash the residue on the filter with about 40 ml of *methanol* and dry to constant weight at 110°.

Arsenic (2.3.10). Mix 3.3 g with 3 g of *anhydrous sodium carbonate*, add 10 ml of *bromine solution* and mix thoroughly. Evaporate to dryness on a water-bath, gently ignite, and dissolve the cooled residue in 16 ml of *brominated hydrochloric acid AsT* and 45 ml of *water*. Remove the excess of bromine with 2 ml of *stannous chloride AsT*. The resulting solution complies with the limit test for arsenic (3 ppm).

Heavy metals (2.3.13). 0.5 g complies with the limit test for heavy metals, Method B (20 ppm).

Total ash (2.3.19). Not more than 4.0 per cent, determined on 1.0 g.

Microbial contamination (2.2.9). 1 g is free from *Escherichia coli* and 10 g is free from *Salmonella*.

Storage. Store protected from moisture.

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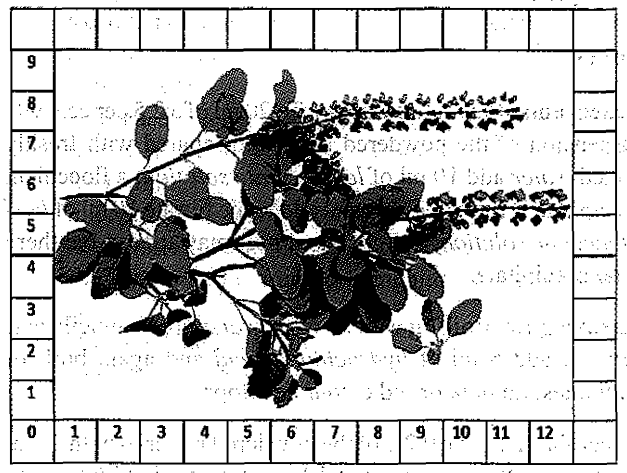
Tulasi

Basil; *Ocimum sanctum*



Tulasi consists of leaves of *Ocimum sanctum* Linn (Fam. Lamiaceae).

Tulasi contains not less than 0.40 per cent w/w of eugenol, calculated on the dried basis.

Category. Antiasthmatic, immunomodulator, expectorant, swasa.

Description. Greyish-black in colour having characteristic odour with slightly pungent and aromatic taste.

Identification

A. *Macroscopic* — Leaves simple, elliptic, 2.7-7.5 cm long, 1-3 cm wide, with acute top, cuneate, obtuse to rounded base, margin entire, undulate or serrate, both surfaces thinly pubescent and dotted; petiole 0.2-3.0 cm long. Flowers are 5-7 mm in length. It has both male and female parts. Calyx: There are 5 sepals and it is greenish in colour. Corolla: There are 5 petals, bilabiate in shape and covered with scattered hairs. Petals whitish-purple.

B. *Microscopic* — Transverse section of leaf shows a pot shaped midrib. Upper epidermis consists of a layer of small, quadrangular transparent cells with thin walls and thin smooth cuticle. On tangential view, these cells are polygonal with straight or wavy walls. Lower epidermis consists of a layer of small, quadrangular transparent cells with thin walls and thin and thin smooth cuticle. Trichomes bent, consisting of 2-6 cells; glandular trichomes short, lamiaceae type, consisting of one stock cell and 2-4 cells with rounded heads. Palisade parenchyma consists of layer of long cylindrical cells contain[illegible] chlorophyll; spongy parenchyma consists of [illegible]ls with thin, straight or slightly wavy side walls. [illegible]es collateral type. Stomata diacytic.

[illegible] thin-layer chromatography (2.4.17), coating [illegible]*ca gel GF254*.

Mobile phase. A mixture of 97 volumes of *toluene* and 3 volumes of *ethyl acetate*.

Test solution. Reflux 2 g of the coarsely powdered substance under examination with 25 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 × 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 10 ml.

Reference solution. Reflux 1 g of *tulasi IPBRS* with 25 ml of *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 × 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat the plate 110° for 10 minutes and examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 3.0 per cent.

Water-soluble extractive (2.6.3). Not less than 10.0 per cent by Method I.

Total ash (2.3.19). Not more than 15.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 5.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by gas chromatography (2.4.13).

Test solution. Reflux 0.5 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water-bath for 15 minutes, cool and filter. Reflux the residue further with *methanol* till the extract turns colourless, cool and filter. Combine all the filtrates and concentrate to a volume slightly less than 100 ml. Dilute to 100.0 ml with *methanol*.

Reference solution. A 0.004 per cent w/v solution of *eugenol IPRS* in *methanol*.

Chromatographic system

- a capillary column 30 m x 0.25 x 0.25 mm coated with 100 per cent dimethylpolysiloxane,
- temperature:
  oven 60° to 260° @10° per minute, (Initially and finally hold for 5 minutes respectively),

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– inlet port at 240° and detector at 280°,
– flow rate: 0.8 ml per minute,
– split flow 20 ml per minute.

Inject 1 µl of the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of eugenol.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Tulasi Dry Extract

Tulasi Dry Extract is obtained from the Tulasi leaves (*Ocimum sanctum* Linn, Fam. Laminaceae) by extraction with *methanol* or other any suitable solvent and evaporation of solvent.

Tulasi Dry Extract contains not less than 90.0 per cent w/w and not more than 120.0 per cent w/w of the stated amount of the ursolic acid, calculated on the dried basis. It may contain suitable added substances.

Category. Antiasthmatic, immunomodulator, expectorant, swasa.

Usual strengths. 0.1 per cent w/w; 0.2 per cent w/w.

Description. A pale green powder.

Identification

A. Determine by thin layer chromatography (2.4.17) coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 95 volumes of *chloroform* and 5 volumes of *methanol.*

Test solution. Shakewell 0.2 g of the extract under examination with 10.0 ml *methanol,* filter.

Reference solution. A 0.02 per cent w/v solution of *ursolic acid IPRS* in the *methanol.*

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and spray with *anisaldehyde-sulphuric acid reagent.* Heat the plate at 110° for 10 minutes and examine under ultraviolet light at 366 nm and in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Ethanol-soluble extractive (2.6.2). Not less than 15.0 per cent.

Water-soluble extractive (2.6.3). Not less than 30.0 per cent by Method I.

Total ash (2.3.19). Not more than 10.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 6.0 per cent determined on 1 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Shakewell about 0.5 g of the extract under examination in 100 ml of the *methanol.*

Reference solution. A 0.03 per cent w/v solution of *ursolic acid IPRS* in the *methanol.*

Chromatographic system
– a stainless steel column, 25 cm x 0.46 mm packed with octadecylsilane bonded to porous (0.5 µm),
– mobile phase: a mixture of 30 volumes of *methanol* and 70 volumes of *acetonitrile,*
– flow rate: 0.6 ml per minute,
– spectrophotometer set at 210 nm,
– injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

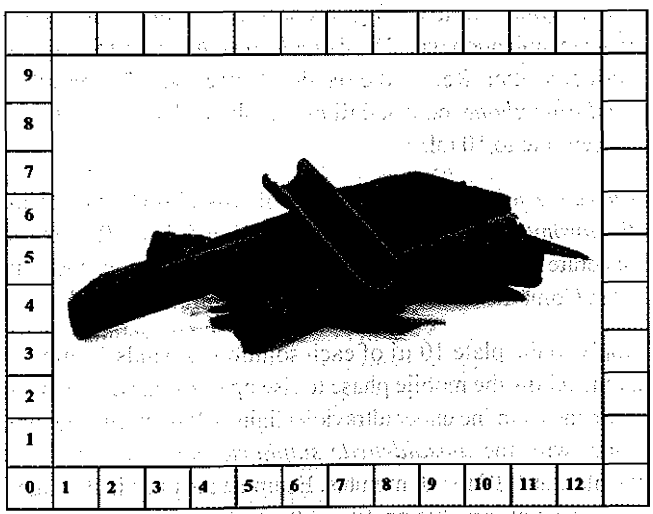
Inject the reference solution and the test solution.

Calculate the content of the ursolic acid in extract.

Storage. Store in a container purged with Nitrogen under the refrigerated condition, protected from heat and moisture.

Tvak

Cinnamomum zeylanicum



Tvak consists of dried inner bark of stem of *Cinnamomum zeylanicum* Blume. (Fam. Lauraceae).

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Tvak contains not less than 1.0 per cent w/w of Cinnamaldehyde, calculated on the dried basis.

Category. Carminative, stimulant, anti-helminthic, antidote, cardioprotective.

Description. The dried stem bark occurs in pieces of variable size and thickness along with rough surface. It is extremely brown in colour with aromatic odour.

Identification

A. *Macroscopic* — Bark pieces about 0.5 mm thick, brittle, occurs as single or double, closely packed compound quills, upto a meter or more in length and upto 1 cm in diameter, outer surface, dull yellowish-brown marked with pale wavy longitudinal lines with occasional small scars or holes, inner surface darker in color, striated with longitudinally elongated reticulation, fracture, splintery, free from all but traces of cork, odour, fragrant, taste, sweet, aromatic with sensation of warmth.

B. *Microscopic*— Transverse section of bark shows except at certain places pericyclic sclerenchyma, 3 or 4 rows or isodiametric cells, sometimes tangentially elongated, some containing starch grains, small group of pericyclic fibres embedded at intervals in the sclerenchyma, phloem of tangential bands of sieve tissue alternating with parenchyma, and containing axially elongated secreting cells containing volatile oil or mucilage.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254.*

Mobile phase. A mixture of 15 volumes of *toluene*, 4.5 volumes of *ethyl acetate* and 1.5 volume of *formic acid.*

Test solution. Reflux 5.0 g of coarsely powdered substance under examination with 25 ml *dichloromethane* for 15 minutes, Cool and filter. Reflux the residue further with 2 x 50 ml of *dichloromethane,* cool and filter. Combine all the filtrates and concentrate to 10 ml.

Reference solution. Reflux 2.0 g of *tvak IPBRS* with 25 ml *dichloromethane* for 15 minutes. Cool and filter. Reflux the macerate further with 2 x 50 ml of *dichloromethane*, cool and filter. Combine all the filtrates and concentrate to 10 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise up to 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, Spray with the *anisaldehyde sulphuric acid reagent.* Heat the plate at 110° for 10 minutes. Examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign Organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 2.0 per cent.

Water-soluble extractive (2.6.3). Not less than 3.0 per cent by Method I.

Total ash (2.3.19). Not more than 6.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by gas chromatography (2.4.14).

Test solution. Weigh about 5.0 g finely powdered of the substance under examination and transfer to a 100 ml beaker. Add 20 ml of *n-hexane*, sonicate for 10 minutes and stir for 30 minutes on magnetic stirrer. Repeat the process one more time and collect all the filtered extracts into a 50 ml volumetric flask and make final volume up to 50 ml with *n-hexane.*

Reference solution. A 0.5 per cent (w/v) solution of *Cinnamaldehyde IPRS* in *n-hexane.*

Chromatographic system

- a capillary column 60 m x 0.25 mm, coated with *macrogel 20000*,
- temperature:
  column: 100° for 0.5 minute, then increased to 180° at a rate of 10° per minute and maintaine at this temperature for 125 minutes,
- inlet port at 200° and detector at 240°,
- flow rate: 1.8 ml per minute, using helium as carrier gas,
- injection volume: 0.2µl,
- split ratio: 1:100.

Inject the reference solution. The test is not valid unless the relative standard deviation for the triplicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the cinnamaldehyde content in the sample using the ratios of the area of the peak corresponding to cinnamaldehyde to the area of the peak due to internal standard in the chromatogram obtained with test solution and the reference solution.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

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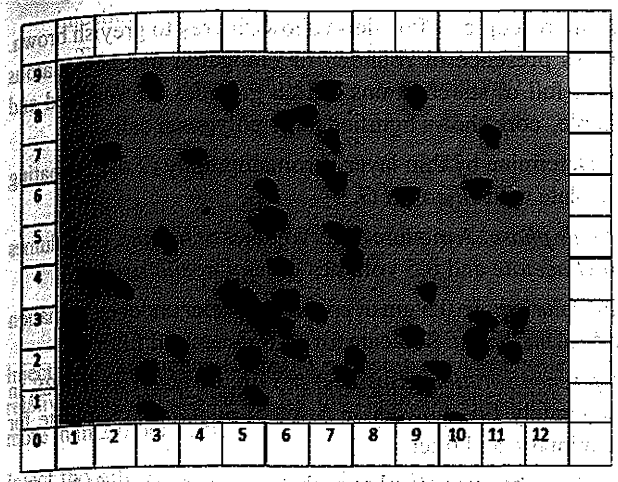
Upakunchika

Nigella sativa Linn.



Upakunchika consists of seeds of *Nigella sativa* Linn. (Fam. Ranunculaceae).

Upakunchika contains of not less than 0.20 per cent w/w of thymoquinone, calculated on the dried basis.

Category. Antidiarrheal, antitumor, anthelmintic.

Description. Seeds, flattened, oblong, angular, rugulose tubercular, small, funnel shaped, 0.2 cm long and 0.1 cm wide, black.

Identification

A. *Macroscopic* — Seeds, flattened, oblong, angular, rugulose tubercular, small, funnel shaped, 0.2 cm long and 0.1 cm wide, black, odour slightly aromatic, taste bitter.

B. *Microscopic* — Transverse section of seed shows single layer of epidermis consisting of elliptical, thick-walled cells covered externally by a papillose cuticle, filled with reddish-brown content, epidermis followed by 2-4 layers of thick-walled, tangentially elongated, parenchymatous cells, followed by a pigmented layer composed of tangentially elongated, cylindrical thick-walled cells filled with reddish-brown pigment, below pigmented layer, parenchyma composed of thick-walled rectangular, radially elongated cells, present in a layer, endosperm consists of moderately thick-walled, rectangular to polygonal cells, a few filled with oil globules, embryo embedded in endosperm.

Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 25 volumes of *toluene*, 7.5 volumes of *ethyl acetate* and 2.5 volumes of *formic acid*.

Test solution. Reflux about 2 g of the coarsely powdered substance under examination, add 100 ml of *methanol*, reflux for 15 minutes, reflux the residue further with 50 ml *methanol* till the extract turns colourless, cool and filter. Combine all the filtrates and concentrate to 100.0 ml.

Reference solution. Reflux 1 g of powdered *upakunchika IPBRS* with 100 ml of *methanol* for 15 minutes, cool and filter, reflux the residue further for two times with 50 ml *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 25 ml.

Apply to the plate 10µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. Spray with the *anisaldehyde sulphuric acid reagent*. Heat the plate at 110° for 10 minutes. Examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 20.0 per cent.

Water-soluble extractive (2.6.3). Not less than 15.0 per cent by Method I.

Total ash (2.3.19). Not more than 6.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.2 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Water (2.3.43). Not more than 10.0 per cent, determined on 0.2 g.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Extract 2 g of the substance under examination (after pulverization of seed) with 25 ml *methanol* by sonication for 15 minutes followed by warming on water-bath for another 15 minutes, cool and filter. Further extract the marc further twice with 25 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate under vacuum to 50.0 ml.

Reference solution. A 0.01 per cent w/v solution of *thymoquinone IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. 0.5 per cent v/v solution of *ortho-phosphoric acid*,
  B. *acetonitrile*,
- a linear gradient programme using the conditions given below,
- flow rate: 1.5 ml per minute,

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– spectrophotometer set at 254 nm,
– injection volume: 10 µl

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	50	50
15	100	0
20	100	0

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

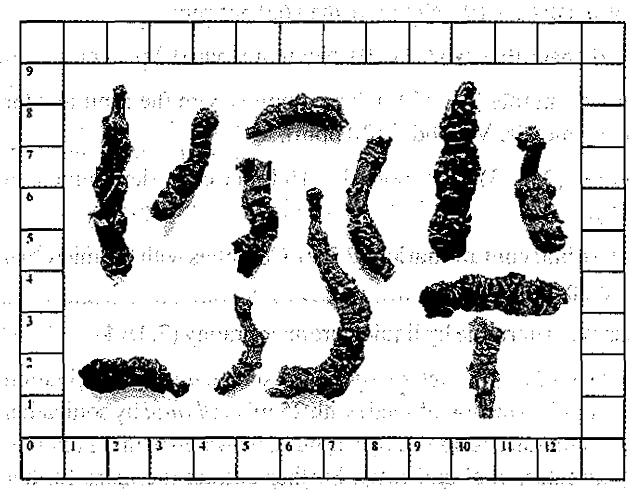
Inject the reference solution and the test solution.

Calculate the content of thymoquinone.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Valerian Root

Valeriana officinalis Root



Valerian consists of dried whole or cut underground parts of *Valeriana officinalis* L or *Valeriana wallichi DC* or a mixture of both including the rhizome, roots and stolons.

Valerian contains not less than 0.02 per cent of sesquiterpenic acid w/w expressed as valerenic acid ($C_{15}H_{22}O_2$) calculated on dried basis.

Description. Yellowish to brownish grey coloured rhizomes with few thick roots with characteristic odour.

Identification

A. Macroscopic — Rhizome is subcylindrical, dorsiventrally flattened slightly curved, unbranched apex showing cup shaped scars. Roots are cylindrical and of the same colour as the rhizome. Fracture is short. The stolons show prominent nodes separated by longitudinally striated internodes.

B. Microscopic — Powder-Yellowish grey to greyish brown, shows presence of rounded or elongated parenchymatous cells, full of starch grains, starch granules are rounded and simple, cork shows lignified large polygonal cells.

C. Determine by thin layer chromatography (2.4.17) coating the plate with *silica gel G.*

Mobile phase. A mixture of 65 volumes of *hexane*, 35 volumes *ethyl acetate* and 0.5 volumes *glacial acetic acid*.

Test solution. To 0.5 gm of coarsely powder under examination in 5 ml *methanol*. Sonicate for 10 minutes and filter.

Reference solution. Suspend 0.5 gm of *valerian IPBRS*, in moderately fine powder in 5 ml of *methanol*. Sonicate for 10 minutes. and filter.

Apply to the plate 10 ml of each solution as band of 10 mm by 2 mm. Allow the mobile phase to rise to 12 cm. Dry the plate in air and spray with *anisaldehyde reagent solution* prepared by mixing 0.5 ml of *anis aldehyde* 10 ml of *glacial acetic acid*, 85 ml of *methanol* and 5 ml of *sulphuric acid*, added in the specified sequence. Heat the plate at 105° for 10 minutes. and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1) Not more than 2 per cent.

Loss on drying (2.4.19). Not more than 12.0 per cent determined on 1 gm dried at 105° to constant weight.

Total ash (2.3.19). Not more than 12.0 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux, 1.5 gm of coarsely powder substance drug in 20 ml of *methanol* for 30 minutes. Reflux the filter residue further two times with 20 ml *methanol*, cool and filter. Combine the filtrates and dilute to 50 ml with *methanol*, rinsing the round bottomed flask and the filter.

Reference solution. Dissolve Valerian standardized dry extract *IPRS* equivalent to 1.0 mg of Valerinic acid in *methanol* and dilute to 10.0 ml with same solvent.

Chromatographic system
– a stainless steel column 25 cm x 4.6 mm packed with *octadecylsilane* bonded to porous silica (5 mm),
– mobile phase. A. a mixture of 20 volumes of *acetonitrile* and 80 volumes of 5.0 w/v solution of *phosphoric acid*,

B. a mixture of 80 volumes of 5.0 w/v solution of *phosphoric acid* and 20 volumes of *acetonitrile*,

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– a gradient programme using the conditions given below,
– flow rate: 1.5 ml per minute,
– spectrophotometer set at 220 nm,
– injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	55	45
5	55	45
18	20	80
22	20	80
23	55	45
35	55	45

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the percentage content of sesquiterpenic acids, expressed as valerenic acid, using the following expression.

$$\frac{(A_1 + A_2) \times M_2 \times P \times 5}{A_3 \times M_1}$$

Where, A_1 = area of the peak due to acetoxyvalerenic acid in the chromatogram obtained with the test solution.

A_2 = area of the peak due to valerenic acid in the chromatogram obtained with the test solution.

A_3 = area of the peak due to valerenic acid in the chromatogram obtained with the reference solution.

M_1 = mass of the drug to be examined, in grams;

M_2 = mass of valerian standardized dry extract *IPRS* used to prepare the reference solution, in gram

P = percentage content of valerenic acid in valerian standardized dry extract *IPRS*

Storage. Store protected from light, heat and moisture and against attack by insects and rodents.

Valerian Dry Extract

Valerian Dry Extract is produced from dried root & rhizomes of valeriana officinalis L or valeriana wallichi DC or a mixture of both by extraction with a suitable solvent using an appropriate procedure.

Valerian Dry Extract contains 90.0 to 110.0 per cent of the labeled amount of sesquiterpenic acid expressed as valerenic acid ($C_{15}H_{22}O_2$), on dried basis.

Usual strength. 0.02 per cent and 0.25 per cent.

Description. Brown hygroscopic powder, with characteristic odour.

Identification

Determine by thin layer chromatograph (2.4.17), coating the plate with silica gel G.

Mobile phase. A mixture of 65 volumes of *hexane*, 35 volumes ethyl *acetate* and 0.5 volumes *glacial acetic acid*.

Test solution. Dissolve 0.5 g of the substance under examination in 5 ml of *methanol*. Sonicate for 10 minutes and filter.

Reference solution. Dissolve 0.5 g of *valerian IPBRS* in 5 ml of *methanol*. Sonicate for 10 minutes and filter.

Apply to the plate 10 ml of each solution as band of 10 mm by 2 mm. Allow the mobile phase to rise to 12 cm. Dry the plate in air and spray with *anisaldehyde* reagent solution prepared by mixing 0.5 ml of *anisaldehyde*, 10 ml of *glacial acetic acid*, 85 ml of *methanol* and 5 ml of *sulphuric acid* added in the specified sequence. Heat the plate at 105° for 10 minutes. and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Loss on drying (2.4.19). Not more than 5.0 per cent determined on 1 g of the dry extract dried at 105° to constant weight.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weight 1.0 g of the substance under examination, into a 50 ml volumetric flask. Add 40 ml of *methanol*, sonicate for 10 minutes, dilute to 50 ml with *methanol* and filter.

Reference solution. Dissolve valerian standardized dry extract *IPRS* equivalent to 1.0 mg of *valerenic acid* in *methanol* and dilute to 10.0 ml with the same solvent.

Chromatographic system

– a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 mm),
– mobile phase. A. a mixture of 20 volumes of *acetonitrile* and 80 volumes of 5.0 w/v solution of *phosphoric acid*,

B. a mixture of 80 volumes of 5.0 w/v solution of *phosphoric acid* and 20 volumes of *acetonitrile*,
– a gradient programme using the conditions given below,
– flow rate: 1.5 ml per minute,
– spectrophotometer set at 220 nm,
– injection volume: 20 µl.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	55	45
5	55	45
18	20	80
22	20	80
23	55	45
35	55	45

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the percentage content of sesquiterpenic acids, expressed as valerenic acid, using the following expression.

$$\frac{(A_1 + A_2) \times M_2 \times P \times 5}{A_3 \times M_1}$$

Where, A_1 = area of the peak due to acetoxyvalerenic acid in the chromatogram obtained with the test solution.

A_2 = area of the peak due to valerenic acid in the chromatogram obtained with the test solution.

A_3 = area of the peak due to valerenic acid in the chromatogram obtained with the reference solution.

M_1 = mass of the dried extract to be examined, in grams;

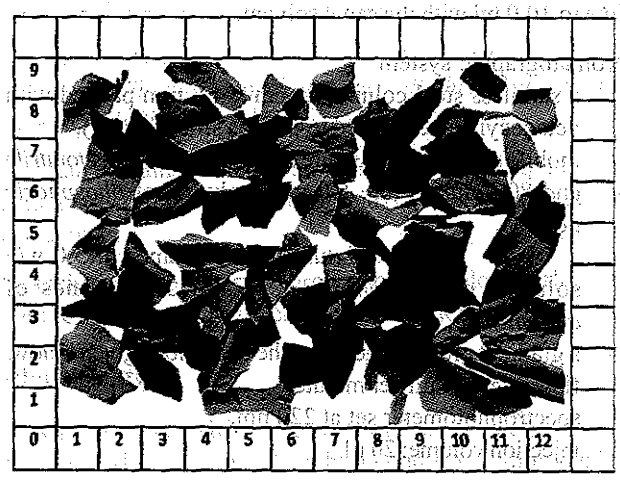
M_2 = mass of valerian standardized dry extract *IPRS* used to prepare the reference solution, in gram

P = percentage content of valerenic acid in valerian stand.ardized dry extract *IPRS*

Storage. Store in well closed containers protected from heat and moisture.

Vasaka

Adulasa; *Adhatoda vasica*



Vasaka consists of the dried mature leaves of *Adhatoda vasica* Nees. (Fam. Acanthaceae).

Vasaka contains not less than 0.6 per cent w/w of vasicine, calculated on the dried basis.

Category. Expectorant, bronchodilator, kustha.

Description. Taste, bitter.

Identification

A. *Macroscopic* — Leaf pieces membranous, brittle, greyish-brown, a few pieces green coloured. Floral bracts leaf-like.

B. *Microscopic* — Stomata diacytic, more on the lower epidermis; glandular and non-glandular hair on both surfaces of leaf; elongated cystoliths present in palisade cells.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 8 volumes of *ethyl acetate*, 2 volumes of *methanol* and 0.2 volume of *strong ammonia solution.*

Test solution. Reflux 1 g of coarsely powdered substance under examination with 50 ml *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 × 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate to 10 ml.

Reference solution. Reflux 0.5 g of *vasaka IPBRS* with 50 ml *methanol* for 15 minutes, cool and filter. Reflux the residue further with 2 × 50 ml of *methanol*, cool and filter. Combine all the filtrates and concentrate to 5 ml.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *Dragendorff's reagent*. Heat the plate at 100° for 5-10 minutes and examine in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 3.0 per cent.

Water-soluble extractive (2.6.3). Not less than 22 per cent by Method I.

Total ash (2.3.19). Not more than 21.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 12.0 per cent, determined on 5 g by drying in an oven at 105°.

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Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Reflux about 2 g of the coarsely powdered substance under examination with 50 ml of *methanol* on a water bath for 15 minutes, cool and filter. Reflux the residue further with *methanol* till the last extract turns colorless, cool and filter. Combine all the filtrates and concentrate to 100.0 ml.

Reference solution. Dissolve 25 mg of *vasicine hydrochloride IPRS* in 50 ml of *methanol*. Dilute 5.0 ml of this solution to 50.0 ml with *methanol*.

Chromatographic system

- a stainless steel column 25 cm × 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 3 volumes of the solution prepared by dissolving 1 g of *sodium hexanesulphonate* in 1000 ml of *water*, 1 volume of *acetonitrile* and 20 volumes of *glacial acetic acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 300 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of vasicine.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Vasaka Extract

Vasaka extract is obtained by extracting Vasaka (*Adhatoda vasica* Nees, Fam. Acanthaceae) dried matured leaves with ethanol or any other suitable solvent. The powdered extract may contain suitable excipients.

Vasaka extract contains not less than 90.0 per cent w/w and not more than 120.0 percent w/w of the stated amount of vasicine.

Category. Expectorant, bronchodilator, kustha.

Usual strengths. 1 per cent w/w; 1.5 per cent w/w.

Description. Light green to greenish brown powder; characteristic odour and bitter taste.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 8 volumes of *ethyl acetate*, 2 volumes of *methanol* and 1 volume of *strong ammonia*.

Test solution. Dissolve 1.0 g of extract under examination with 25 ml of *methanol* and filter.

Reference solution. A 0.1 per cent w/v solution of *vasicine hydrochloride IPRS* in *methanol*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. Spray the plate with *Dragendorff reagent*. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Loss of drying (2.4.19). Not more than 6.0 per cent, determined on 2.0 g when dried at 105° for 3 hours.

Total ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Microbial contamination (2.2.9). Complies with the microbial contamination test.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 1.5 g of the extract containing 20 mg of vasicine in 100 ml of *methanol* by gently heating, and filter.

Reference solution. A 0.025 per cent w/v solution of *vasicine hydrochloride IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 250 cm x 4.6 mm packed with silica bonded with cyanopropyl groups (5 µm),
- *mobile phase*: A mixture of 92 volumes of buffer solution prepared by dissolving 1.36 g of *potassium dihydrogen ortho phosphate* in 500 ml of *water* and 2 ml of *orthophosphoric acid*, dilute to 1000 ml with *water*, 5 volumes of *acetonitrile* and 3 volumes of *tetrahydrofuran*,
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 300 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the refernce solution and the test solution.

Calculate the content of vasicine.

Storage. Store protected from heat and moisture.

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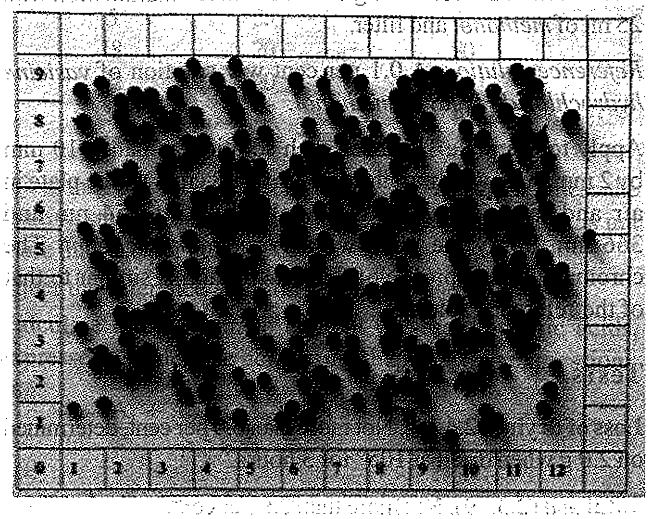
Vidanga

Bhabhiranga, Vayavidanga; *Embelia ribes*



Vidanga consists of dried mature fruits of *Embelia ribes* burm. f. (Fam. Myrsinaceae).

Vidanga contains not less than 2.0 per cent w/w of embelin, calculated on the dried basis.

Category. Vermicidal, antiallergic, anthelmintic, krmiroga.

Description. The fruit is brownish-black in colour with a wrinkled surface about 2 to 4 mm in diameter.

Identification

A. *Macroscopic* — Fruit brownish-black, globular, 2-4 mm in diameter,with anastomosing calyx surface, beak like projection of the remnants of the style at the apex and occasionally with remains of 2-3 lobes at the base; pericarp brittle enclosing a single or occasionally two seeded fruit, seed brownish black with longitudinal furrows . Embryo lies at the centre, just above the basal cavity of the seed.

B. *Microscopic* — Transverse section of fruit shows epicarp consisting of single row of tubular cells of epidermis, usually obliterated, in surface view cells rounded with wrinkled cuticle; mesocarp consists of a number of layers of reddish-brown coloured cells and numerous fibrovascular bundles and rarely a few prismatic crystals of calcium oxalate; inner part of mesocarp and endodermis composed of stone cells; endodermis consisting of single layered thick-walled, large, palisade like stone cells; seed coat composed of 2-3 layered reddish-brown coloured cells; endosperm cells irregular in shape, thick-walled, containing fixed oil and proteinous masses; embryo small when present otherwise most of the seeds sterile.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 5 volumes of *chloroform*, 4 volumes of *ethyl acetate*, and 1 volume of *formic acid*.

Test solution. Reflux 1 g of coarsely powdered substance under examination with 25 ml of *chloroform* for 30 minutes, cool and filter. Reflux the residue further with 25 ml of *chloroform*, cool and filter. Combine the filtrates and evaporate to dryness. Dissolve the residue in *methanol* and dilute to 10 ml with *methanol*. Dilute 2 ml of this solution and make up the volume to 10 ml with *methanol*.

Reference solution. Reflux 1 g of *vidanga IPBRS* with 25 ml of *chloroform* for 30 minutes, cool and filter. Reflux the residue further with 25 ml of *chloroform*, cool and filter. Combine the filtrates and evaporate to dryness. Dissolve the residue in 10 ml of *methanol*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 366 nm. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 2.0 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 8.5 per cent.

Water-soluble extractive (2.6.3). Not less than 8.5 per cent.

Total ash (2.3.19). Not more than 4.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 1.28 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss on drying (2.4.19). Not more than 7.8 per cent, determined on 5.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14)

Test solution. Reflux 50.0 g of the coarsely powdered substance under examination with 250 ml of *chloroform* at 70° for 60 minutes, cool and filter. The filtered extract was concentrated under reduced pressure to 5 ml.

Reference solution. A 0.05 per cent w/v solution of *embelin IPRS* in *methanol*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 88 volumes of *methanol* and 12 volumes of 0.1 per cent *TFA solution* in 1000 ml *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 288 nm,

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injection volume: 10 µl.

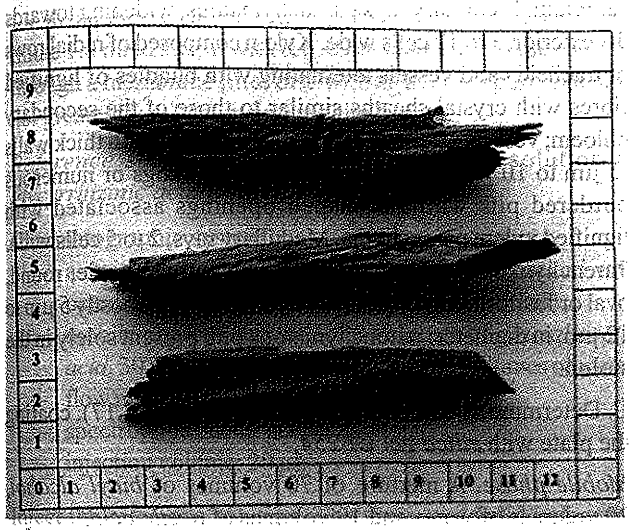
Inject the reference solution and the test solution.

Calculate the content of embelin.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Vijayasara

Asana; *Pterocarpus marsupium*



Vijayasara consists of heart-wood of *Pterocarpus marsupium* Roxb. (Fam; Fabaceae).

Vijayasara contains not less than 0.30 per cent w/w of pterostilbene, calculated on the dried basis.

Category. Antirheumatic, antidiabetic.

Description. Irregular pieces of varying thickness and length of heart-wood, golden-brown in colour. Brittle and difficult to break; taste astringent.

Identification

A. *Macroscopic* — Drug occurs as irregular pieces of variable size and thickness, golden yellowish-brown with darker streaks; on soaking in water gives yellow colour solution with blue fluorescence strong, tough, very hard, moderately heavy, difficult to break but brittle; taste, astringent.

B. *Microscopic* — Transverse section shows alternating bands of smaller polygonal cells consisting of tracheids, fibre tracheids, xylem parenchyma and transverse by xylem rays; numerous xylem vessels distributed throughout, in singles or in groups of 2-3, showing tyloses filled with tannin; in isolated preparations, vessels, drum or barrel shaped with well-marked perforation rims and bordered pits; tracheids numerous, long, thick-walled with tapering ends and simple pits; fibre tracheids elongated, thick walled with narrow lumen and simple pit, paratracheal, surrounding vessels; xylem rays uni-to-biseriate, 3-5-7 cells high; prismatic crystals of calcium oxalate present in crystal fibres; starch absent.

C. Determine by thin layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 10 volumes of *diethyl ether*, 1 volume of *toluene*, and 10 volumes of *10.0 per cent acetic acid*.

Test solution. Reflux 1 g of coarsely powdered substance under examination with 50 ml *methanol* for 30 minutes. Reflux the residue again two times with 50 ml *methanol*, 30 minutes for each extraction. Cool and filter. Combine the extracts and concentrate under vacuum to dryness. Dissolve the residue in 10 ml *methanol*.

Reference solution. Reflux 1 g of *vijayasara IPBRS* with 50 ml *methanol* for 30 minutes. Reflux the residue again two times with 50 ml *methanol*, 30 minutes for each extraction. Cool and filter. Combine the extracts and concentrate under vacuum to dryness. Dissolve the residue in 10 ml *methanol*.

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry plate in air and examine under ultraviolet light at 254 nm and 366 nm. Spray the plate with *liebermann-burchard reagent*. Dry and examine the plate under ultraviolet light at 366 nm and in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

Tests

Foreign organic matter (2.6.1). Not more than 0.6 per cent.

Ethanol-soluble extractive (2.6.2). Not less than 14.0 per cent.

Water-soluble extractive (2.6.3). Not less than 3.5 per cent.

Total ash (2.3.19). Not more than 3.0 per cent.

Acid-insoluble ash (2.3.19). Not more than 0.4 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

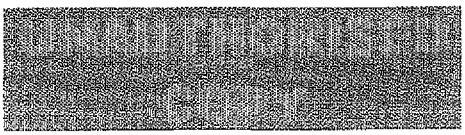
Loss on drying (2.4.19). Not more than 6.0 per cent, determined on 5 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 7 volumes of *toluene* and 3 volumes of *ethyl acetate*.

Test solution. To 1 g of the powdered substance under examination, add 50 ml of *methanol* and reflux for 30 minutes.



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Extract the residue two times with 50 ml of *methanol*, 30 minutes for each extraction. Cool and filter. Combine the extracts and concentrate under vacuum to dryness. Dissolve the residue in 10 ml of *methanol*.

Reference solution. A 0.1 per cent w/v solution of *pterostilbene IPRS* in *methanol*.

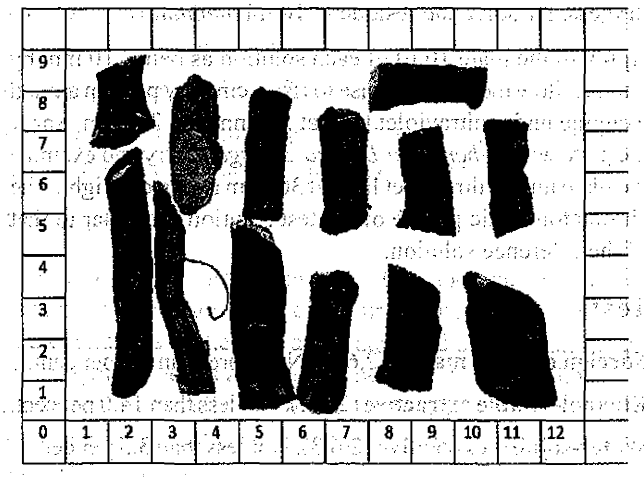
Apply to the plate, 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry plate in air and scan the plate in absorbance mode at 366 nm. Record the chromatograms and measure the responses for the analyte peak.

Calculate the content of pterostilbene.

Storage. Store protected from heat, moisture and against attack by insects and rodents.

Yasti

Liquorice root; Mulethi; *Glycyrrhiza glabra*



Yasti consists of the dried, unpeeled roots and stolons of *Glycyrrhiza glabra* Linn. (Fam. Leguminosae).

Yasti contains not less than 3.0 per cent w/w of glycyrrhizinic acid.

Category. Expectorant, antiulcer, stomachic, antiinflammatory, kasa.

Description. Odour, characteristic and slightly aromatic; taste, very sweet and faintly astringent; the bark is not bitter.

Identification

A. *Macroscopic* — Root with few branches, up to 1 m long and 0.5 to 3 cm in diameter. Bark, brownish-grey to brown with longitudinal striations, bearing traces of lateral roots. Stolons, cylindrical, 1 to 2 cm in diameter and up to several meters long, but may be cut into lengths of 10 to 15 cm; similar in external appearance to the root but with occasional small buds. Fracture of the root and stolon, granular and fibrous. Cork layer, thin; secondary phloem region, wide, light yellow with radial striations; xylem, compact, yellow, with radiate structure. The stolon has a central pith which is absent from the root.

B. *Microscopic* — Cork and phelloderm are narrow. Phloem consisting of bundles of thick-walled, yellow fibres with narrow lumina surrounded by cells each containing a calcium oxalate prism, alternating in the external layers with areas of strongly hyaline keratenchyma; functional sieve tissue near the cambium. Medullary rays parenchymatous, widening towards the exterior, 3 to 12 cells wide. Xylem composed of radial rows of tracheids and vessels alternating with bundles of lignified fibres with crystal sheaths similar to those of the secondary phloem; vessels 30 µm to 150 µm in diameter with thick walls (5 µm to 10 µm) having reticulate thickenings or numerous bordered pits with slit-shaped openings associated with lignified xylem parenchyma. Medullary rays, 2 to 5 cells wide. Parenchymatous cells throughout containing simple, round, oval or fusiform starch granules 2 µm to 20 µm, mostly 5 µm to 12 µm, in diameter; parenchymatous pith present solely in the stolon.

C. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *butyl alcohol*, 20 volumes of *water* and 10 volumes of *acetic acid*.

Test solution. Add 10 ml of 70 per cent v/v *methanol* to 1 g of dried yasti powder, heat by shaking on a water bath for 5 minutes, cool and filter.

Reference solution. Add 10 ml of 70 per cent v/v *methanol* to 1 g of dried *yasti IPBRS* powder, heat by shaking on a water bath for 5 minutes, cool and filter

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm, spray with *anisaldehyde sulphuric acid reagent*. Heat the plate at 105° for 10 minutes and examine the plate in day light. The chromatographic profile of the test solution is similar to that of the reference solution.

D. Mix a small quantity, in powder, with 0.05 ml of *sulphuric acid*; the powder particles become orange-yellow and some fragments change, more slowly, to pinkish red.

Tests

Curcuma. When examined under a microscope, none of the fragments of the powder in *sulphuric acid* (see Identification test D) should immediately take on a carmine-red colour.

Water-soluble extractive (2.6.3). Not less than 20 per cent, determined by the following method. Mix 2.5 g of the finely

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powdered drug with 50 ml of *water* and allow to stand for 2 hours, shaking frequently. Filter, evaporate 10.0 g of the filtrate to dryness on a water-bath, dry the residue at 105° and weigh.

Acid-insoluble ash (2.3.19). Not more than 2.0 per cent.

Sulphated ash (2.3.18). Not more than 10.0 per cent.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Weigh accurately 1.0 g of the coarsely powdered substance under examination in a 250-ml conical flask, add 100 ml of *0.1 M ammonia* and mix with the aid of ultrasound for 30 minutes. Centrifuge a part of the supernatant liquid and dilute 1.0 ml to 5.0 ml with *0.1 M ammonia*. Filter the solution through a membrane filter disc with an average pore diameter not greater than 1.0 µm and use the filtrate.

Reference solution. A 0.005 per cent w/v solution of *glycyrrhizinic acid IPRS* in *0.1 M ammonia*.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 6 volumes of *glacial acetic acid*, 30 volumes of *acetonitrile* and 64 volumes of *water*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of glycyrrhizinic acid.

Storage. Store protected from light and moisture.

Yasti Dry Extract

Yasti Dry Extract is obtained by extracting Yasti with *water* or aqueous *ethanol* or any other suitable solvent.

Yasti Dry Extract contains not less than 90.0 per cent w/w and not more than 120.0 per cent w/w of the stated amount of glycyrrhizinic acid.

Category. Expectorant, antiulcer, stomachic, antiinflammatory, kasa.

Description. A yellowish-brown to dark brown powder.

Identification

A. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel GF254*.

Mobile phase. A mixture of 70 volumes of *butanol*, 10 volumes of *acetic acid* and 20 volumes of *water*.

Test solution. Dissolve 200 mg of the extract under examination with 50 ml of *methanol* (70 per cent) and filter.

Reference solution. A 0.1 per cent w/v solution of *glycyrrhizin ammonical hydrate IPRS* in *methanol* (70 per cent).

Apply to the plate 10 µl of each solution as bands 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. Spray the plate with *anisaldehyde sulphuric acid reagent* and heat the plate at 105° for 10 minutes and examine the plate in day light. The chromatogram obtained with test solution shows a band corresponding to the band obtained with the reference solution indicating the presence of glycyrrhizin.

Tests

Acid insoluble ash (2.3.19). Not more than 2.0 per cent.

Heavy metals (2.3.13). 1.0 g complies with the limit test for heavy metals, Method B (20 ppm).

Loss of drying (2.4.19). Not more than 5.0 per cent, determined on 1.0 g by drying in an oven at 105°.

Microbial contamination (2.2.9). Complies with the microbial contamination tests.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 500 mg of the extract or a quantity containing 7 mg of glycyrrhizinic acid and dissolve in the mobile phase by mixing for 30 minutes with the aid of ultrasound and dilute to 100.0 ml with the mobile phase and filter.

Reference solution. A 0.01 per cent w/v solution of *glycyrrhizin ammonical hydrate IPRS* in the mobile phase.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: a mixture of 1 volume of *glacial acetic acid*, 67 volumes of *methanol* and 33 volumes of *0.2 M ammonium acetate* in *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 250 nm,
- injection volume. 20 µl.

Inject the reference solution. The test is not valid unless the relative standard deviation for the replicate injections is not more than 2.0 per cent.

Inject the reference solution and the test solution.

Calculate the content of glycyrrhizinic acid in the extract.

Storage. Store protected from heat and moisture.

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VACCINES AND IMMUNOSERA FOR HUMAN USE

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General Requirements

Vaccines

Vaccines are preparations of antigenic substances that are administered for the purpose of inducing in the recipient a specific and active immunity against the infective agent or toxin produced by it.

Vaccines may contain living micro-organisms suitably treated to attenuate their virulence but retain their immunogenicity or they may consist of pathogenic organisms which have been killed or inactivated. Some vaccines consist of antigenic fractions or substances produced by the same pathogenic organisms but rendered harmless whilst retaining their immunogenicity. Vaccines may be prepared from one species only or from a mixture of two or more species.

Vaccines may be prepared by the method described in the individual monographs or by the general methods given below or in any other manner provided the identity of the antigens is maintained and the vaccines are free from microbial contamination and extraneous agents. Suitable adjuvants may be added during the preparation but streptomycin, penicillin or other β-lactam antibiotics may not be added at any stage of manufacture or in the final vaccine. A suitable microbicide may be added to sterile and inactivated vaccines. The final products are distributed aseptically into sterile containers which are then sealed to exclude extraneous micro-organisms. Unless otherwise indicated in the monograph, the final vaccine may be filled into single dose or multiple dose containers but vaccines in multiple dose containers must invariably contain a microbicide.

Bacterial Vaccines. Bacterial vaccines are either sterile suspensions of live or killed bacteria or sterile extracts of derivatives of bacteria. They may be simple vaccines prepared from one species or may be mixed vaccines prepared by blending two or more simple vaccines from different species or strains.

Bacterial vaccines may be prepared from cultures grown on suitable solid or liquid media. The whole culture or parts of it may be used in preparing the vaccine. The identity, antigenic potency and purity of each bacterial culture must be carefully controlled.

Vaccines containing killed organisms may be prepared by killing the organisms by chemical or physical means provided the immunogenicity of the vaccine is preserved. Vaccines containing living bacteria may be prepared from strains which are avirulent for humans but can stimulate the production of antibodies active against pathogenic strains of the same species. The final vaccines must be free from any substance known to cause toxic, allergic or other undesirable immunological reactions in humans.

Bacterial vaccines are suspensions of varying degrees of opacity in colourless or slightly coloured liquids or they may be freeze-dried so that the water content is not more than 3.0 per cent w/w unless otherwise stated in the individual monograph. They may be standardized in terms of interopacity units or, where appropriate, by numbers of living or killed bacteria determined by direct cell count or by viable count.

Bacterial toxoids. Bacterial toxoids are toxins or material derived therefrom, the toxicity of which has been reduced to a very low level or completely eliminated by chemical or physical means without destroying their immunizing potency. The toxins are obtained from selected strains of specific micro-organisms, grown in media free from ingredients known to cause toxic, allergic or other undesirable immunological reactions in humans. Toxoids produced by the action of formaldehyde are known as formol toxoids.

Bacterial toxoids may be liquid or may be prepared by adsorbing on mineral carriers such as *aluminium phosphate, aluminium hydroxide* or any other suitable adsorbent; the adsorbed product may be separated, washed and suspended in a *saline* or other appropriate solution isotonic with blood.

Bacterial toxoids are clear or slightly opalescent liquids, colourless or slightly yellow. Adsorbed toxoids may be white or greyish white suspensions or pale-yellow liquids with a sediment at the bottom of the container. Freeze-dried preparations are greyish white or yellowish white powders or pellets.

Viral and rickettsial vaccines. Viral and rickettsial vaccines are suspensions of viruses or rickettsiae and are prepared from infected tissues or blood obtained from artificially infected animals, from cultures in fertile eggs, or from cell or tissue culture. Viral vaccines may be live or killed and they may be freeze-dried. Live vaccines are usually prepared using attenuated strains of the specific organisms. Killed vaccines may be inactivated by suitable chemical or physical means.

Mixed Vaccines. Mixed vaccines are mixtures of two or more vaccines. A suitable antibacterial substance may be added to inactivated or live viral and rickettsial vaccines provided that it has no action against the specific organisms.

Production

General provisions. Requirements for production including in-process testing are included in individual monographs. Where justified and authorized, certain tests may be omitted where it can be demonstrated, for example by validation studies, that the production process consistently ensures compliance with the test.

Unless otherwise justified and authorized, vaccines are produced using a seed-lot system. The methods of preparation are designed to maintain adequate immunogenic properties,

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to render the preparation harmless and to prevent contamination with extraneous agents.

Unless otherwise justified and authorized, in the production of a final lot of vaccine, the number of passages of a virus, or the number of subcultures of a bacterium, from the master seed lot shall not exceed that used for production of the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

Vaccines are as far as possible free from ingredients known to cause toxic, allergic or other undesirable reactions in man. Suitable additives, including stabilizers and adjuvants may be incorporated. Penicillin and streptomycin are neither used at any stage of production nor added to the final product; however, master seed lots prepared with media containing penicillin or streptomycin may, where justified and authorized, be used for production.

Substrates for propagation. Substrates for propagation comply with the relevant requirements of the Pharmacopoeia or in the absence of such requirements with those of the competent authority. Processing of cell banks and subsequent cell cultures is done under aseptic conditions in an area where no other cells are being handled. Serum and trypsin used in the preparation of cell suspensions shall be shown to be free from extraneous agents.

Seed lot. The strain of bacterium or virus used in a master seed lot is identified by historical records that include information on the origin of the strain and its subsequent manipulation. No micro-organism other than the seed strain shall be present in a seed lot.

Culture media. Culture media are as far as possible free from ingredients known to cause toxic, allergic or other undesirable reactions in man; if inclusion of such ingredients is necessary, it shall be demonstrated that the amount present in the final lot is reduced to such a level as to render the product safe. Approved animal (but not human) serum may be used in the growth medium for cell cultures but the medium used for maintaining cell growth during virus multiplication shall not contain serum, unless otherwise stated. Cell culture media may contain a pH indicator such as *phenol red* and approved antibiotics at the lowest effective concentration although it is preferable to have a medium free from antibiotics during production.

Propagation and harvest. The seed cultures are propagated and harvested under defined conditions. The purity of the harvest is verified by suitable tests as defined in the monograph.

Control cells. For vaccines produced in cell cultures, control cells are maintained and tested as prescribed. In order to provide a valid control, these cells must be maintained in conditions that are rigorously identical with those used for the production cell cultures, including use of the same batches of media and media changes.

Control eggs. For live vaccines produced in SPF eggs, control eggs are incubated and tested as prescribed in the monograph.

Purification. Where applicable, validated purification procedures may be applied.

Inactivation. Inactivated vaccines are produced using a validated inactivation process whose effectiveness and consistency have been demonstrated. Where there are recognised potential contaminants of a harvest, for example in vaccines produced in eggs from healthy, non-SPF flocks, the inactivation process is also validated with respect to the potential contaminants. A test for inactivation is carried out as soon as possible after the inactivation process, unless otherwise justified and authorised.

Intermediates. Where applicable, the stability of intermediates in given storage conditions shall be evaluated and a period of validity established.

Carrier Protein. Being intermediate stage, test for bioburden shall be done.

Final bulk. The final bulk is prepared by aseptically blending the ingredients of the vaccine.

Adsorbents. Vaccines may be adsorbed on *aluminium hydroxide, aluminium phosphate, calcium phosphate* or other suitable adsorbent; the adsorbents are prepared in special conditions which confer the appropriate physical form and adsorptive properties.

Antimicrobial preservative. A suitable antimicrobial preservative may be included in sterile and inactivated vaccines and is invariably added if these preparations are issued in multidose containers, unless otherwise stated. If an antimicrobial preservative is used, it shall be shown that it does not impair the safety or efficacy of the vaccine and its effectiveness throughout the period of validity shall be demonstrated.

Final lot. For vaccines for parenteral administration, the final lot is prepared by aseptically distributing the final bulk into sterile tamper-proof containers which, after freeze-drying where applicable, are closed so as to exclude contamination. For vaccines for administration by a non-parenteral route, the final lot is prepared by distributing the final bulk under suitable conditions into sterile, tamper-proof containers.

Stability. Maintenance of potency of the final lot throughout the period of validity shall be demonstrated by validation studies; the loss of potency in the recommended storage conditions is assessed and excessive loss even within the limits of acceptable potency may indicate that the vaccine is unacceptable.

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Degree of adsorption. During development of an adsorbed vaccine, the degree of adsorption is evaluated as part of the consistency testing. A release specification for the degree of adsorption is established in the light of results found for batches used in clinical testing. From the stability data generated for the vaccine it must be shown that at the end of the period of validity the degree of adsorption will not be less than for batches used in clinical testing. For tetanus, diphtheria and their combination vaccines, determine the degree of adsorption of each of the antigens as parameters for consistency, release and stability.

NOTE —The degree of adsorption test may be omitted for routine release specifications once the consistency in production has been well established to the satisfaction of the National Regulatory Authority. Thereafter, the test is to be performed at the final bulk stage of the vaccine. However, each final lot should comply with the test for degree of adsorption, when tested by National Control Laboratory.

Tests

Vaccines, reconstituted where necessary, comply with the following requirements unless otherwise stated in the individual monograph.

Phenol (*If present*) (2.3.36). Not more than 0.25 per cent w/v.

Thiomersal (*If present*) (2.3.48). Between 0.005 per cent w/v and 0.02 per cent w/v.

Free formaldehyde (*if present*) (2.3.20). Use either method A or method B. Not more tan 0.02 per cent w/v.

Aluminium (*If present*) (2.3.9). Not more than 1.25 mg per dose.

Sterility (2.2.11). Unless otherwise stated all vaccines comply with tests for sterility, except that for living bacterial vaccines, growth of the organism from which the vaccine was prepared is permitted (sterility means absence of bacterial and fungal contaminants except where specified in the individual monograph). Antimicrobial preservatives containing vaccines, preparations of 4 ml or more but less than 20 ml in each container minimum quantity of 2 ml to be used for each culture.

Abnormal toxicity (2.2.1). Unless otherwise stated, all vaccines comply with the test for abnormal toxicity, Method B. In vaccines containing phenol as preservative, the test in mice may be inappropriate.

The abnormal toxicity test may be omitted for routine lot release once the consistency in production has been well established to the satisfaction of the National Regulatory Authority and when good manufacturing practices are in place. Each lot, if tested by the National Control Laboratory should pass the test for abnormal toxicity.

NOTE — The statements given in this general chapter is intended to be read in conjunction with the monographs on the individual vaccine in this Pharmacopoeia which refer to preparations for human use; they do not necessarily apply to vaccines for use in veterinary practice.

Storage. Liquid vaccines must be stored at a temperature between 2° and 8° and should not be allowed to freeze unless otherwise specified in the individual monograph. Freeze-dried preparations must be stored at temperatures between 2° and 8° and for long term storage a temperature of -20° protected. At higher temperatures vaccines deteriorate rapidly.

Labelling. The label states (1) for liquid vaccines, the total number of ml in the container and, for freeze dried vaccines, the number of doses in the container; (2) unless otherwise indicated the minimum number of Units per dose or per ml or, for viral vaccines, the minimum viral titre; (3) the dose and route of administration; (4) the name and proportion of any antibacterial preservative or other auxiliary substances added to the vaccine; (5) the date after which the vaccine is not intended to be used; (6) the conditions under which it should be stored; (7) for freeze dried vaccines, the liquid to be used for reconstitution and its volume; (8) that the vaccine should be used immediately after reconstitution; (9) unless otherwise directed, that the vaccine should be shaken well before use; (10) any contraindication to the use of the vaccine.

Antisera

Immunosera

Antisera for Human use are liquid or freeze dried preparations containing purified Immunoglobulins or immunoglobulin fragments obtained from serum or plasma of immunized animals of different species that have prophylactic or therapeutic action. They contain specific neutralizing or binding antibodies against the antigen used for immunization. The antigens may include microbial or other toxins/toxoids, human antigens, bacterial and viral antigens, venoms of snakes and scorpions. These preparations are intended for parenteral (intravenous or intramuscular or subcutaneous) use, after dilution where applicable, to provide passive immunity.

Antisera are prepared by injecting antigens into healthy animals such as equines or any other animal species approved by the competent authority so as to produce in them antibodies which are normally associated with the globulin fraction of plasma or serum. Sub-lethal amounts of the antigen blended with a suitable adjuvant (if required) are injected periodically in gradually increasing doses into animals. Specific antitoxins are formed in the serum and the animals become hyper immune. During the process of plasma or serum harvesting, if the animals are treated with antibiotics they need to be withdrawn from the immunization and bleeding

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cycle temporarily. Use of topical antibiotics during immunization and bleeding is permissible. When a satisfactory degree of the immunity is produced, specified volumes of blood are withdrawn from the animals and the plasma or serum is processed to produce specific antisera.

The animals used are of a species (Equine or other suitable species) approved by the competent authority and are healthy. Each animal shall be free from defined list of infectious agents such as Glanders, Babesiosis, Trypanosomiasis, Equine Infectious Anemia Strangles etc, if Glanders infection found, Isolate the animals immediately from area OR Euthanized as per CPCSEA guideline animal shall not be used and herd shall be tested again. For other infections, animals shall be treated and tested for absence of infection. Animals shall be screened for notifiable diseases such as Glanders as per current guidelines before introduction into the production herd, the animals need to be quarantined, vaccinated against diseases, treated for parasitic infections and certified by Veterinary specialist.

Antigens commonly used for this purpose are toxins, toxoids, venoms, bacterial and viral vaccines.

The antigens used should be identified and characterized. Tests for venoms such as SDS PAGE, Total protein, potency in terms of LD50 or other suitable term, whichever applicable could be used. Venoms/Immunosera used for immunization need to be identified by their names.

Date of Immunization, Batch of antigen/Reference number, Antigen Dose minimum to maximum (limit or range), Volume, Route of injection and details of preparation need to be recorded.

Adjuvants or stimulators may be used to boost response.

Traceability of the immunization process is important for production of quality antisera. Immunized animal should be identified properly and immunization step needs to be recorded precisely.

If suitable, animals used for one project eg. Rabies antisera production, Tetanus antisera production, etc. may be used to produce other antibodies unless they are used for specific projects such as immunosuppressive antibodies. There shall be washout period of minimum 3 months between the projects.

During the process of plasma or serum harvesting, if the animals are treated with antibiotics they need to be withdrawn from the immunization and bleeding cycle temporarily for a period depending on the elimination kinetics of the drug concerned.

When several types of antisera are produced by a single production facility, a system to identify each batch of antiserum should be established. Identity tests may include biological assays as well as physiochemical and immunological tests. Double Immuno diffusion assays, is to be used. In case of animals of various species used for raising anti sera, equines orcaprinae, an immunological identity test should be used to identify the species used for antivenom production.

Animals are bled by vein puncture. The animals may be anaesthetized under conditions that do not influence the quality of the end product, where ever suitable. Unless otherwise prescribed, an antimicrobial preservative may be added to the separated or pooled plasma. The blood or plasma is collected aseptically in sterilized containers. Several single plasma or serum samples may be pooled Eg. Monovalents/ Bivalents/ Polyvalents before or after purification process. Test bleed shall be performed before collection of blood.

Labels on all blood or plasma containers should be marked with Identification Numbers (Eg. Animal Number on the blood collection container), specific product name, Date of Collection.

A veterinarian needs to certify the animals prior to bleeding indicating good health. Antisera in liquid form are distributed under aseptic conditions into sterile containers which are then sealed so as to exclude micro-organisms. A suitable antibacterial substance may be added during processing and is invariably added when the final product is filled in multiple dose containers. The product may be freeze-dried by a procedure which reduces the residual moisture content of the final product to less than 3.0 per cent w/w. Antisera are almost colourless or very faintly yellow liquids free from turbidity. Freeze-dried antisera consist of white or pale-yellow powders or friable masses which dissolve in water to form colourless or pale yellow liquids having the same characteristics as the corresponding liquid preparations.

PLASMA OR POOLED PLASMA

Single or pooled plasma are tested before purification for the following tests:

Tests

Potency. Carry out the assay as indicated in the monograph. A validated in vitro/ bioassay method may also be used, wherever applicable.

Protein Content. Not less than 5.0 per cent when determined by carrying out the determination of nitrogen, Method C (2.3.30) and multiplying the result by 6.25 or by biuret method A (2.3.49).

pH (2.4.24). 6.0 to 7.0.

Antisera Purification

The Immunoglobulins or their fragments may be purified by multiple techniques such as enzyme treatment, fractional precipitation, chromatography, immunoadsorption or by other chemical or physical methods.

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The methods are selected and validated to avoid formation of protein aggregates that affect characteristics of the product and do not generate additional components that compromise the quality and safety of the product or concentrated bulk. The process need to be validated for removal of agents used in plasma fractionation and precipitation agents such as Ammonium sulphate, Sodium sulphate, caprylic acid and digestion agents such as pepsin and papain.

INTERMEDIATE PRODUCT/CONCENTRATED BULK

Intermediate products or concentrated bulk that complies the following requirements may be used for the preparation of the final bulk.

Identity. Carry out the identity by using method prescribed for assay as indicated in the monograph. A validated in vitro method may also be used where ever appropriate based product specific.

Purity. Examine by non-reducing polyacrylamide gel electrophoresis by comparison with the reference preparation.

Potency. Carry out the assay as indicated in the monograph. A validated in vitro method may also be used where ever appropriate.

Protein Content. Not more than 17.0 per cent when determined by carrying out the determination of nitrogen, Method C (2.3.30) and multiplying the result by 6.25 or by biuret method A (2.3.49).

Microbial Contamination/ Sterility. It complies with test for microbial contamination (2.2.9). Total viable count not more than 10 CFU per 100 ml. It complies with the test for Sterility (2.2.11), if it goes for final bulk

FINAL BULK

The final bulk is prepared from a single intermediate product or from a pool of intermediate products (Sub-lots). During formulation of the final bulk, addition of salts, preservatives and excipients and adjustment of pH may be considered to improve the stability of the product and prevent aggregation.

Test for Purity by HPLC or suitable validated method with established limit for purity needs to be performed.

Tests

Purity. HPLC or suitable validated method

Antimicrobial Preservatives. Where ever applicable, determine the amount of antimicrobial preservative by a suitable Physico-chemical method. It should not be more than 0.25 per cent of label claim of the amount stated on the label.

Sterility/Microbial contamination. It complies with the test for Sterility (2.2.11), if the final bulk is stored for future use. It complies with test for microbial contamination (2.2.9). Total viable count not more than 10 CFU per 100 ml, if the final bulk is used immediately for formulation of the product

FINAL LOT

Antisera in liquid form are distributed under aseptic conditions into sterile containers which are then sealed so as to exclude micro-organisms. A suitable antibacterial substance may be added and is invariably added when the final product is filled in multiple dose containers. The product may be freeze-dried by a procedure which reduces the residual moisture content of the final product to less than 3.0 per cent w/w. Only a final lot that complies with the requirements prescribed below under Identification, Tests and Assay may be released for use. Provided that the tests for osmolality, protein content, molecular-size distribution, antimicrobial preservative, stabiliser, purity, foreign proteins and albumin and the assay have been carried out with satisfactory results on the final bulk, they may be omitted on the final lot.

The following tests refer to liquid antisera and to the reconstituted freeze-dried preparations.

Identification. Complies with the biological activity as described under potency in individual monographs or other suitable immunological tests may also be established.

Appearance. Antisera are clear to opalescent or pale yellow liquids free from suspended particles. Freeze dried anti sera products are white or pale yellow powder or solid masses which dissolve in water to form colourless or pale yellow liquids having the same characteristics of the corresponding liquid preparations.

Solubility (Only for Freeze dried preparation). The time from the addition of solvent to the complete dissolution of the freeze dried antisera under gentle mixing should be determined. Antisera should be completely dissolved within 10 minutes at room temperature. The solution should not be cloudy post complete dissolution. Shaking of the container should be avoided to prevent the formation of foam.

Residual Moisture Content. It can be determined by methods such as gravimetric method or thermo gravimetric method or Karl fisher titration. Residual moisture content of NMT 3 per cent is recommended for freeze dried anti sera products.

Reconstitution time (For lyophilised product only). The time from the addition of solvent to the complete dissolution of freeze-dried antisera, under gentle mixing, should be determined. Antisera should be completely dissolved within 10 minutes at room temperature. The solution should not be cloudy post complete dissolution. Shaking of the container should be avoided to prevent the formation of foam.

pH (2.4.24). 6.0 to 7.0.

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Osmolality. Not less than 240 mosmol/Kg after dilution, where applicable.

Total protein. Not more than 17.0 per cent for Gas gangrene Antitoxin, tetanus antitoxin, Diphtheria antitoxin. Not more than 10 per cent for Rabies antiserum, Scorpion antisera and Snake venom Antisera and other antisera preparations, when determined by using Determination of nitrogen, Method C (2.3.30) and multiplying the result by 6.25 or by biuret method A (2.3.49).

Molecular size distribution. Examine by liquid chromatography(2.4.14), the sum of peak areas of the immunoglobulin or immunoglobulin fragments and dimers should not be less than 90 per cent of the total area of all peaks in the chromatogram and the sum of peak areas of polymers, small molecular weight fragments and aggregates should not be more than 10 per cent of the total area of all peaks in the chromatogram.

Purity. Determine by sodium Dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) (2.4.12) under reducing condition and non-reducing conditions.

No additional bands are found for the preparation to be examined when compared with that of reference preparation.

This analysis provides qualitative information on Intact IgG digestion products and High molecular weight oligomers & low molecular weight contaminants.

Assay. Carry out a biological assay as indicated in the monograph. A validated in vitro method may also be used with the approval of competent authority.

Foreign proteins. When examined by precipitation tests with specific antisera only protein from the declared animal species is shown to be present unless otherwise prescribed for Eg. Where material of human origin is used during production.

Albumin. Albumin content should not be more than 3 per cent of the Total protein content using any suitable and validated method.

Antimicrobial preservative. Concentration of suitable preservatives (phenols or cresols) should not exceed 0.25 per cent w/v. Phenol concentration can be determined spectrophotometrically, cresols and Thiomersal by suitable method.

Excipients or Stabilizers. The concentration of various excipients or stabilizers should be determined by using appropriate chemical methods. The amount of stabilizers should not be less than 80 per cent and not more than 120 per cent of the quantity stated on the label.

Sterility (2.2.11). Comply with the tests for sterility.

Test for Pyrogens. Unless otherwise justified and authorized, it complies with the test for Pyrogens. Inject 1 ml/kg body weight of the rabbit.

Abnormal toxicity (2.2.1). Unless otherwise stated, all antisera comply with the test for abnormal toxicity, Method B. In antisera containing phenol as preservative, the test in mice may be inappropriate. The abnormal toxicity test may be omitted for routine lot release once the consistency in production has been well-established to the satisfaction of NRA and when good manufacturing practices are in place. Each lot, if tested by National Control Laboratory should pass test for abnormal toxicity.

Storage. Liquid antisera should be stored at a temperature between 2° and 8°, protected from light. Do not freeze liquid preparations.

Lyophilized Preparation should be stored in a cool and dark place, protected from light and stored at below 25°.

NOTE — The statements given in this general monograph are intended to be read in conjunction with the monographs on the individual antiserum in the Indian Pharmacopoeia which refer to preparations for human use; they do not necessarily apply to antisera for use in veterinary practice.

Labelling. The label states (1)the number of International Units per millilitre, where applicable or Potency as per Individual Monograph; (2) for freeze-dried preparations: the name and volume of the reconstituting liquid to be added, if applicable; (3) that the immunosera is to be used immediately after reconstitution, if applicable; (4) reconstitution time; (5) the route of administration; the storage conditions; (6) the expiry date; (7) the animal species of origin; (8) the name and limit of any antimicrobial preservative, name of any stabilizer and any other excipients.

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Adsorbed Diphtheria, Tetanus and Hepatitis B (rDNA) Vaccine

Diphtheria, Tetanus and Hepatitis B (rDNA) Vaccine (Adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; hepatitis B surface antigen (HBsAg); a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

HBsAg is a component protein of hepatitis B virus; the antigen is obtained by recombinant DNA technology.

Production

General provisions

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for antisera and vaccines, and with the following test for specific toxicity of the diphtheria and tetanus components: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing between 250 and 350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

The content of bacterial endotoxins in the bulk purified diphtheria toxoid and tetanus toxoid is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxin is less than the limit approved for the particular vaccine and in any case the contents are such that the final vaccine contains less than 100 IU per single human dose.

Reference vaccine(s)

Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

Production of the components

The production of the components complies with the requirements of the monographs on Diphtheria Vaccine (Adsorbed), Tetanus Vaccine (Adsorbed) and Hepatitis B Vaccine (rDNA).

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid and HBsAg onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the test for antimicrobial preservative and the assays for the diphtheria and tetanus components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the content of free formaldehyde has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

If an *in vivo* assay is used for the hepatitis B component, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Osmolality (2.4.23). The osmolality of the vaccine is within the limits approved for the particular preparation.

Identification

A. Diphtheria toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine under

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examination sufficient *sodium citrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The assay or, where applicable, the electrophoretic profile, serves also to identify the hepatitis B component of the vaccine.

Tests

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Complies with the test for sterility.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject the equivalent of 1 human dose into each rabbit or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be less than 100 EU per Single Human Dose.

Assay

Diphtheria component

Carry out one of the prescribed methods for the assay as stated under Diphtheria Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than 30 IU per single human dose.

Tetanus component

Carry out one of the prescribed methods for the assay as stated under Tetanus Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than 40 IU per single human dose.

Hepatitis B component

It complies with the assay of Hepatitis B Vaccine.

Labelling. The label states (1) the minimum number of International Units of diphtheria and tetanus toxoid per single human dose; (2) the amount of HBsAg per single human dose; (3) the type of cells used for production of the HBsAg component; (4) where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults; (5) the name and the amount of the adsorbent; (6) that the vaccine must be shaken before use; (7) that the vaccine is not to be frozen.

Adsorbed Diphtheria, Tetanus, Pertussis (Acellular Component) and *Haemophilus influenzae* Type b Conjugate Vaccine

Diphtheria, Tetanus, Pertussis (Acellular Component) and *Haemophilus influenzae* Type b Conjugate Vaccine (Adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; polyribosylribitol phosphate (PRP) covalently bound to a carrier protein; a mineral absorbent such as aluminium hydroxide or hydrated aluminium phosphate. The product may be presented with the *Haemophilus influenzae* type b component in a separate container, the contents of which are mixed with the other components immediately before use.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the toxin harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The acellular pertussis component may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter two antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* Type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

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Production

General provisions

The production method shall have been shown to yield consistently the vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

If the vaccine is presented with the haemophilus component in a separate vial, as part of consistency studies the assays of the diphtheria, tetanus and pertussis components are carried out on a suitable number of batches of vaccine reconstituted for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

The content of bacterial endotoxins in bulk purified diphtheria toxoid, tetanus toxoid, pertussis components and PRP conjugate is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine; if the vaccine is presented with the haemophilus component in a separate container, the contents of the diphtheria, tetanus and pertussis antigens are in any case such that the final vial for these components contains less than 100 IU per single human dose.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for antisera and vaccines.

During development studies and wherever revalidation is necessary, it shall be demonstrated by tests in animals that the vaccine induces a T-cell dependent B-cell immune response to PRP.

Reference vaccine

Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

Production of the components

The production of the components complies with the tests of the monographs on Diphtheria Vaccine (Adsorbed), Tetanus Vaccine (Adsorbed), Pertussis Vaccine (Acellular Component, Adsorbed) and *Haemophilus influenzae* Type b Conjugate Vaccine.

FINAL BULK VACCINE

Different methods of preparation may be used: a final bulk vaccine may be prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and PRP conjugate onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate; or 2 final bulks may be prepared and filled separately, one containing the diphtheria, tetanus and pertussis components, the other containing the haemophilus component, which may be freeze-dried. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality shown below and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the tests for absence of residual pertussis toxin, irreversibility of pertussis toxoid and antimicrobial preservative and the assays have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Osmolality (2.4.23). The osmolality of the vaccine, reconstituted where applicable, is within the limits approved for the particular preparation.

pH (2.4.24). The pH of the vaccine, reconstituted if necessary, is within the range approved for the particular product.

Free PRP. Unbound PRP is determined after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography (2.4.16), ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

Identification

If the vaccine is presented with the haemophilus component in a separate vial: identification tests A, B and C are carried out using the vial containing the diphtheria, tetanus and pertussis components; identification test D is carried out on the vial containing the haemophilus components.

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A. Diphtheria toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine under examination sufficient *sodium citrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in Identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis components are identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in Identification test A reacts with a specific antisera to the pertussis components of the vaccine.

D. The haemophilus component is identified by a suitable immunochemical method (2.2.14) for PRP.

Tests

If the product is presented with the haemophilus component in a separate container: the tests for absence of residual pertussis toxin, irreversibility of pertussis toxoid, aluminium, free formaldehyde, antimicrobial preservative and sterility are carried out on the container with the diphtheria, tetanus and pertussis components; the tests for PRP content, water (where applicable), sterility and pyrogens are carried out on the container with the haemophilus component.

If the haemophilus component is freeze-dried, some tests may be carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component under test.

Absence of residual pertussis toxin and irreversibility of pertussis toxoid

This test is not necessary for the product obtained by genetic modification. Use 3 groups each of not less than 5 histamine-sensitive mice. Inject intraperitoneally into each mouse of the first group twice the single human dose of the vaccine stored at 2° to 8°. Inject intraperitoneally into each mouse of the second group twice the single human dose of the vaccine incubated at 37° for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 hours. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If 1 mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5.0 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously three-fold dilutions of a *reference pertussis toxin* preparation in *phosphate-buffered saline solution* containing 0.2 per cent w/v of *gelatin* and challenge with *histamine* as above; the strain is suitable if more than 50 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine shows symptoms of sensitisation.

PRP. Minimum 80.0 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (2.7.1) or phosphorus (2.7.1), by an immunochemical method (2.2.14) or by anion-exchange liquid chromatography with pulsed-amperometric detection.

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Water (2.3.43). Maximum 3.0 per cent for the freeze-dried haemophilus component.

Sterility (2.2.11). Complies with the test for sterility.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject per kg of the rabbit's mass a quantity of the vaccine equivalent to: 1 mg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 mg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 mg of PRP for a vaccine with OMP as carrier or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be Less than 100 EU per Single Human Dose; for haemphilus component less than 25 IU per mcg of PRP. If any component of the vaccine prevents the determination of the endotoxin, a test for Pyrogen should be carried out.

Assay

Diphtheria component

Carry out one of the prescribed methods for the assay as stated under Diphtheria Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than the minimum potency stated on the label.

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Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

Tetanus component

Carry out one of the prescribed methods for the assay as stated under Tetanus Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than 40 IU per single human dose.

Pertussis component

The vaccine complies with the assay as the stated Adsorbed Pertussis Vaccine (Acellular Component).

Labelling. The label states (1) the minimum number of International Units of diphtheria and tetanus toxoid per single human dose; (2) the names and amounts of the pertussis components per single human dose; (3) the number of micrograms of PRP per single human dose; (4) the type and nominal amount of carrier protein per single human dose; (5) where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults; (6) the name and the amount of the adsorbent; (7) that the vaccine must be shaken before use; (8) that the vaccine is not to be frozen; (9) where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification.

Adsorbed Diphtheria, Tetanus, Pertussis (Acellular Component) and Hepatitis B (rDNA) Vaccine

Diphtheria, Tetanus, Pertussis (Acellular Component) and Hepatitis B (rDNA) Vaccine (Adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; hepatitis B surface antigen; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties, produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the latter harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

Hepatitis B surface antigen is a component protein of hepatitis B virus; the antigen is obtained by recombinant DNA technology.

Production

General provisions

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The content of bacterial endotoxins in the bulk purified diphtheria toxoid, tetanus toxoid and pertussis components is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine.

Reference vaccine(s)

Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

Production of the components

The production of the components complies with the requirements of the monographs on Diphtheria Vaccine (Adsorbed), Tetanus Vaccine (Adsorbed), Pertussis Vaccine (Acellular Component, Adsorbed) and Hepatitis B Vaccine (rDNA).

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and hepatitis B surface antigen onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

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Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the tests for absence of residual pertussis toxin, irreversibility of pertussis toxoid and antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the content of free formaldehyde has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

If an *in vivo* assay is used for the hepatitis B component, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Osmolality (2.4.23). The osmolality of the vaccine is within the limits approved for the particular preparation.

Identification

A. Diphtheria toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine under examination sufficient *sodium citrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis components are identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with a specific antisera to the pertussis components of the vaccine.

D. The assay or, where applicable, the electrophoretic profile, serves also to identify the hepatitis B component of the vaccine.

Tests

Absence of residual pertussis toxin and irreversibility of pertussis toxoid

This test is not necessary for the product obtained by genetic modification. Use 3 groups each of not less than 5 histamine-sensitive mice. Inject intraperitoneally into each mouse of the first group twice the single human dose of the vaccine stored at 2° to 8°. Inject intraperitoneally into each mouse of the second group twice the single human dose of the vaccine incubated at 37° for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 hours. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If 1 mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously threefold dilutions of a reference pertussis toxin preparation in phosphate-buffered saline solution containing 0.2 per cent w/v of gelatin and challenge with histamine as above; the strain is suitable if more than 50.0 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine shows symptoms of sensitisation.

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Complies with the test for sterility.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject the equivalent of 1 human dose into each rabbit or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be less than 100 EU per Single Human Dose.

Assay

Diphtheria component

Carry out one of the prescribed methods for the assay as stated under Diphtheria Vaccine (Adsorbed).

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The lower confidence limit (P = 0.95) of the estimated potency is not less than the minimum potency stated on the label.

Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

Tetanus component

Carry out one of the prescribed methods for the assay as stated under Tetanus Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than 40 IU per single human dose.

Pertussis component

The vaccine complies with the assay as stated under Adsorbed Pertussis Vaccine (Acellular Component).

Hepatitis B component

The vaccine complies with the assay as stated under Hepatitis B Vaccine (rDNA).

Labelling. The label states (1) the minimum number of International Units of diphtheria and tetanus toxoid per single human dose; (2) the names and amounts of the pertussis components per single human dose; (3) the amount of HBsAg per single human dose; (4) the type of cells used for production of the hepatitis B component; (5) where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults; (6) the name and the amount of the adsorbent; (7) that the vaccine must be shaken before use; (8) that the vaccine is not to be frozen (9) where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification.

Adsorbed Diphtheria, Tetanus, Pertussis (Acellular Component), Inactivated Poliomyelitis Vaccine and *Haemophilus influenzae* Type b Conjugate Vaccine

Diphtheria, Tetanus, Pertussis (Acellular Component), Inactivated Poliomyelitis Vaccine and *Haemophilus influenzae* Type b Conjugate Vaccine (Adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; suitable strain of human polioviruses 1,2 and 3 grown in suitable cell cultures and inactivated by validated method; polyribosylribitol phosphate (PRP) covalently bound to a carrier protein; a mineral absorbent such as aluminium hydroxide or hydrated aluminium phosphate. The product may be presented with the *Haemophilus influenzae type b* component in a separate container, the contents of which are mixed with the other components immediately before use.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the toxin harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The acellular pertussis component may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* Type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

Production

General provisions

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The content of bacterial endotoxins in bulk purified diphtheria toxoid, tetanus toxoid, pertussis components, purified, inactivated monovalent poliovirus harvests and bulk PRP conjugate is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine and, in any case, the contents are such that the final vaccine contains less than 100 IU per single human dose.

The production method is validated to demonstrate that the product, if tested, would comply with the following test. Inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing between 250 and 350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria, toxaemia or tetanus, the vaccine does

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not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

During development studies and wherever revalidation is necessary, it shall be demonstrated by tests in animals that the vaccine induces a T-cell dependent B-cell immune response to PRP.

As part of consistency studies the assays of the diphtheria, tetanus, pertussis and poliomyelitis components are carried out on a suitable number of batches of vaccine reconstituted for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

Reference vaccine(s)

Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

Production of the components

The production of the components complies with the requirements of the monographs on Diphtheria Vaccine (Adsorbed), Tetanus Vaccine (Adsorbed), Pertussis Vaccine (Acellular Component, Adsorbed), Poliomyelitis Vaccine (Inactivated) and *Haemophilus influenzae* Type b Conjugate Vaccine.

FINAL BULK VACCINE

The final bulk of the diphtheria, tetanus, pertussis and poliomyelitis components is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, bulk purified tetanus toxoid and bulk purified acellular pertussis components onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate and admixture of suitable quantities of purified, monovalent harvests of human polioviruses 1, 2 and 3 or a suitable quantity of a trivalent pool of such monovalent harvests. Suitable antimicrobial preservatives may be added.

The final bulk of the haemophilus component is prepared by dilution of the bulk conjugate to the final concentration with a suitable diluent. A stabiliser may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bovine serum albumin. Determine on the poliomyelitis components by a suitable immunochemical method (2.2.14) during preparation of the final bulk vaccine, before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will not be more than 50 ng per single human dose.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk of the haemophilus component is freeze-dried. Only a final lot that is satisfactory with respect to the test for osmolality shown below and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the test for absence of residual pertussis toxin and irreversibility of pertussis toxoid, the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided that the free formaldehyde content has been determined on the bulk purified antigens and the purified monovalent harvests or the trivalent pool of polioviruses or the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Provided that the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Osmolality (2.4.23). The osmolality of the vaccine, reconstituted where applicable, is within the limits approved for the particular preparation.

Free PRP

Unbound PRP is determined on the haemophilus component after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography (2.4.16), ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

Identification

Identification tests A, B, C and D are carried out using the vial containing the diphtheria, tetanus, pertussis and poliomyelitis

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components; identification test E is carried out on the vial containing the haemophilus component.

A. Diphtheria toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine under examination sufficient *sodium citrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis components are identified by suitable immunochemical methods (2.2.14). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with specific antisera to the pertussis components of the vaccine.

D. The vaccine is shown to contain human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.2.14), such as determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

E. The haemophilus component is identified by a suitable immunochemical method (2.2.14) for PRP.

Tests

The tests for absence of residual pertussis toxin, irreversibility of pertussis toxoid, aluminium, free formaldehyde, antimicrobial preservative and sterility are carried out on the container with the diphtheria, tetanus, pertussis and poliomyelitis components; the tests for PRP content, water, sterility and pyrogens are carried out on the container with the haemophilus component.

Some tests for the haemophilus component may be carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component under test.

Absence of residual pertussis toxin and irreversibility of pertussis toxoid

This test is not necessary for the product obtained by genetic modification. Use 3 groups each of not fewer than 5 histamine-sensitive mice. Inject intraperitoneally into each mouse of the first group twice the single human dose of the vaccine stored at 2° to 8°. Inject intraperitoneally into each mouse of the second group twice the single human dose of the vaccine incubated at 37° for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 hours. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following *histamine* challenge. If 1 mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously threefold dilutions of a *reference pertussis toxin preparation* in *phosphate-buffered saline solution* containing 0.2 per cent w/v of *gelatin* and challenge with *histamine* as above; the strain is suitable if more than 50.0 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

PRP. Minimum 80.0 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (2.7.1) or phosphorus (2.7.1), by an immunochemical method (2.2.14) or by anion-exchange liquid chromatography with pulsed-amperometric detection.

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Water (2.3.43). Maximum 3.0 per cent for the haemophilus component.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject per kg of the rabbit's mass a quantity of the vaccine equivalent to: 1 mg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 mg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 mg of PRP for a vaccine with OMP as a carrier or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be Less than 100 EU per Single Human Dose; for haemphilus component less than 25 IU per mcg of PRP. If any component of the vaccine prevents the determination of the endotoxin, a test for pyrogens should be carried out.

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Assay

Diphtheria component

Carry out one of the prescribed methods for the assay as stated under Diphtheria Vaccine (Adsorbed).

Unless otherwise justified and authorised, the lower confidence limit (P = 0.95) of the estimated potency is not less than 30 IU per single human dose.

Tetanus component

Carry out one of the prescribed methods for the assay as stated under Tetanus Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than 40 IU per single human dose.

Pertussis component

It complies with the assay as stated under Adsorbed Pertussis Vaccine (Acellular Component).

Poliomyelitis component

D-antigen content

As a measure of consistency of production, determine the D-antigen content for human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.2.14) using a reference preparation calibrated in units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) reference preparation* is calibrated in Units and intended for use in the assay of D-antigen. The Unit and the International Unit are equivalent.

In vivo test

The vaccine complies with the *in vivo* assay as stated under Inactivated Poliomyelitis Vaccine.

Labelling. The label states (1) the minimum number of International Units of diphtheria and tetanus toxoid per single human dose (2) the names and amounts of the pertussis components per single human dose; (3) the nominal amount of poliovirus of each type (1, 2 and 3), expressed in units of D-antigen per single human dose; (4) the type of cells used for production of the poliomyelitis component; (5) the number of micrograms of PRP per single human dose; (6) the type and nominal amount of carrier protein per single human dose; (7) where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults; (8) the name and the amount of the adsorbent; (9) that the vaccine must be shaken before use; (10) that the vaccine is not to be frozen; (11) where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification.

Adsorbed Diphtheria, Tetanus, Pertussis (Acellular Component), Hepatitis B (rDNA), Poliomyelitis (Inactivated) and *Haemophilus influenzae* Type b Conjugate Vaccine

Diphtheria, Tetanus, Pertussis (Acellular Component), Hepatitis B (rDNA), Poliomyelitis (Inactivated) and *Haemophilus influenzae* Type b Conjugate Vaccine (Adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; hepatitis B surface antigen (HBsAg); human poliovirus types 1, 2 and 3 grown in suitable cell cultures and inactivated by a suitable method; polyribosylribitol phosphate (PRP) covalently bound to a carrier protein. The antigens in the vaccine may be adsorbed on a mineral carrier such as aluminum hydroxide or hydrated aluminum phosphate. The product is presented either as a hexavalent liquid formulation in the same container, or as a pentavalent liquid formulation with the haemophilus component in a separate container, the contents of which are mixed with the other components immediately before or during use.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the toxin harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The acellular pertussis component may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

Hepatitis B surface antigen is a component protein of hepatitis B virus; the antigen is obtained by recombinant DNA technology.

PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

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Production

General provisions

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

Specific toxicity of the diphtheria and tetanus components. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

The content of bacterial endotoxins in bulk purified diphtheria toxoid, tetanus toxoid and pertussis components, hepatitis B surface antigen, purified, inactivated monovalent poliovirus harvests and bulk PRP conjugate is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is not greater than the limit approved.

During development studies, it shall be demonstrated that the vaccine consistently induces a T-cell-dependent B-cell immune response to PRP. If the manufacturing process is modified, it shall be demonstrated by appropriate in vitro methods that the characteristic properties of the conjugate are not affected. If the vaccine is presented with the haemophilus component in a separate container, as part of consistency studies the assays of the diphtheria, tetanus, pertussis, hepatitis B and poliomyelitis components are carried out on a suitable number of batches of vaccine reconstituted as for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

The stability of the final lot and relevant intermediates is evaluated using one or more indicator tests. For the haemophilus component, such tests may include determination of molecular size, determination of free PRP in the conjugate and kinetics of depolymerisation. Taking account of the results of the stability testing, release requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

Reference vaccine(s)

Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of differences in composition between the monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilized by a method that has been shown to have no effect on the assay procedure.

Production of the components

The production of the components complies with the requirements of the monographs Diphtheria vaccine (adsorbed), Tetanus vaccine (adsorbed), Pertussis vaccine (acellular, component, adsorbed), Hepatitis B vaccine (rDNA), Poliomyelitis vaccine (inactivated) and Haemophilus type b conjugate vaccine.

FINAL BULK VACCINE

Vaccine with all components in the same container. The final bulk is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and hepatitis B surface antigen onto a mineral carrier such as aluminum hydroxide or hydrated aluminum phosphate and admixture of a suitable quantity of PRP conjugate and suitable quantities of purified and inactivated, monovalent harvests of human poliovirus types 1, 2 and 3 or a suitable quantity of a trivalent pool of such monovalent harvests. Suitable antimicrobial preservatives may be added.

Vaccine with the haemophilus component in a separate container. The final bulk of diphtheria, tetanus, pertussis, hepatitis B and poliovirus component is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and hepatitis B surface antigen onto a mineral carrier such as aluminum hydroxide or hydrated aluminum phosphate and admixture of suitable quantities of purified and inactivated, monovalent harvests of human poliovirus types 1, 2 and 3 or a suitable pool of such monovalent harvests. This final bulk is filled separately. Suitable antimicrobial preservatives may be added.

The final bulk of the haemophilus component is Prepared by dilution of the bulk conjugate to the final concentration with a suitable diluent. A stabiliser may be added.

Only a final bulk vaccine that complies with the Following requirements may be used in the preparation of the final lot.

Bovine serum albumin. Determined on the poliomyelitis components by a suitable immunochemical method (2.2.14) after purification of the harvests and before preparation of the

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final bulk vaccine, before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85.0 per cent and not greater than 115.0 per cent of the intended content.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml for each sterility medium.

FINAL LOT

Where the haemophilus component is in a separate container, the final bulk of the haemophilus component is freeze-dried. Only a final lot that is satisfactory with respect to the test for osmolality shown below and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the test for osmolality, the test for residual pertussis toxin and irreversibility of pertussis toxoid, the test for antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens and the purified monovalent harvests or the trivalent pool of polioviruses or the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Provided that the test for bovine serum albumin has been carried out with satisfactory results on the trivalent pool of inactivated monovalent harvests of polioviruses or on the final bulk vaccine, it may be omitted on the final lot.

If an *in vivo* assay is used for the hepatitis B component, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Provided that the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

For each poliovirus type that the acceptance criteria for the D-antigen determination are such that it yields the same result as the *in vivo* assay in terms of acceptance or rejection of a batch. This demonstration must include testing of subpotent batches, produced experimentally if necessary, for example by heat treatment or other means of diminishing the immunogenic activity. Where there is a significant change in the manufacturing process of the antigens or their formulation, any impact on the *in vivo* and *in vitro* assays must be evaluated, and the need for revalidation considered.

Free PRP

For vaccines with all components in the same container, the free PRP content is determined on the non-absorbed fraction. Unbound PRP is determined on the haemophilus component after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography(2.4.16), ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

Identification

If the vaccine is presented with the haemophilus component in a separate container: Identification tests A, B, C, D and E are carried out using the container with the diphtheria, tetanus, pertussis, hepatitis B and poliomyelitis components; identification test F is carried out on the container with the haemophilus components.

A. Diphtheria toxoid is identified by a suitable immunochemical method (2.2.14). The following method applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (2.2.14). The following method applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis components are identified by suitable immunochemical methods (2.2.14).The following method applicable to certain vaccines, is given as an example.The clear supernatant obtained during identification test A reacts with specific Antisera to the pertussis components of the vaccine.

D. The hepatitis B component is identified by a suitable immunochemical method, for example the *in vitro* assay, or by a suitable electrophoretic method.

E. The vaccine is shown to contain human poliovirus types 1, 2 and 3 by a suitable immunochemical method (2.2.14), such as determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

F. the Haemophilus component is identified by a suitable immunochemical method (2.2.14), for PRP.

Tests

If the product is presented with the haemophilus component in a separate container, the tests for residual pertussis toxin and irreversibility of pertussis toxoid, free formaldehyde, aluminum, antimicrobial preservative and sterility are carried out on the container with the diphtheria, tetanus,

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pertussis, poliomyelitis and hepatitis B components; the tests for PRP, water, antimicrobial preservative (where applicable), aluminum (where applicable) and sterility are carried out on the container with the haemophilus component.

Some tests for the haemophilus component are carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component under test.

Absence of residual pertussis toxin and irreversibility of pertussis toxoid

This test is not necessary for the product obtained by genetic modification. Use 3 groups each of not fewer than 5 histamine-sensitive mice. Inject intraperitoneally into each mouse of the first group twice the single human dose of the vaccine stored at 2° to 8°. Inject intraperitoneally into each mouse of the second group twice the single human dose of the vaccine incubated at 37° for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 hours. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following *histamine* challenge. If 1 mouse dies in either or both of thefirst and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously three fold dilutions of a *reference pertussis toxin preparation in phosphate-buffered saline solution* containing 0.2 per cent w/v of *gelatin* and challenge with *histamine* as above; the strain is suitable if more than 50.0 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with *histamine*show symptoms of sensitisation.

This test may be omitted if justified and approved by the competent authority

PRP. minimum 80.0 per cent of the amount of PRP stated on the label, for a vaccine with the haemophilus component in a separate container.

For a vaccine with all components in the same container: the PRP content determined on the non-absorbed fraction is not less than that approved for the product.

PRP is determined either by assay of ribose (2.7.1) or phosphorus (2.7.1), by an immunochemical method (2.2.14) or by anion-exchange liquid chromatography with pulsedamperometric detection.

Aluminum (2.3.9). Maximum 1.25 mg per single human dose, ifaluminum hydroxide or hydrated aluminum phosphate is used as the adsorbent.

Free formaldehyde (2.3.20).Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Water (2.3.43).Maximum 3.0 per cent for the freeze-dried haemophilus component.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of for each sterility medium.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject per kg of the rabbit's mass a quantity of the vaccine equivalent to: 1 mg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 mg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 mg of PRP for a vaccine with OMP as a carrier or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be within the limits approved by the National Regulatory authority for the haemophilus componentof the particular product. If any components of the vaccine prevents the determination of the endotoxins, a test for pyrogens should be carried out.

Assay

Diphtheria component

Carry out the prescribed methods for the assay of diphtheria vaccine (adsorbed).

Unless otherwise justified and authorized, the lower confidence limit (P = 0.95) of the estimated potency is not less than 30 IU per single human dose.

Tetanus component

Carry out one of the prescribed methods for the assay asstated under Tetanus Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potencyis not less than 40 IU per single human dose.

Pertussis component

It complies with the assay as stated under Adsorbed PertussisVaccine (Acellular Component).

The capacity of the vaccine to induce antibodies for each included acellular pertussis antigen is within the limits approved for the particular product.

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Hepatitis B component

The vaccine complies with the assay of hepatitis B vaccine.

Poliomyelitis component

D-antigen content

As a measure of consistency of production, determine the D-antigen content for human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.2.14) using a reference preparation calibrated in units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) reference preparation is* calibrated in Units and intended for use in the assay of D-antigen. The Unit and the international Unit are equivalent.

In vivo test

The vaccine complies with the *in vivo* assay as stated under Inactivated Poliomyelitis Vaccine.

Labelling. The label states (1) the minimum number of International Units of diphtheria and tetanus toxoid per single human dose; (2) the names and amounts of the pertussis components per single human dose; (3) the amount of HBsAg per single human dose; (4) the nominal amount of poliovirus of each type (1, 2 and 3), expressed in D-antigen per single human dose; (5) the types of cells used for production of the poliomyelitis and the hepatitis B components; (6) the number of micrograms of PRP per single human dose; (7) the type and nominal amount of carrier protein per single human dose; (8) where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults; (9) the name and the amount of the adsorbent (10) that the vaccine must be shaken before use; (11) that the vaccine is not to be frozen; (12) where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification.

Adsorbed Diphtheria, Tetanus, Pertussis (Acellular Component) and Inactivated Poliomyelitis Vaccine

Diphtheria, Tetanus, Pertussis (Acellular Component) and Poliomyelitis (Inactivated) Vaccine is a combined vaccine containing: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; suitable strains of human polioviruses 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the toxin harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be copurified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

Production

General provisions

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for antisera and vaccines.

The content of bacterial endotoxins in bulk purified diphtheria toxoid, tetanus toxoid, pertussis components and purified, inactivated monovalent poliovirus harvests is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine and, in any case, the contents are such that the final vaccine contains less than 100 IU per single human dose.

Reference vaccine(s)

Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

Production of the components

The production of the components complies with the requirements of the monographs on Diphtheria Vaccine

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(Adsorbed), Tetanus Vaccine (Adsorbed), Pertussis Vaccine (Acellular Component, Adsorbed) and Poliomyelitis Vaccine (Inactivated).

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and admixture of suitable quantities of purified monovalent harvests of human polioviruses 1, 2 and 3 or a suitable quantity of a trivalent pool of such purified monovalent harvests. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bovine serum albumin. Determine on the poliomyelitis components by a suitable immunochemical method (2.2.14) after virus harvest and before addition of the adsorbent in the preparation of the final bulk vaccine, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the tests for absence of residual pertussis toxin, irreversibility of pertussis toxoid and antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Provided that the determination of D-antigen content has been carried out with satisfactory results during preparation of the final bulk before addition of the adsorbent, it may be omitted on the final lot.

Provided that the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Osmolality (2.4.23). The osmolality of the vaccine is within the limits approved for the particular preparation.

Identification

A. Diphtheria toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine under examination sufficient *sodium citrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hour and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in Identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis components are identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in Identification test A reacts with a specific antisera to the pertussis components of the vaccine.

D. The vaccine is shown to contain human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.2.14) such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

Tests

Absence of residual pertussis toxin and irreversibility of pertussis toxoid

This test is not necessary for the product obtained by genetic modification. Use 3 groups each of not less than 5 histamine-sensitive mice. Inject intraperitoneally into each mouse of the first group twice the single human dose of the vaccine stored at 2° to 8°. Inject intraperitoneally into each mouse of the second group twice the single human dose of the vaccine incubated at 37° for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 hours. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If 1 mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than

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5.0 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously three-fold dilutions of a *reference pertussis toxin preparation* in *phosphate-buffered saline solution* containing 0.2 per cent w/v of *gelatin* and challenge with *histamine* as above; the strain is suitable if more than 50.0 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine shows symptoms of sensitisation.

Aluminium (2.3.9). Maximum 1.25 mg per single human dose if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject the equivalent of 1 human dose into each rabbit or a validated test for bacterial endotoxin (2.2.3) may be used instead of the test for pyrogens. If used, the content should be less than 100 EU per Single Human Dose.

Assay

Diphtheria component

Carry out one of the prescribed methods for the assay as stated under Diphtheria Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than the minimum potency stated on the label.

Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

Tetanus component

Carry out one of the prescribed methods for the assay as stated under Tetanus Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than 40 IU per single human dose.

Pertussis component

The vaccine complies with the assay as stated under Adsorbed Pertussis Vaccine (Acellular Component).

Poliomyelitis component

D-antigen content

As a measure of consistency of production, determine the D-antigen content for human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.2.14) following desorption using a reference preparation calibrated in units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) reference preparation* is calibrated in Units and intended for use in the assay of D-antigen. The Unit and the International Unit are equivalent.

In vivo test

The vaccine complies with the *in vivo* assay as stated under Inactivated Poliomyelitis Vaccine.

Labelling. The label complies with the requirements stated under Vaccine and also states (1) the minimum number of International Units of diphtheria and tetanus toxoid per single human dose; (2) the names and amounts of the pertussis components per single human dose; (3) the nominal amount of poliovirus of each type (1, 2 and 3), expressed in units of D-antigen per single human dose; (4) the type of cells used for production of the poliomyelitis component; (5) the number of micrograms of PRP per single human dose; (6) the type and nominal amount of carrier protein per single human dose; (7) where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults; (8) the name and the amount of the adsorbent; (9) that the vaccine must be shaken before use; (10) that the vaccine is not to be frozen; (11) where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification.

Adsorbed Diphtheria, Tetanus, Pertussis and Poliomyelitis (Inactivated) Vaccine

Diphtheria, Tetanus, Pertussis and Poliomyelitis (Inactivated) Vaccine (Adsorbed) is a combined vaccine containing: diphtheria formol toxoid; tetanus formol toxoid; an inactivated suspension of *Bordetella pertussis*; suitable strains of human polioviruses 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

Production

General provisions

The production method shall have been shown to yield consistently the vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

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The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for antisera and vaccines, and with the following test for specific toxicity of the diphtheria and tetanus components : inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing between 250 and 350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

Reference vaccine(s)

Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

Production of the components

The production of the components complies with the requirements of the monographs on Diphtheria Vaccine (Adsorbed), Tetanus Vaccine (Adsorbed), Pertussis Vaccine (Adsorbed) and Poliomyelitis Vaccine (Inactivated).

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, separately or together, of suitable quantities of bulk purified diphtheria toxoid and bulk purified tetanus toxoid and admixture of suitable quantities of an inactivated suspension of *B. pertussis* and purified monovalent harvests of human polioviruses 1, 2 and 3 or a suitable quantity of a trivalent pool of such purified monovalent harvests. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Specific toxicity

Use not less than 5 healthy mice each weighing between 14 and 16 g for the vaccine group and for the saline control. Use mice of the same sex or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 hours before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 ml, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 ml of a 0.9 per cent sterile solution of *sodium chloride*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection and 72 hours and 7 days after the injection. The vaccine complies with the test if: (a) at the end of 72 hours the total mass of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of the vaccinated mice die during the test. The test may be repeated and the results of the tests combined.

Bovine serum albumin. Determine on the poliomyelitis components by a suitable immunochemical method (2.2.14) during preparation of the final bulk vaccine; before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

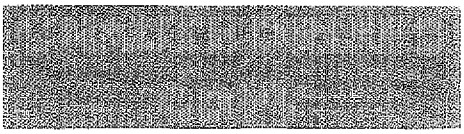
FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for specific toxicity and antimicrobial preservative, and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided that the free formaldehyde content has been determined on the bulk purified antigens, the inactivated *B. pertussis* suspension and the purified monovalent harvests or the trivalent pool of polioviruses or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Provided that the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.



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Osmolality (2.4.23). The osmolality of the vaccine is within the limits approved for the particular preparation.

Identification

A. Diphtheria toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine under examination sufficient *sodium citrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The centrifugation residue obtained in identification A may be used. Other suitable methods for separating the bacteria from the adsorbent may also be used. Identify pertussis vaccine by agglutination of the bacteria from the resuspended precipitate by antisera specific to *B. pertussis* or by the assay of the pertussis component prescribed under Assay.

D. The vaccine is shown to contain human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.2.14) such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

Tests

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination: Complies with the test for sterility (2.2.11).

Assay

Diphtheria component

Carry out one of the prescribed methods for the assay as stated under Diphtheria Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than 30 IU per single human dose.

Tetanus component

Carry out one of the prescribed methods for the assay as stated under Tetanus Vaccine (Adsorbed).

If the test is carried out in guinea pigs, the lower confidence limit (P = 0.95) of the estimated potency is not less than 40 IU per single human dose; if the test is carried out in mice, the lower confidence limit (P = 0.95) of the estimated potency is not less than 60 IU per single human dose.

Pertussis component

Carry out the assay as stated under Pertussis Vaccine.

The estimated potency is not less than 4 IU per single human dose and the lower confidence limit (P = 0.95) of the estimated potency is not less than 2 IU per single human dose.

Poliomyelitis component

D-antigen content

As a measure of consistency of production, determine the D-antigen content for human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.2.14) using a reference preparation calibrated in Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) reference preparation* is calibrated in Units and is intended for use in the assay of D-antigen. The Unit and the IU are equivalent.

In vivo test

The vaccine complies with the *in vivo* assay as stated under Poliomyelitis Vaccine (Inactivated).

Labelling. The label states (1) the minimum number of International Units of diphtheria and tetanus toxoid per single human dose; (2) the minimum number of International Units of pertussis vaccine per single human dose; (3) the nominal amount of poliovirus of each type (1, 2 and 3), expressed in units of D-antigen per single human dose; (4) the type of cells used for production of the poliomyelitis component; (5) where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults; (6) the name and the amount of the adsorbent; (7) that the vaccine must be shaken before use; (8) that the vaccine is not to be frozen.

Adsorbed Diphtheria, Tetanus, Pertussis, Poliomyelitis (Inactivated) and *Haemophilus influenzae* Type b Conjugate Vaccine

Diphtheria, Tetanus, Pertussis, Poliomyelitis (Inactivated) and *Haemophilus influenzae* Type b Conjugate Vaccine (Adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; an inactivated

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suspension of *Bordetella pertussis*; suitable strains of human polioviruses 1, 2 and 3 grown in suitable cell cultures and inactivated by a suitable method; polyribosylribitol phosphate (PRP) covalently bound to a carrier protein; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate. The product is presented with the haemophilus component in a separate container, the contents of which are mixed with the other components immediately before use.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate [$(C_{10}H_{19}O_{12}P)_n$], with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

Production

General provisions

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for antisera and vaccines, and with the following test for specific toxicity of the diphtheria and tetanus components: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing between 250 and 350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

During development studies and wherever revalidation is necessary, it shall be demonstrated by tests in animals that the vaccine induces a T-cell dependent B-cell immune response to PRP.

As part of consistency studies the assays of the diphtheria, tetanus, pertussis and poliomyelitis components are carried out on a suitable number of batches of vaccine reconstituted for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

Reference vaccine(s)

Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

Production of components

The production of the components complies with the requirements of the monographs on Diphtheria Vaccine (Adsorbed), Tetanus Vaccine (Adsorbed), Pertussis Vaccine (Adsorbed), Poliomyelitis Vaccine (Inactivated) and *Haemophilus influenzae* Type b Conjugate Vaccine.

FINAL BULK VACCINE

The final bulk of the diphtheria, tetanus, pertussis and poliomyelitis components is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, and bulk purified tetanus toxoid onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate and admixture of suitable quantities of an inactivated suspension of *B. pertussis* and of purified, monovalent harvests of human polioviruses 1, 2 and 3 or a suitable quantity of a trivalent pool of such monovalent harvests. Suitable antimicrobial preservatives may be added.

The final bulk of the haemophilus component is prepared by dilution of the bulk conjugate to the final concentration with a suitable diluent. A stabiliser may be added.

Only final bulk that complies with the following requirements may be used in the preparation of the final lot.

Specific toxicity

Use not less than 5 healthy mice each weighing between 14 and 16 g, for the vaccine group and for the saline control. Use mice of the same sex or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 hours before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 ml, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 ml of a 0.9 per cent sterile solution of *sodium chloride*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection and 72 hours and 7 days after the injection. The vaccine complies with the test if (a) at the end of 72 hours the total mass of the group of vaccinated mice is not less than that

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preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of the vaccinated mice die during the test. The test may be repeated and the results of the tests combined.

Bovine serum albumin. Determine on the poliomyelitis components by a suitable immunochemical method (2.2.14) during preparation of the final bulk vaccine, before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will not be more than 50 ng per single human dose.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk of the haemophilus component is freeze-dried.

Only a final lot that is satisfactory with respect to the test for osmolality shown below and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for specific toxicity and antimicrobial preservative, and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided that the free formaldehyde content has been determined on the bulk purified antigens, the inactivated *B. pertussis* suspension and the purified monovalent harvests or the trivalent pool of polioviruses or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Provided that the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Osmolality (2.4.23). The osmolality of the vaccine, reconstituted where applicable, is within the limits approved for the particular preparation.

Free PRP

Unbound PRP is determined on the haemophilus component after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography (2.4.16), ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

Identification

Identification tests A, B, C and D are carried out using the vial containing the diphtheria, tetanus, pertussis and poliomyelitis components; identification test E is carried out on the vial containing the haemophilus component.

A. Diphtheria toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine under examination sufficient *sodium citrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The centrifugation residue obtained in identification A may be used. Other suitable methods for separating the bacteria from the adsorbent may also be used. Identify pertussis vaccine by agglutination of the bacteria from the resuspended precipitate by antisera specific to *B. pertussis* or by the assay of the pertussis component prescribed under Assay.

D. The vaccine is shown to contain human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.2.14), such as determination of D-antigen by enzyme linked immunosorbent assay (ELISA).

E. The haemophilus component is identified by a suitable immunochemical method (2.2.14) for PRP.

Tests

The tests for specific toxicity, aluminium, free formaldehyde, antimicrobial preservative and sterility are carried out on the container with diphtheria, tetanus, pertussis and poliomyelitis components; the tests for PRP content, water, sterility and pyrogens are carried out on the container with the haemophilus component.

Some tests for the haemophilus component may be carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component under test.

PRP. Minimum 80.0 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (2.7.1) or phosphorus (2.7.1), by an immunochemical method (2.2.14) or by anion-exchange liquid chromatography with pulsed-amperometric detection.

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

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Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Water (2.3.43). Maximum 3.0 per cent for the haemophilus component.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject per kg of the rabbit's mass a quantity of the vaccine equivalent to 1 mg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 mg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 mg of PRP for a vaccine with OMP as carrier or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be within the limits approved by the National Regulatory Authority for the haemophilus component of the particular product. If any components of the vaccine prevents the determination of the endotoxin, a test for Pyrogen should be carried out.

Assay

Diphtheria component

Carry out one of the prescribed methods for the assay as stated under Diphtheria Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than 30 IU per single human dose.

Tetanus component

Carry out one of the prescribed methods for the assay as stated under Tetanus Vaccine (Adsorbed).

If the test is carried out in guinea-pigs, the lower confidence limit (P = 0.95) of the estimated potency is not less than 40 IU per single human dose; if the test is carried out in mice, the lower confidence limit (P = 0.95) of the estimated potency is not less than 60 IU per single human dose.

Pertussis component

Carry out the assay as stated under Pertussis Vaccine.

The estimated potency is not less than 4 IU per single human dose and the lower confidence limit (P = 0.95) of the estimated potency is not less than 2 IU per single human dose.

Poliomyelitis component

D-antigen content

As a measure of consistency of production, determine the D-antigen content for human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.2.14) using a reference preparation calibrated in Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) reference preparation* is calibrated in Units and intended for use in the assay of D-antigen. The Unit and the IU are equivalent.

In vivo test

The vaccine complies with the *in vivo* assay as stated under Poliomyelitis Vaccine (Inactivated).

Labelling. The label states (1) the minimum number of International Units of diphtheria and tetanus toxoid per single human dose; (2) the minimum number of International Units of pertussis vaccine per single human dose; (3) the nominal amount of poliovirus of each type (1, 2 and 3) expressed in Units of D-antigen per single human dose; (4) the type of cells used for production of the poliomyelitis component; (5) the number of micrograms of PRP per single human dose; (6) the type and nominal amount of carrier protein per single human dose; (7) where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults; (8) the name and the amount of the adsorbent; (9) that the vaccine must be shaken before use; (10) that the vaccine is not to be frozen.

Adsorbed Pertussis Vaccine (Acellular Component)

Pertussis Vaccine (Acellular Component, Adsorbed) is a preparation of individually prepared and purified antigenic components of *Bordetella pertussis* adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate.

The vaccine contains either pertussis toxoid or a pertussis toxin, like protein free from toxic properties, produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the latter harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be copurified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

Production

General provisions

The production method shall have been shown to yield consistently the vaccines comparable with the vaccine of

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proven clinical efficacy and safety in man. Where a genetically modified form of B. Pertussis is used, production consistency and genetic stability shall be in conformity with the requirements prescribed by the National Regulatory Authority.

Reference vaccine

A batch of vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine is preferably stabilised by a method that has been shown to have no significant effect on the assay procedure when the stabilised and non-stabilised batches are compared.

CHARACTERISATION OF COMPONENTS

During development of the vaccine, the production process shall be validated to demonstrate that it yields consistently individual components that comply with the following requirements; after demonstration of consistency, the tests need not be applied routinely to each batch.

Adenylate cyclase. Not more than 500 ng in the equivalent of 1 dose of the final vaccine, determined by immunoblot analysis or another suitable method.

Tracheal cytotoxin. Not more than 2 pmol in the equivalent of 1 dose of the final vaccine, determined by a suitable method such as a biological assay or liquid chromatography (2.4.14).

Absence of residual dermonecrotic toxin. Inject intradermally into each of 3 unweaned mice, in a volume of 0.1 ml, the amount of component or antigenic fraction equivalent to 1 dose of the final vaccine. Observe for 48 hours. No dermonecrotic reaction is demonstrable.

Specific properties. The components of the vaccine are analysed by one or more of the methods shown below in order to determine their identity and specific properties (activity per unit amount of protein) in comparison with reference preparations.

Pertussis toxin

It may be demonstrated by Chinese hamster ovary (CHO) cell-clustering effect and haemagglutination as *in vitro* methods; lymphocytosis-promoting activity, histamine-sensitising activity and insulin secretory activity as *in vivo* methods. The toxin shows ADP- ribosyl transferase activity using transducin as the acceptor.

Filamentous haemagglutinin

It may be demonstrated by Haemagglutination and inhibition by specific antibody.

Pertactin, fimbrial-2 and fimbrial-3 antigens may be demonstrated by reactivity with specific antibody.

Pertussis toxoid

The toxoid induces in animals production of antibodies capable of inhibiting all the properties of pertussis toxin.

PURIFIED COMPONENTS

Production of each component is based on a seed-lot system. The seed cultures from which toxin is prepared are managed to conserve or where necessary restore toxinogenicity by deliberate selection.

None of the media used at any stage contains blood or blood products of human origin. Media used for the preparation of seed lots and inocula may contain blood or blood products of animal origin.

Pertussis toxin and, where applicable, filamentous haemagglutinin and pertactin are purified and, after appropriate characterisation, detoxified using suitable chemical reagents, by a method that avoids reversion of the toxoid to toxin, particularly on storage or exposure to heat. Other components such as fimbrial-2 and fimbrial-3 antigens are purified either separately or together, characterised and shown to be free from toxic substances. The purification procedure is validated to demonstrate appropriate clearance of substances used during culture or purification.

The content of bacterial endotoxins is determined to monitor the purification procedure and to limit the amount in the final vaccine. The limits applied for the individual components are such that the final vaccine contains less than 100 IU per single human dose.

Before detoxification, the purity of the components is determined by a suitable method such as polyacrylamide gel electrophoresis (PAGE) or liquid chromatography. SDS-PAGE or immunoblot analysis with specific monoclonal or polyclonal antibodies may be used to characterise subunits. Requirements are established for each individual product.

Only purified components that comply with the following requirements may be used in the preparation of the final bulk vaccine.

Sterility (2.2.11). Carry out the test for sterility using for each medium a quantity of purified component equivalent to not less than 100 doses.

Absence of residual pertussis toxin

This test is not necessary for the product obtained by genetic modification. Use a group of not fewer than 5 histamine-sensitive mice each weighing between 18 and 26 g. Inject into each mouse the equivalent of 1 human dose intravenously or twice the human dose intraperitoneally, diluted to not more than 0.5 ml with *phosphate-buffered saline solution* containing 0.2 per cent w/v of *gelatin*. Inject diluent into a second group of control mice. After 5 days, inject 2 mg of *histamine base*

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intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 hours. If no animal dies, the preparation complies with the test.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject three-fold dilutions of a *reference pertussis toxin preparation* in *phosphate-buffered saline solution* containing 0.2 per cent w/v of *gelatin* and challenge with *histamine* as above; the strain is suitable if more than 50 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

A validated test based on the clustering effect of the toxin for Chinese Hamster Ovary (CHO) cells may be used instead of the test in mice.

Residual detoxifying agents and other reagents

The content of residual detoxifying agents and other reagents is determined and shown to be below approved limits unless validation of the process has demonstrated acceptable clearance.

Antigen content

Determine the antigen content by a suitable immunochemical method (2.2.14) and protein nitrogen by sulphuric acid digestion (2.2.30) or another suitable method. The ratio of antigen content to protein nitrogen is within the limits established for the product.

FINAL BULK VACCINE

The vaccine is prepared by adsorption of suitable quantities of purified components, separately or together, onto aluminium hydroxide or hydrated aluminium phosphate. A suitable antimicrobial preservative may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for absence of residual pertussis toxin and irreversibility of pertussis toxoid, antimicrobial preservative, free formaldehyde and the assay have been carried out with satisfactory results on the final bulk vaccine, these tests may be omitted on the final lot.

Identification

Subject the vaccine to a suitable desorption procedure such as the following: dissolve in the vaccine under examination sufficient *sodium citrate* to give a 1 per cent w/v solution; maintain at 37° for about 16 hours and centrifuge until a clear supernatant liquid is obtained. Examined by a suitable immunochemical method (2.2.14), the clear supernatant liquid reacts with specific antisera to the components stated on the label.

Tests

Absence of residual pertussis toxin and irreversibility of pertussis toxoid

This test is not necessary for the product obtained by genetic modification. Use 3 groups each of not fewer than 5 histamine-sensitive mice. Inject intraperitoneally into the first group twice the single human dose of the vaccine stored at 2° to 8°. Inject intraperitoneally into the second group twice the single human dose of the vaccine incubated at 37° for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of *histamine base* intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 hours. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If 1 mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5.0 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously threefold dilutions of a reference pertussis toxin preparation in *phosphate-buffered saline solution* containing 0.2 per cent w/v of *gelatin* and challenge with *histamine* as above; the strain is suitable if more than 50.0 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

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Sterility (2.2.11). Complies with the test for sterility.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject the equivalent of 1 human dose into each rabbit or a validated test for bacterial endotoxin (2.2.3) may be used instead of the test for pyrogens. If used, the content should be less than 100 EU per Single Human Dose.

Assay

The capacity of the vaccine to induce the formation of specific antibodies is compared with the same capacity of a reference preparation examined in parallel; antibodies are determined using suitable immunochemical methods (2.2.14) such as enzyme-linked immunosorbent assay (ELISA). The test on mice shown below uses a three-point model but, after validation, for routine testing a single-dilution method used or any other validated serological assay in guinea pigs or mice as approved by National Regulatory Authority may also be used.

Where a single dilution assay is used production and test consistency over time shall be monitored via suitable indicators and carrying out a full multidilution assay periodically for example every two years.

Requirement

The capacity to induce antibodies is not significantly ($P = 0.95$) less than that of the reference vaccine.

The following test model is given as an example of a method that has been found to be satisfactory.

Selection and distribution of test animals

Use in the test healthy mice (for example, CD1 strain) of the same stock 4 to 8 weeks old. Distribute the animals in 6 groups of a number appropriate to the requirements of the assay. Use 3 dilutions of the vaccine under examination and 3 dilutions of a *reference preparation* and attribute each dilution to a group of mice. Inject intraperitoneally or subcutaneously into each mouse 0.5 ml of the dilution attributed to its group.

Collection of serum samples

4 to 5 weeks after vaccination, bleed the mice individually under anaesthesia. Store the sera at -20° until tested for antibody content.

Antibody determination

Assay the individual sera for content of specific antibodies to each component using a validated method such as the ELISA test shown below.

ELISA

Microtitre plates (polyvinyl chloride or polystyrene as appropriate for the specific antigen) are coated with the purified antigen at a concentration of 100 ng per well. After washing, unreacted sites are blocked by incubating with a *solution of bovine serum albumin* and then washed. Two-fold dilutions of sera from mice immunised with test or reference vaccines are made on the plates. Reference antiserum shall be included in each plate. After incubation at 22° to 25° for 1 hour, the plates are washed. A suitable solution of *anti-mouse IgG enzyme conjugate* is added to each well and incubated at 22° to 25° for 1 hour. After washing, a substrate is added from which the bound enzyme conjugate liberates a chromophore which can be quantified by measurement of absorbance. The test conditions are designed to obtain a linear response for absorbance with respect to antibody content over the range of measurement used and absorbance values within the range 0.1 to 2.0.

A reference antiserum of assigned potency is used in the test and serves as the basis for calculation of the antibody levels in test sera. A standardised control serum is also included in the test.

The test is not valid if (a) the value found for the control serum differs by more than 2 standard deviations from the assigned value; (b) the confidence interval of the potency estimate is greater than 50.0 per cent to 200.0 per cent.

Calculation

The antibody titres in the sera of mice immunised with reference and test vaccines are calculated and from the values obtained the potency of the test vaccine in relation to the reference vaccine is calculated by the usual statistical methods.

Labelling. The label states (1) the names and amounts of the components present in the vaccine; (2) where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification; (3) the name and amount of the adsorbent; (4) that the vaccine must be shaken before use; (5) that the vaccine is not to be frozen.

Adsorbed Pertussis Vaccine (Acellular, Co-Purified)

Pertussis Vaccine (Acellular, Co-Purified, Adsorbed) is a preparation of antigenic components of *Bordetella pertussis* adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate.

The vaccine contains an antigenic fraction purified without separation of the individual components. The antigenic fraction is treated by a method that transforms pertussis toxin to toxoid, rendering it harmless while maintaining adequate immunogenic properties of all the components and avoiding reversion to toxin. The antigenic fraction is composed of pertussis toxoid,

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filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. It may contain residual pertussis toxin up to a maximum level approved by the competent authority. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

Production

General provisions

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

Reference vaccine. A batch of vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine is preferably stabilised, by a method that has been shown to have no significant effect on the assay procedure when the stabilised and non-stabilised batches are compared.

CHARACTERISATION OF COMPONENTS

During development of the vaccine, the production process shall be validated to demonstrate that it yields consistently an antigenic fraction that complies with the following requirements; after demonstration of consistency, the tests need not be applied routinely to each batch.

Adenylate cyclase. Not more than 500 ng in the equivalent of 1 dose of the final vaccine, determined by immunoblot analysis or another suitable method.

Tracheal cytotoxin. Not more than 2 pmol in the equivalent of 1 dose of the final vaccine, determined by a suitable method such as a biological assay or liquid chromatography (2.4.14).

Absence of residual dermonecrotic toxin. Inject intradermally into each of 3 unweaned mice, in a volume of 0.1 ml, the amount of antigenic fraction equivalent to 1 dose of the final vaccine. Observe for 48 hours. No dermonecrotic reaction is demonstrable.

Specific properties. The antigenic fraction is analyzed by one or more of the methods shown below in order to determine the identity and specific properties (activity per unit amount of protein) of its components in comparison with reference preparations.

Pertussis toxin

Chinese Hamster Ovary (CHO) cell-clustering effect and haemagglutination as *in vitro* methods; lymphocytosis-promoting activity, histamine-sensitising activity and insulin secretory activity as *in vivo* methods. The toxin shows ADP-ribosyl transferase activity using transducin as the acceptor.

Filamentous haemagglutinin

Haemagglutination and inhibition by specific antibody.

Pertactin, fimbrial-2 and fimbrial-3 antigens. Reactivity with specific antibody.

Pertussis toxoid

The toxoid induces in animals the production of antibodies capable of inhibiting all the properties of pertussis toxin.

PURIFIED ANTIGENIC FRACTION

Production of the antigenic fraction is based on a seed-lot system. The seed cultures are managed to conserve or, where necessary, restore toxinogenicity by deliberate selection.

None of the media used at any stage contains blood or blood products of human origin. Media used for the preparation of seed batches and inocula may contain blood or blood products of animal origin.

The antigenic fraction is purified and, after appropriate characterisation, detoxified using suitable reagents by a method that ensures minimal reversion of toxoid to toxin, particularly on or exposure to heat. The purification procedure is validated to demonstrate appropriate clearance of substances used during culture or purification.

The content of bacterial endotoxins is determined to monitor the purification procedure and to limit the amount in the final vaccine. The limits applied are such that the final vaccine contains not more than 100 IU per single human dose.

Before detoxification, the purity of the antigenic fraction is determined by a suitable method such as polyacrylamide gel electrophoresis (PAGE) (2.4.12) or liquid chromatography (2.4.14). SDS-PAGE or immunoblot analysis with specific monoclonal or polyclonal antibodies may be used to characterise subunits. Requirements are established for each individual product.

Only a purified antigenic fraction that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Sterility (2.2.11). Carry out the test for sterility using for each medium a quantity of purified antigenic fraction equivalent to not less than 100 doses of the final vaccine.

Test for residual pertussis toxin. Use 3 groups of not fewer than 5 histamine-sensitive mice each weighing between 18 and 26 g. Using *phosphate-buffered saline* containing 0.2 per

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cent of *gelatin*, prepare a series of dilutions of the *purified antigenic fraction* that have been shown to yield a graded response and attribute each dilution to a separate group of mice. Inject intraperitoneally into each mouse the dilution attributed to its group. Inject *diluent* into a fourth group of control mice. After 5 days, inject intraperitoneally into each mouse 1 mg of *histamine base* in a volume not exceeding 0.5 ml. Record the number of animals that die within 24 hours of histamine challenge. Calculate the weight or volume of a preparation that sensitises 50.0 per cent of the mice injected using a suitable statistical method such as probit analysis. The residual activity of pertussis toxin does not exceed that of batches shown to be safe in clinical studies.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject threefold dilutions of a *reference pertussis toxin preparation* in *phosphate-buffered saline* solution containing 0.2 per cent w/v of *gelatin* and challenge with *histamine* as described above; the strain is suitable if more than 50 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

A validated test based on the clustering effect of the toxin for Chinese Hamster Ovary (CHO) cells may be used instead of the test on mice.

Residual detoxifying agents and other reagents. The content of residual detoxifying agents and other reagents is determined and shown to be below approved limits unless validation of the process has demonstrated acceptable clearance.

Antigen content. Determine the complete quantitative antigen composition of the antigenic fraction by suitable immunochemical methods (2.2.14) and protein nitrogen by sulphuric acid digestion or another suitable method. The ratio of total antigen content to protein nitrogen is within the limits established for the product.

FINAL BULK VACCINE

The vaccine is prepared by adsorption of a suitable quantity of the antigenic fraction onto aluminium hydroxide or hydrated aluminium phosphate. A suitable antimicrobial preservative may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for residual pertussis toxin, reversibility of toxoid, antimicrobial preservative, free formaldehyde and the assay have been carried out with satisfactory results on the final bulk vaccine, these tests may be omitted on the final lot.

Identification

Subject the vaccine to a suitable desorption procedure such as the following: dissolve in the vaccine under examination sufficient *sodium citrate* to give a 10 per cent w/v solution; maintain at 37° for about 16 hours and centrifuge until a clear supernatant is obtained. Examine by a suitable immunochemical method (2.2.14), the clear supernatant reacts with specific antisera to the components in the vaccine.

Tests

Test for residual pertussis toxin. Use 3 groups of not fewer than 5 histamine-sensitive mice (see under Production) each weighing between 18 and 26 g. Using *phosphate-buffered saline* containing 0.2 per cent w/v of *gelatin*, prepare a series of dilutions of the vaccine under examination that have been shown to yield a graded response and attribute each dilution to a separate group of mice. Inject intraperitoneally into each mouse the dilution attributed to its group. Inject *diluent* into a fourth group of control mice. After 5 days, inject intraperitoneally into each mouse 1 mg of *histamine base* in a volume not exceeding 0.5 ml. Note the number of animals that die within 24 hours of histamine challenge. Calculate the weight or volume of a preparation that sensitises 50 per cent of the mice injected using a suitable statistical method such as probit analysis. The residual activity of pertussis toxin does not exceed that of batches shown to be safe in clinical studies.

Reversibility of toxoid. Carry out the test for residual pertussis toxin described above using the vaccine incubated at 37° for 4 weeks in parallel with a sample stored at 2° to 8°. The degree of reversibility does not exceed that of batches shown to be safe in clinical studies.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Sterility (2.2.11). Complies with the test for sterility.

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Pyrogens (2.2.8). Complies with the test for pyrogens. Inject the equivalent of 1 human dose into each rabbit or a validated test for bacterial endotoxin (2.2.3) may be used instead of the test for pyrogens. If used, the content should be less than 100 EU per Single Human Dose.

Assay

The vaccine complies with the assay as stated under Adsorbed Pertussis Vaccine (Acellular Component).

Labelling. The label states (1) the names and amounts of the antigenic components present in the vaccine; (2) the maximum amount of residual pertussis toxin present in the vaccine; (3) the maximum degree of reversion of toxoid to toxin during the period of validity; (4) the name and amount of the adsorbent; (5) that the vaccine must be shaken before use; (6) that the vaccine is not to be frozen.

Bacillus Calmette-Guerin Vaccine (Freeze-Dried)

Freeze-dried BCG Vaccine is a preparation of live bacteria derived from a culture of the bacillus of Calmette and Guérin (*Mycobacterium bovis* BCG) capacity of which to protect against tuberculosis has been established.

Vaccine complies with the requirements stated under Vaccines with the following modifications.

Production

General provisions

The production method is validated to demonstrate that the product, if tested, would comply with the tests for safety and efficacy.

BCG vaccine shall be produced by a staff consisting of healthy persons who do not work with other infectious agents; in particular they shall not work with virulent strains of *Mycobacterium tuberculosis*, during the course of production cycle nor shall they be exposed to a known risk of tuberculosis infection. Staff involved in production and testing of BCG vaccine shall be examined periodically for tuberculosis. BCG vaccine is susceptible to sunlight: the procedures for the preparation of the vaccine shall be so designed that all cultures and vaccines are protected from direct sunlight and from ultraviolet light at all stages of manufacture, testing and storage.

Production of the vaccine is based on a seed-lot system. The production method shall have been shown to yield consistently BCG vaccines that induce adequate sensitivity to tuberculin in man, that have acceptable protective potency in animals and are safe. The vaccine is prepared from cultures which are derived from the master seed lot by as few subcultures as possible and in any case not more than 12 subcultures e.g. If the secondary seed lot is 4 passages from the primary seed lot, the number of passages from the secondary seed lot must not exceed 8. During the course of these subcultures the preparation is not freeze dried for more than once.

The capacity of the working seed lot to induce sensitivity to tuberculin in guinea-pigs is demonstrated.

If a bioluminescence test or other biochemical method is used instead of viable count, the method is validated against the viable count for each stage of the process at which it is used.

SEED LOT

The strain used to establish the master seed lot is chosen for and maintained to preserve its characteristics, stability, its capacity to sensitise man and guinea-pigs to tuberculin and to protect animals against tuberculosis, and its relative absence of pathogenicity for man and laboratory animals. The strain used shall be identified by historical records that include information on its origin and subsequent manipulation.

A suitable batch of vaccine is prepared from the first working seed lot and is reserved for use as the comparison/ in-house reference vaccine. When a new working seed lot is established, a suitable test for delayed hypersensitivity in guinea-pigs is carried out on a batch of vaccine prepared from the new working seed lot; the vaccine is shown to be not significantly different in activity from the comparison vaccine.

Only a working seed lot that complies with the following requirements may be used for propagation.

Identification

The bacteria in the working seed lot are identified as *Mycobacterium bovis* BCG using microbiological techniques, which may be supplemented by molecular biology techniques (for example, nucleic acid amplification and restriction-fragment-length polymorphism).

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11), carried out using 10 ml for each medium. The working seed lot complies with the test for sterility except for the presence of mycobacteria.

Virulent mycobacteria

Examine the working seed lot as prescribed under Tests, using 10 guinea pigs.

PROPAGATION AND HARVEST

The bacteria are grown in a suitable medium for not more than 21 days by surface or submerged culture. The culture medium

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shall contain no substances known to cause toxic or allergic reactions in human beings or to cause the bacteria to become virulent for guinea-pigs. The culture is harvested and suspended in a sterile liquid medium that protects the viability of the organism as determined by a suitable method of viable count.

Test for purity. Purity is checked by acid fast staining.

FINAL BULK VACCINE

The final bulk vaccine is prepared from a single harvest or by pooling a number of single harvests. A stabiliser may be added; if the stabiliser interferes with the determination of bacterial concentration on the final bulk vaccine, the determination is carried out before addition of the stabiliser.

Only final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Virulent mycobacteria. Examine as prescribed under Tests.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11) using 10 ml for each medium except for the presence of mycobacteria.

Count of viable units

Determine the number of viable units per ml on solid medium using a method suitable for the vaccine under examination or by determination of adenosine triphosphate by a bioluminescence reaction. Carry out the test in parallel on a *reference preparation* of the same strain.

Bacterial concentration

Determine the total bacterial concentration by a suitable method, either directly by determining the mass of the micro-organisms, or indirectly by an opacity method that has been calibrated in relation to the mass of the organisms; if the bacterial concentration is determined before addition of a stabiliser, the concentration in the final bulk vaccine is established by calculation. The total bacterial concentration is within the limits approved for the particular product by National Regulatory Authority.

The ratio of the count of viable units to the total bacterial concentration is not less than that approved for the particular product by National Regulatory Authority.

FINAL LOT

The final bulk vaccine is distributed into sterile containers and freeze-dried to a moisture content favourable to the stability of the vaccine; the containers are closed either under vacuum or under a gas that is not deleterious to the vaccine.

Except where the filled and closed containers are stored at a temperature of -20° or lower, the expiry date is not later than 4 years from the date of harvest.

Only a final lot that complies with the following requirement for count of viable units and with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for virulent mycobacteria has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot. Provided the test for excessive dermal reactivity has been carried out with satisfactory results on the working seed lot and on 5 consecutive final lots produced from it, the test may be omitted on the final lot.

Count of viable units

Determine the number of viable units per ml of the reconstituted vaccine by viable count on solid medium using a method suitable for the vaccine under examination or by determination of adenosine triphosphate by a bioluminescence reaction. The ratio of the count of viable units after freeze-drying to that before is not less than that approved for the particular product.

Identification

BCG vaccine is identified by microscopic examination of the bacilli in stained smears demonstrating their acid-fast property and by the characteristic appearance of colonies grown on solid medium. Alternatively, molecular biology techniques (like nucleic acid amplification) may be used.

Tests

Virulent mycobacteria

If this test is satisfactory at final bulk stage it can be omitted at the final lot.

Inject subcutaneously or intramuscularly into each of 6 guinea-pigs, each weighing between 250 and 400 g and having received no treatment likely to interfere with the test, a quantity of vaccine equivalent to at least 50 human doses. Observe the animals for at least 42 days. At the end of this period, kill the guinea-pigs and examine by autopsy for signs of infection with tuberculosis, ignoring any minor reactions at the site of injection. Animals that die during the observation period are also examined for signs of tuberculosis. The vaccine complies with the test if none of the guinea-pigs shows signs of tuberculosis and if not more than one animal dies during the observation period. If 2 animals die during this period and autopsy does not reveal signs of tuberculosis repeat the test on 6 other guinea-pigs. The vaccine complies with the test if not more than one animal dies during the 42 days following the injection and autopsy does not reveal any sign of tuberculosis.

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.2.11) except for the presence of mycobacteria.

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Excessive dermal reactivity

Use 6 healthy white or pale-coloured guinea-pigs, each weighing not less than 250 g and having received no treatment likely to interfere with the test. Inject intradermally into each guinea-pig, according to a randomised plan, 0.1 ml of the reconstituted vaccine and of 2 tenfold serial dilutions of the vaccine and identical doses of the comparison vaccine. Observe the lesions formed at the site of the injection for 4 weeks. The vaccine complies with the test if the reaction it produces is not markedly different from that produced by the comparison vaccine.

Temperature stability

The temperature stability test is performed to demonstrate the consistency of vaccine production. The requirement of this test should be at the discretion of the National Regulatory Authority. If the production consistency is demonstrated, this test may be omitted on the final lot after getting approval from National Regulatory Authority.

If required each final lot should be tested for temperature stability by validated method as approved by National Regulatory Authority.

To perform temperature stability test maintain samples of the freeze-dried vaccine at 37° for 4 weeks. Determine the number of viable units in the heated vaccine and in unheated vaccine kept at 2-8° as described below in Assay. The number of viable units in the heated vaccine is not less than 20.0 per cent of that in unheated vaccine.

Water (2.3.43). Not more than 3.0 per cent, determined by the semi-micro determination of water.

Assay

Determine the number of viable units in the reconstituted vaccine by viable count on solid medium (2.2.5) or using a suitable validated biochemical method for the vaccine under examination. Add a suitable surfactant such as Tween 80 in diluent used for reconstituting the vaccine. The number is within the range stated on the label. Determine the number of viable units in the comparison vaccine in parallel. The number of viable units determined on unheated vaccine kept at 2-8° during temperature stability test may be considered as assay for viability.

Labelling. The label states (1) the minimum and maximum number of viable units per ml in the reconstituted vaccine; (2) that the vaccine must be protected from direct sunlight; (3) that the vaccine is to be used immediately after broaching the container; (4) the age group for which the vaccine is intended; (5) the dose for each age group; (6) follow instructions as mentioned in the product insert/leaflet.

BCG for Immunotherapy

BCG for Immunotherapy (intravesical) is a freeze-dried preparation of live bacteria derived from a culture of the bacillus of Calmette and Guerin (*Mycobacterium bovis* BCG) whose capacity for treatment of carcinoma *in situ* and papilloma tumors of the urinary bladder has been established.

Production

General Provisions

BCG for Immunotherapy shall be produced by a staff consisting of healthy persons who do not work with other infectious agents; in particular they shall not work with virulent strains of *Mycobacterium tuberculosis*, nor shall they be exposed to a known risk of tuberculosis infection. Staff involved in production and testing of BCG for Immunotherapy shall be examined periodically for tuberculosis. BCG for immunotherapy is susceptible to sunlight: the procedures for production shall be so designed that all products are protected from direct sunlight and from ultraviolet light at all stages of manufacture, testing and storage.

Production is based on a seed-lot system. The production method shall have been shown to yield consistently BCG products that can be used for treatment of superficial bladder cancer and are safe. The product is prepared from cultures which are derived from the master seed lot by as few subcultures as possible and in any case not more than 12 subcultures e.g. If the secondary seed lot is 4 passages from the primary seed lot, the number of passages from the secondary seed lot must not exceed 8. During the course of these subcultures the preparation is not freeze-dried more than once.

If a bioluminescence test or other biochemical method is used instead of viable count, the method is validated against the viable count for each stage of the process at which it is used.

SEED LOT

The strain used to establish the master seed lot is chosen for and maintained to preserve its characteristics stability, its capacity to treat and prevent superficial bladder cancer, and its relative absence of pathogenicity for man and laboratory animals. The strain used shall be identified by historical records that include information on its origin and subsequent manipulation.

Before establishment of a working seed lot a batch is prepared and reserved for use as the comparison product. When a new working seed lot is established, a suitable test for delayed hypersensitivity in guinea-pigs is carried out on a batch of product prepared from the new working seed lot; the product is shown to be not significantly different in activity from the

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comparison product. Antimicrobial agent sensitivity testing is also carried out.

Only a working seed lot that complies with the following requirements may be used for propagation.

Identification

The bacteria in the working seed lot are identified as *Mycobacterium bovis BCG* using microbiological techniques, which may be supplemented by molecular biology techniques (for example, nucleic acid amplification and restriction-fragment-length polymorphism).

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11), using 10 ml for each medium. The working seed lot complies with the test for sterility, except for the presence of mycobacteria.

Virulent mycobacteria. Examine the working seed lot as prescribed under Tests, using 10 guinea-pigs.

PROPAGATION AND HARVEST

The bacteria are grown in a suitable medium for not more than 21 days by surface or submerged culture. The culture medium does not contain substances known to cause toxic or allergic reactions in human beings or to cause the bacteria to become virulent for guinea-pigs. The culture is harvested and suspended in a sterile liquid medium that protects the viability of the culture as determined by a suitable method of viable count.

FINAL BULK

The final bulk is prepared from a single harvest or by pooling a number of single harvests. A stabilizer may be added; if the stabilizer interferes with the determination of bacterial concentration on the final bulk, the determination is carried out before addition of the stabilizer.

Only final bulk that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11), using 10 ml of final bulk for each medium. The final bulk complies with the test for sterility, except for the presence of mycobacteria.

Count of viable units

Determine the number of viable units per milliliter on solid medium using a method suitable for the product (Using not less than 5 containers before freeze drying) to be examined or by a suitable biochemical method. Carry out the test in parallel on a reference preparation of the same strain.

Bacterial concentration

Determine the total bacterial concentration by a suitable method, either directly by determining the mass of the micro-organisms, or indirectly by an opacity method that has been calibrated in relation to the mass of the micro-organisms; if the bacterial concentration is determined before addition of a stabilizer, the concentration in the final bulk is established by calculation. The total bacterial concentration is within the limits approved for the particular product.

The ratio of the count of viable units to the total bacterial concentration is not less than that approved for the particular product by National Regulatory Authority.

Virulent mycobacteria. Examine the final bulk as prescribed under Test using 6 guinea-pigs.

FINAL LOT

The final bulk is distributed into sterile containers and freeze-dried to moisture content favorable to the stability of the product; the containers are closed either under vacuum or under an inert gas.

Except where the filled and closed containers are stored at a temperature of – 20° or lower, the expiry date is not later than four years from the date of harvest.

Only a final lot that complies with the following requirement for count of viable units and with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the test for virulent Mycobacterium has been carried out with satisfactory results, it may be omitted on the final lot.

Count of viable units

Determine the number of viable units per milliliter of the reconstituted product on solid medium using a method suitable for the product to be examined, or by a suitable biochemical method. The ratio of the viable units after freeze-drying to that before is not less than that approved for the particular product.

Reconstituted product contains 1- 19.2 x 10^8 colony forming units per vial.

Identification

BCG for immunotherapy is identified by microscopic examination of the bacilli in stained smears demonstrating their acid-fast property and by the characteristic appearance of colonies grown on solid medium. Alternatively, molecular biology techniques (for example, nucleic acid amplification) may be used.

Tests

Water (2.3.43). Not more than the limit approved for the particular product, determined by a suitable method.

Virulent mycobacteria. Inject subcutaneously or intramuscularly into each of 6 guinea-pigs, each weighing

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250 to 400 g and having received no treatment likely to interfere with the test, a quantity of the product to be examined equivalent to at least 1/25 of 1 human dose. Observe the animals for at least 42 days. At the end of this period, euthanise the guinea-pigs and examine by autopsy for signs of infection with tuberculosis, ignoring any minor reactions at the site of injection. Animals that die during the observation period are also examined for signs of tuberculosis. The product complies with the test if none of the guinea-pigs shows signs of tuberculosis and if not more than 1 animal dies during the observation period. If 2 animals die during this period and autopsy does not reveal signs of tuberculosis, repeat the test on 6 other guinea-pigs. The product complies with the test if not more than 1 animal dies during the 42 days following the injection and autopsy does not reveal any sign of tuberculosis.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11). The reconstituted product complies with the test for sterility, except for the presence of mycobacteria.

Assay

Potency. Determine the number of viable units in the reconstituted product on solid medium (2.2.5) using a method suitable for the product to be examined or by a suitable validated biochemical method. Add a suitable surfactant such as Tween 80 in the diluent used for reconstituting the vaccine. The number is within the range stated on the label. Determine the number of viable units in the comparison control in parallel.

Labelling. The label states (1) the minimum and the maximum number of viable units per vial in the reconstituted product; (2) that the product must be protected from direct sunlight at a temperature between 2° and 8°; (3) for intravesical instillation only; not intended for immunization; (4) to be used immediately after reconstitution.

Bivalent Poliomyelitis Vaccine Type 1 and 3, Live (Oral)

Bivalent Oral Poliomyelitis Vaccine Type 1 and 3 is a preparation of approved strains of live attenuated poliovirus type 1and 3 grown in *in vitro* cultures of approved cells, containing Polioviruses of Type 1 and 3 (-Sabin strains), prepared in a form suitable for oral administration. The vaccine is a clear liquid that may be colored owing to the presence of a pH indicator.

Production

General provisions

The vaccine strains and the production method should consistently yield vaccines that are both immunogenic and safe in man.

The production of vaccine is based on a virus seed-lot system. Cell lines are used according to a cell-bank system. If primary monkey kidney cells are used, production complies with the requirements indicated below. Unless otherwise justified and authorized, the virus in the final vaccine shall not have undergone more than two passages from the master seed lot.

Substrate for virus propagation

The virus is propagated in human diploid cells (2.7.2) or in continuous cell lines (2.7.2) or in primary monkey kidney cells (including serially passaged cells from primary monkey kidney cells). Continuous cell lines are approved by the competent authority.

Primary monkey kidney cells. *The following special requirements for the substrate for virus propagation apply to primary monkey kidney cells.*

Monkeys used for preparation of kidney cell cultures and for testing of virus. If the vaccine is prepared in monkey kidney cell cultures, animals of a species approved by the competent authority, in good health, and not previously employed for experimental purpose shall be used.

The monkeys shall be kept in well-constructed and adequately ventilated animal rooms in cages spaced as far apart as possible. Adequate precautions shall be taken to prevent cross-infection between cages. Not more than two monkeys shall be housed per cage and cage-mates shall not be interchanged. The monkeys shall be kept in the country of manufacture of the vaccine in quarantine groups for a period of not less than 6 weeks before use. A quarantine group is a colony of selected, healthy monkeys kept in one room, with separate feeding and cleaning facilities, and having no contact with other monkeys during the quarantine period. If at any time during the quarantine period the overall death rate of a shipment consisting of one or more groups reaches 5 per cent (excluding deaths from accidents or where the cause was specifically determined not to be an infectious disease), monkeys from the entire shipment shall continue in quarantine from that time for a minimum of 6 weeks. The groups shall be kept continuously in isolation, as in quarantine, even after completion of the quarantine period, until the monkeys are used. After the last monkey of group has been taken, the room that housed the group shall be thoroughly cleaned and decontaminated before being used for fresh group. If kidneys from near-term monkeys are used, the mother is quarantined for the term of pregnancy.

Monkeys from which kidneys are to be removed shall be anaesthetized and thoroughly examined, particularly for evidence of tuberculosis and *Cercopithecid herpesvirus1 (B virus)* infection.

If a monkey shows any pathological lesion relevant to the use of its kidneys in the preparation of seed lot or vaccine, it shall

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not be used, nor shall any of the remaining monkeys of the quarantine group concerned be used unless it is evident that their use will not impair the safety of the product.

All the operations described in this section shall be conducted outside the areas where the vaccine is produced.

The monkeys used shall be shown to be free from antibodies to simian virus 40 (SV40) and simian immunodeficiency virus, If *Macaca spp*. Are used for production, the monkeys shall also be shown to be free from antibodies to cercopithecid herpesvirus 1 (B virus). Human herpesvirus has been used as an indicator for freedom from B virus antibodies on account of the danger of handling cercopithecide herpesvirus 1 (B virus).

Monkey kidney cell cultures for vaccine production. Kidneys that show no pathological signs are used for preparing cell cultures. If the monkeys are from a colony maintained for vaccine production, serially passaged monkey kidney cell cultures from primary monkey kidney cells may be used for virus propagation, otherwise the monkey kidney cells are not propagated in series. Virus for the preparation of vaccine is grown by aseptic methods in such cultures. If animal serum is used in the propagation of the cells, the maintenance medium after virus inoculation shall contain no added serum.

Each group of cell cultures derived from a single monkey or from fetuses from no more than ten near-term monkeys is prepared and tested as an individual group.

SEED LOT

The strains of poliovirus used shall be identified by historical records that include information on the origin and subsequent manipulation of the strains.

Working seed lots are prepared by a single passage from a master seed lot and at an approved passage level from the original Sabin virus. Virus seed lots are prepared in large quantities and stored at a temperature below – 60°.

Only a virus seed lot that complies with the following requirements may be used for virus propagation.

Identification

Each working seed lot is identified as poliovirus of the given type, using specific antibodies.

Virus concentration. Determined by the method described below, the virus concentration is the basis for the quantity of virus used in the neurovirulence test.

Extraneous agents (2.7.3). If the working seed lot is produced in human diploid cells (2.7.2) or in continuous cell lines (2.7.2) it complies with the requirements for seed lot for virus vaccines. If the working seed lot is produced in primary monkey cells, it complies with the requirement given below under Propagation and Harvest and Monovalent Pooled Harvest and with the tests in adult mice, suckling mice and guinea-pigs given under Test for extraneous agents in viral vaccines for human use.

Working seed lot shall be free from detectable DNA sequences from simian virus 40 (SV40)

Neurovirulence (2.7.6). Each master and working seed lot complies with the test for neurovirulence of poliomyelitis vaccine (oral) in monkeys. Furthermore, the seed lot shall cease to be used in vaccine production if the frequency of failure of the monovalent pooled harvests produced from it is greater than predicted statistically. This statistical prediction is calculated after each test on the basis of all the monovalent pooled harvests tested; it is equal to the probability of false rejection on the occasion of a first test (i.e. 1 per cent), the probability of false rejection on retest being negligible. If the test is carried out only by the manufacturer, the test slides are provided to the control authority for assessment.

Genetic markers. Each working seed lot is tested for its replicating properties at temperatures ranging from 36° to 40° as described under Monovalent Pooled Harvest.

PROPAGATION AND HARVEST

All processing of cell-banks and subsequent cell-cultures is done under aseptic conditions in an area where no other cells are handled. Approved animal (but not human) serum may be used in the media, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from live extraneous agents. The cell-culture medium may contain a pH indicator such a phenol red and approved antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. Not less than 5 per cent and not more than 1000 ml of the cell cultures employed for vaccine production are set aside as uninfected cell cultures (control cells); special requirements, given below, apply to control cells when the vaccine is produced in primary monkey cells. The virus suspension is harvested not later than 4 days after virus inoculation. After inoculation of the production cell culture with the virus working seed lot, inoculated cells are maintained at a fixed temperature, shown to be suitable, within the range 33° to 35° the temperature is maintained constant to ± 0.5° control cell cultures are maintained at 33° to 35° for the relevant incubation periods. Only a single virus harvest hat complies with the following requirement may be used in the preparation of the monovalent pooled harvest.

Virus concentration. The virus concentration of virus harvests is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

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Extraneous agents (2.7.3).Complies with tests for extraneous agents.

Control cells. The control cells of the production cell cultures from which the virus harvest is derived comply with a test for identity and with the requirements for extraneous agents or, where primary monkey cells are used, as shown below:

Primary monkey kidney cells.*The following special requirements apply to virus propagation and harvest in primary monkey kidney cells.*

Cell cultures. On the day of inoculation with virus seed, each cell culture is examined for degeneration caused by an infective agent. If, in this examination, evidence is found of the presence in a cell culture of any extravenous agent, the entire group of cultures concerned shall be rejected.

On the day of inoculation with the virus working seed lot, a sample of at least 30 ml of the pooled fluid removed from the cell cultures of the kidneys of each single monkey or from fetuses from not more than ten near-term monkeys is divided into two equal portions. One portion of the pooled fluid is tested in monkey kidney cell cultures prepared from the same species, but not the same animal, as that used for vaccine production. The other portion of the pooled fluid is, where necessary, tested in monkey kidney cell cultures from another species so that tests on the pooled fluids are done in cell cultures from at least one species known to be sensitive to SV40. The pooled fluid is inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet is at least 3 cm^2 per ml of pooled fluid. At least one bottle of each kind of cell culture remains uninoculated to serve as a control. If the monkey species used for vaccine production is known to be sensitive to SV40, a test in a second species is not required. Animal serum may be used in the propagation of the cells, provided that it does not contain SV40 antibody, but the maintenance medium after inoculation of test material contains no added serum except as described below.

The cultures are incubated at a temperature of 35° to 37° and are observed for a total period of at least 4 weeks. During the observation period and after not less than 2 weeks incubation, at least one subculture of fluid is made from each of these cultures in the same cell culture system. The subcultures are also observed for at least 2 weeks.

Serum may be added to the original culture at the time of sub culturing, provided that the serum does not contain SV 40 antibody.

Fluorescent-antibody techniques may be useful for detecting SV40 virus and other viruses in the cells.

A further samples of at least 10 ml of the pooled fluid is tested for cercopithecid herpesvirus 1 (B virus) and other viruses in rabbit kidney cell cultures. Serum used in the nutrient medium of these cultures shall have been shown to be free from inhibitors of B virus. Human herpesvirus has been used as an indicator for freedom from B virus inhibitors on account of the danger of handling cercopithecid herpesvirus 1 (B virus). The sample is inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet is at least 3 cm^2 per ml of pooled fluid. At least one bottle of the cell cultures remains uninoculated to serve as a control.

The cultures are incubated at a temperature of 35° to 37° and observed for at least 2 weeks.

A further sample of 10 ml of the pooled fluid removed from the cell cultures on the day of inoculation with the seed lot virus is tested for the presence of extraneous agents by inoculation into human cell cultures sensitive to measles virus.

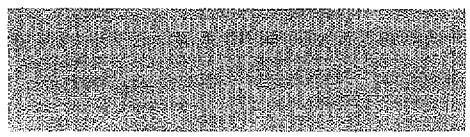
The tests are not valid if more than 20 per cent of the culture vessels have been discarded for non-specific accidental reasons by the end of the respective test periods.

If, in these tests, evidence is found of the presence of an extraneous agent, the single harvest from the whole group of cell cultures concerned is rejected.

If the presence of *Cercopithecid herpesvirus* 1 (B virus) is demonstrated, the manufacture of oral poliomyelitis vaccine shall be discontinued and the competent authority shall be informed. Manufacturing shall not be resumed until a thorough investigation has been completed and precautions have been taken against any reappearance of the infection, and then only with the approval of the competent authority.

If these tests are not done immediately, the samples of pooled cell-culture fluid shall be kept at a temperature of -60° or below, with the exception of the sample for the test for B virus, which may be held at 4°, provided that the test is done not more than 7 days after it has been taken.

Control cell cultures. On the day of inoculation with the virus working seed lot 25 per cent (but not more than 2500 ml) of the cell suspension obtained from the kidneys of each single monkey or from not more than ten near-term monkeys is taken to prepare uninoculated control cell cultures. These control cell cultures are incubated in the same conditions as the inoculated cultures for at least 2 weeks and are examined during this period for evidence of cytopathic changes. The tests are not valid if more than 20 per cent of the control cell cultures have been discarded for non-specific accidental reasons. At the end of the observation period, the control cell cultures are examined for degeneration caused by an infectious agent. If this examination or any of the tests required in this section shows evidence of the presence in a control culture of any extraneous agent, the poliovirus grown in the corresponding inoculated cultures from the same group shall be rejected.



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Tests for haemadsorbing viruses. At the time of harvest or within 4 days of inoculation of the production cultures with the virus working seed lot, a sample of 4 percent of the control cell cultures is taken and tested for haemadsorbing viruses. At the end of the observation period, the remaining control cell cultures are similarly tested. The Tests are made as described in (2.7.3). *Tests for extraneous agents in viral vaccines for human use.*

Tests for other extraneous agents. At the time of harvest, or within 7 days of the day of inoculation of the production cultures with the working seed lot, a sample of at least 20 ml of the pooled fluid from each group of control cultures is taken and tested in two kinds of monkey kidney cell culture, as described above.

At the end of the observation period for the original control cell cultures, similar samples of the pooled fluid are taken and the tests referred to in this section in the two kinds of monkey kidney cell culture and in the rabbit cell cultures are repeated, as described above under Cell cultures.

If the presence of *Cercopithecid herpesvirus 1* (B virus) is demonstrated, the production cell cultures shall not be used and the measures concerning vaccine production described above must be undertaken.

The fluids collected from the control cell cultures at the time of virus harvest and at the end of the observation period may be pooled before testing for extraneous agents. A sample of 2 per cent of the pooled fluid is tested in each of the cell culture systems specified.

Single harvests

Tests for neutralized single harvests in monkey kidney cell cultures. A sample of at least 10 ml of each single harvest is neutralized by a type-specific poliomyelitis antiserum prepared in animals other than monkeys. In preparing antisera for this purpose, the immunizing antigens used shall be prepared in non-simian cells.

Half of the neutralized suspension (corresponding to at least 5 ml of single harvest) is tested in monkey kidney cell cultures prepared from the same species, but not the same animal, as that used for vaccine production. The other half of the neutralized suspension is tested, if necessary, in monkey kidney cell cultures from another species so that the tests on the neutralized suspension are done in cell cultures from at least one species known to be sensitive to SV40.

The neutralized suspensions are inoculated into bottles of these cell cultures in such a way that the dilution of the suspension in the nutrient medium does not exceed 1 in 4. The area of the cell sheet is at least 3 cm^2 per ml of neutralized suspension. At least one bottle of each type of cell culture remains uninoculated to serve as a control and is maintained by nutrient medium containing the same concentration of the specific antiserum used for neutralization.

Animal serum may be used in the propagation of the cells, provided that it does not contain SV40 antibody, but the maintenance medium, after the inoculation of the test material, contains no added serum other than the poliovirus neutralizing antiserum, except as described below.

The cultures are incubated at a temperature of 35° to 37° and observed for a total period of at least 4 weeks. During this observation period and after not less than 2 weeks incubation, at least on subculture of fluid is made from each of these cultures in the same cell-culture system. The subcultures are also observed for at least 2 weeks.

Serum may be added to the original cultures at the time of sub culturing, provided that the serum does not contain SV40 antibody.

Additional tests are made for extraneous agents on a further sample of the neutralized single harvests by inoculation of 10 ml into human cell cultures sensitive to measles virus.

Fluorescent-antibody techniques may be useful for detecting SV40 virus and other viruses in the cells.

The tests are not valid if more than 20 per cent of the culture vessels have been discarded for non-specific accidental reasons by the end of the respective test periods.

If any cytopathic changes occur in any of the cultures, the causes of these changes are investigated. If the cytopathic changes are shown to be due to unneutralised poliovirus, the test is repeated. If there is evidence of the presence of SV40 or other extraneous agents attributable to the single harvest that single harvest is rejected.

MONOVALENT POOLED HARVEST

Monovalent pooled harvests are prepared by pooling a number of satisfactory single harvests of the same virus type. Monovalent pooled harvests from continuous cell lines may be purified. Each monovalent pooled harvest is filtered through a bacteria-retentive filter.

Only a monovalent pooled harvest that complies with the following requirement may be used in the preparation of the final bulk vaccine.

Identification

Each monovalent pooled harvest is identified as poliovirus of the given type, using specific antiserum.

Virus concentration

The virus concentration is determined by the method described below and serves as the basis for calculating the

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dilutions for preparation of the final bulk, for the quantity of virus used in the neurovirulence test and to establish and monitor production consistency.

Genetic markers. A ratio of the replication capacities of the virus in the monovalent pooled harvest is obtained over a temperature range between 36° and 40° in comparison with the seed lot or a reference preparation for the marker tests and with appropriate rct/40- and rct/40+ strains of poliovirus of the same type. The incubation temperatures used in this test are controlled to within ± 0.1°. The monovalent pooled harvest passes the test if, for both the virus in the harvest and the appropriate reference material, the titre determined at 36° is at least 5.0 log greater than that determined at 40°. If growth at 40° is so low that a valid comparison cannot be established, a temperature in the region of 39.0° to 39.5° is used, at which temperature the reduction in titre of the reference material must be in the range 3.0 to 5.0 log of its value at 36°; the acceptable minimum reduction is determined for each virus strain at a given temperature. If the titres obtained for one or more of the reference viruses are not concordant with the expected values, the test must be repeated.

Neurovirulence (2.7.6). Each monovalent pooled harvest complies with the test for neurovirulence of poliomyelitis vaccine (oral). If the test is carried out only by the manufacturer, the test slides are provided to the competent authority for assessment. The TgPVR21 transgenic mouse model provides a suitable alternative to the monkey neurovirulence test for neurovirulence testing of types 1and 3 vaccines once a laboratory qualifies as being competent to perform the test and the experience gained is to be satisfaction of the competent authority. The test is carried out using a standard operating procedure approved by the competent authority. A suitable procedure (*Neurovirulence test of type 1, and 3 live poliomyelitis vaccine (oral) in transgenic mice susceptible to poliovirus)* is available from WHO, Quality and Safety of Biologicals, Geneva.

Primary monkey kidney cells. *The following special requirements are applicable to monovalent pooled harvests derived from primary monkey kidney cells.*

Retroviruses. The monovalent pooled harvest is examined using a reverse transcriptase assay. No indication of the presence of retrovirus is found.

Test on rabbits. A sample of the monovalent pooled harvest is tested for *cercopithecid* herpesvirus 1 (B virus) and other viruses by injection of not less than 100 ml into not fewer than 10 healthy rabbits each weighing between 1.5 kg and 2.5 kg. Each rabbit receives not less than 10 ml and not more than 20 ml, of which 1 ml is given intradermally at multiple sites and the remainder subcutaneously. The rabbits are observed for at least 3 weeks for death or signs of illness.

All rabbits that die after the first 24 h of the test and those showing signs of illness are examined by autopsy, and the brain and organs removed for detailed examination to establish the cause of death.

The test is not valid if more than 20.0 per cent of the inoculated rabbits show signs of intercurrent infection during the observation period. The monovalent pooled harvest passes the test if none of the rabbits shows evidence of infection with B virus or with other extraneous agents or lesions of any kind attributable to the bulk suspension.

If the presence of B virus is demonstrated, the measure concerning vaccine production described above under Cell cultures are taken.

Test on guinea pigs. Administer to not less than five guinea-pigs, each weighing between 350 g and 450 g, 0.1 ml of the monovalent pooled harvest by intracerebral injection and 0.5 ml by intraperitoneal injection. Measure the rectal temperature of each animal on each working day for 6 weeks. At the end of the observation period, carry out autopsy on each animal.

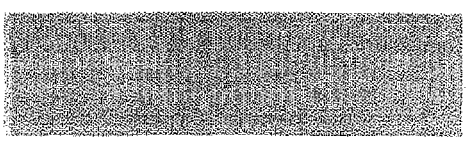
In addition, administer to not fewer than five guinea-pigs 0.5 ml by intraperitoneal injection and observe as described above for 2 to 3 weeks. At the end of the observation period, carry out a passage from these animals to not fewer than five guinea pigs using blood and a suspension of liver or spleen tissue. Measure the rectal temperature of the latter guinea pigs for 2 to 3 weeks. Examine by autopsy all animals that, after the first day of the test, die or are killed because they show disease or show for three consecutive days a body temperature higher than 39°; carry out histological examination to detect infection with Marburg virus; in addition, inject a suspension of liver or spleen tissue or of blood intraperitoneally into not fewer than three guinea pigs. If any signs of infection with Marburg virus are noted,confirmatory serological tests are carried out on the blood of the affected animals. The monovalent pooled harvest complies with the test if not fewer than 80.0 per cent of the guinea pigs survive to the end of the observation period and remain in good health and no animal shows signs of infection with filoviruses virus.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more satisfactory monovalent pooled harvests of Poliovirus Type 1 and 3. Suitable flavouring substances and stabilizers may be added.

Only a final bulk vaccine comply that complies with the following requirement may be used in the preparation of the final lot.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).



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FINAL LOT

Only a final lot that complies with the following requirement for thermal stability and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Thermal stability. Expose samples of the final lot at 37° for 48 hours. Determine the total virus concentration as described under assay in parallel for the heated vaccine and for unheated vaccine. The estimated difference between the total virus concentration of the unheated and heated vaccines is not greater than 0.5 $\log_{10}$ infectious virus units ($CCID_{50}$) per single dose.

Identification

The vaccine is shown to contain poliovirus of each type stated on the label, using specific antibodies.

Tests

Bacterial and fungal contamination. The vaccine complies with the test for sterility (2.2.11).

Assay

Titrate for infectious virus using no fewer than three separate vials of vaccine, using the method described below. Use an appropriate virus reference preparation to validate each assay. Titrate each type separately, using appropriate type-specific antiserum (or preferably a monoclonal antibody) to neutralize each of the other types present.

In Bivalent vaccine the estimated mean virus titres must be: not less than $1 \times 10^{6.0}$ infectious virus units ($CCID_{50}$) per single human dose for type 1 and not less than $1 \times 10^{5.8}$ infectious virus units ($CCID_{50}$) for type 3 per single human dose.

Method. Groups of eight to twelve flat-bottomed wells in a microtitre plate are inoculated with 0.05 ml of each of the selected dilutions of virus followed by a suitable cell suspension of the Hep-2 (Cincinnati) line. The plates are incubated at a suitable temperature. Examine the cultures on days 7 to 9.

The assay is not valid if (a) the confidence interval (P=0.95) of the logarithm of the virus concentration is greater than ±0.3; (b) the virus concentration of the reference preparation differs by more than 0.5 log $CCID_{50}$ from the assigned value.

Labelling. The label states (1) the types of poliovirus contained in the vaccine; (2) the minimum amount of virus of each type contained in one single human doses; (3) the cell substrate used for the preparation of the vaccine; (4) that the vaccine is not to be injected.

Cholera Vaccine (Inactivated, Oral)

Cholera vaccine (inactivated, oral) is a homogeneous suspension of inactivated suitable strains of *Vibrio cholerae* serogroup O1 alone or in combination with O139, representing serotypes and biotypes of epidemic strains. The vaccine may contain the B subunit of cholera toxin (CTB). As required, just prior to ingestion, one dose of vaccine suspension is mixed with a suitable buffer as stated on the label.

Production

General provisions

The production method must be validated to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in human.

The production process must be validated to show that no clinically significant quantities of active toxin are present in the product.

CHOICE OF VACCINE STRAIN

The vaccine consists of a mixture of epidemic *V. cholerae* strains inactivated by a suitable method such as heat or formalin inactivation representative of various serogroups (O1 and O139), serotypes (Ogawa and Inaba) and biotypes (classical or ElTor). Preferably the selected strains express smooth lipopolysaccharide (LPS) and are low cholera-toxin producers. The CTB is produced by recombinant DNA technology in a strain that lacks the gene for cholera toxin subunit A (*ctxA*).

The national regulatory authority may recommend new vaccine strains or antigens that may be used if necessary, in accordance with the regulations in force.

SEED LOTS

The strains of *V. cholerae* used shall be identified by historical records that include information on the origin of the strains and by its biochemical and serological characteristics; and their subsequent manipulation if recombinant strains are used. Characterisation and maintenance of the recombinant strains and plasmids used for production of the recombinant B subunit of cholera toxin (rCTB) and the origin of the gene for cholera toxin subunit B (*ctxB*) are documented. The stability of the rCTB plasmid in the recombinant strain during storage and beyond the passage level used in production is confirmed.

Characterisation of the rCTB is undertaken using a variety of analytical techniques including determination of molecular size, charge and amino acid composition. Techniques suitable for such purposes include sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and different liquid chromatographies. The identity of the product is confirmed by at least partial *N*-terminal and *C*-terminal amino acid sequencing.

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Master seed lots are grown on agar plates which may contain appropriate antibiotics, and the colonies are used to produce working seed lots in liquid media that are free from antibiotics. Cultures derived from the working seed lot must have the same characteristics as the cultures of the strain from which the master seed lot was derived.

Only a seed lot that complies with the following requirements may be used in the preparation of the monovalent cell harvest.

Identification. Master seed lots are identified by colony morphology, and by biochemical characterization, using suitable molecular assays or immunoassays. Working seed lots are identified by colony morphology and by molecular assays or immunoassays.

Purity. Purity of master seed lots and working seed lots is verified by methods of suitable sensitivity.

PROPAGATION AND HARVEST

Each strain is grown separately from the working seed lot in suitable liquid medium.

Cultures are checked at different stages of fermentation (subcultures and main culture) for purity, identity, cell opacity and pH and biochemical characteristics.

Production cultures are shown to be consistent in respect of growth rate, pH and yield of cells or cell products.

MONOVALENT CELL HARVEST

Only a monovalent harvest that complies with established specifications for the following tests may be used.

pH (2.4.24). Within the range approved for the particular product. This test can be performed either in the monovalent cell harvest or in the inactivated monovalent cell harvest.

Identification. Relevant antigenic characteristics are verified by suitable immunological or biochemical assays. This test can be performed either in the monovalent cell harvest or in the inactivated monovalent cell harvest.

Purity. Samples of culture are examined by microscopy of Gram-stained smears, by inoculation of appropriate culture media or by another suitable procedure.

Opacity. The absorbance at 590/600nm is within the range established for the product.

INACTIVATED MONOVALENT CELL BULK

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. Bacteria are inactivated after washing, either by treatment with formaldehyde or another detoxifying agent or by heating under conditions that ensure inactivation.

Only an inactivated monovalent cell bulk that complies with established specifications for the following tests may be used in the preparation of the final bulk.

pH (2.4.24). Within the range approved for the product.

Identification. Should show agglutination with strain specific antiserum.

Inactivation. Complete inactivation is verified by a suitable culture method and no viable cells should be detected.

Bacterial and fungal contamination. It complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

Opacity. The inactivation process may affect the accuracy of opacity measurements.

Purity. Samples of culture are examined by microscopy of Gram-stained smears, by inoculation of appropriate culture media or by another suitable procedure.

LPS content. It is verified by a suitable immunoassay (2.2.14).

Residual cholera toxin content. The residual cholera toxin content is verified by a suitable immunoassay (2.2.14) or biochemical assay, and the limit to be assigned as approved by national regulatory authority.

Free formaldehyde(2.3.20). To be determined where formaldehyde is used for inactivation and residual concentration is not more than 0.02 per cent w/v.

Preservative. Content to be determined, if used.

PURIFIED rCTB

Production of the rCTB follows the guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant technology. Prior to harvest, the cell culture is checked for purity and opacity. rCTB is harvested by suitable filtration, concentrated by diafiltration, purified by chromatography, filter-sterilised and stored under suitable conditions. The pH of the pooled eluate is adjusted prior to buffer exchange.

Only purified rCTB that complies with established specifications for the following tests may be used in the preparation of the final bulk.

pH (2.4.24). Within the range approved for the particular product.

Purity. Verified by SDS-PAGE and an appropriate liquid chromatography method.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

rCTB. The amount of rCTB is determined by a suitable immunoassay (2.2.14).

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FINAL BULK

The final bulk vaccine is prepared by aseptically mixing a suitable buffer with monovalent cell bulks. Where used, the rCTB bulk is added in appropriate amounts. Preservatives, if used, may be added at this stage.

Only a final bulk that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. Complies with the test for sterility, carried out using 10 ml for each medium.

Free formaldehyde (2.3.20). Not more than 0.02 per cent where applicable.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not more than 115 per cent of the intended amount. This test can be performed either in the final bulk or in the final lot.

FINAL LOT

The final bulk is mixed to homogeneity and filled aseptically into suitable containers.

Only a final lot that is within the limits approved for the particular product and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Identification

Serotypes are detected by a suitable immunoassay (2.2.14) or molecular assay. rCTB is detected by a suitable immunoassay. The antigen-content assays may also serve as an identity test.

Tests

pH (2.4.24). Within the range approved for the product.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Assay

Antigen content. The total amount of LPS and where applicable, the amount of rCTB, is within the limits approved for the particular product, determined by a suitable immunoassay (2.2.14). For LPS, *the cumulative antigen content of all the constituent strains shall not be less than 2,100 lipo-polysaccharide ELISA units for 1 SHD.*

Labelling. The label states (1) the method of inactivation; (2) the serogroup, serotypes and biotypes of vaccine strains; (3) the number of bacteria per human dose; (4) the amount of rCTB; (5) the preservative content; (6) that the vaccine is for oral use, only; (7) storage temperature; (8) manufacturing and expiry date.

Diphtheria Antitoxin

Diphtheria Antitoxin is a preparation containing the specific antitoxic globulins or their derivatives obtained by purification of hyperimmune serum or plasma of healthy horses or other suitable animals and having the specific activity of neutralising the toxin formed by *Corynebacterium diphtheriae*. The liquid preparation may contain a suitable antimicrobial preservative.

Diphtheria Antitoxin has a potency of not less than 1000 Units per ml when obtained from horse serum and not less than 500 Units per ml when obtained from other animals.

Description. A clear, colourless or pale yellow liquid or a freeze-dried, cream-coloured powder or pellet.

Identification

Specifically neutralises and renders the toxin formed by *C. diphtheriae* harmless to susceptible animals or by any other suitable *in-vitro* test.

Tests

Potency. Carry out the biological assay of diphtheria antitoxin described below.

Biological Assay of Diphtheria Antitoxin

The potency of diphtheria antitoxin is determined by comparing the dose necessary to protect guinea-pigs or rabbits against the erythrogenic effects of a fixed dose of the Standard Preparation of diphtheria antitoxin necessary to give the same protection. For this purpose, a suitable preparation of diphtheria toxin is required to be used as a test toxin. The test dose of the toxin is determined in relation to the Standard Preparation. The potency of the preparation under examination is then determined in relation to the Standard Preparation using the test toxin.

Standard Preparation

The Standard Preparation is the 1st International Standard for Diphtheria antitoxin, equine, consisting of the dried hyperimmune horse serum and glycerin, or another suitable preparation the potency of which has been determined in relation to the International Standard.

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Suggested Method

Test toxin. Prepare diphtheria toxin by filtering through bacteria-proof filter the medium in which a toxigenic strain of *C. diphtheriae* has grown. Store at a temperature of 2° and 8°.

Selection of test toxin. In selecting a toxin for use as the test toxin determine the following.

Lr/100 dose — This is the smallest quantity of the toxin which, when mixed with 0.01 Unit of antitoxin and injected intracutaneously into guinea-pigs or rabbits causes a characteristic reaction at the site of the injection within 48 hours.

Minimal reacting dose — This is the smallest quantity of toxin which, when injected intracutaneously into guinea-pigs or rabbits, causes a characteristic reaction at the site of injection within 48 hours.

A suitable toxin is one which contains at least 200 minimal reacting doses in the Lr/100 dose. The test toxin is allowed to stand for some months before being used for the assay of samples of antitoxin. During this time its toxicity declines and the Lr/100 dose may be slightly increased. When experiment shows that the Lr/100 dose is constant, the test toxin is ready for use and may be used for a long period. Determine the minimal reacting dose and the Lr/100 dose at frequent intervals. Store the test toxin in the dark at a temperature between 0° and 5°. Maintain its sterility by the addition of *toluene* or other antimicrobial preservative which does not cause a rapid decline in specific toxicity.

Determination of test dose of toxin (*Lr/100 dose*). Prepare a solution of the Standard Preparation with *saline solution* such that 1 ml contains 0.1 Unit. Prepare mixtures such that 2.0 ml of each mixture contains 1.0 ml of the dilution of the Standard Preparation (0.1 Unit) and one of a series of graded volumes of the test toxin. Dilute each mixture with *saline solution* to the same final volume (2.0 ml). Allow the mixtures to stand at room temperature, protected from light, for 15 to 60 minutes and inject intracutaneously 0.2 ml of each mixture at suitably spaced sites into the shaven or depilated flanks of two animals. Observe the animals for 48 hours.

The test dose (Lr/100) of the toxin is the amount present in 0.2 ml of that mixture which causes at the site of injection a small, characteristic reaction in the skin of the guinea-pig or rabbit. Mixtures containing larger amounts of toxin cause larger reaction and necrosis and mixtures containing smaller amount of toxin cause no reaction.

Determination of potency of the antitoxin. Dilute the test toxin with *saline solution* so that 1.0 ml contains 10 times the test dose. Prepare mixtures such that 2.0 ml of each mixture contains 1.0 ml of the dilution of the toxin and one of a series of graded volumes of the preparation under examination. Prepare further mixtures such that 2.0 ml of each contains 1.0 ml of the solution of the test toxin and 0.1 Unit of antitoxin. Dilute each mixture with *saline solution* to the same final volume (2.0 ml). Allow the mixtures to stand at room temperature, protected from light, for 15 to 60 minutes. Inject a dose of 0.2 ml of each mixture into the animals under the conditions described in the determination of the Lr/100 dose of the toxin.

The mixture of the preparation under examination that contains 0.01 Unit of antitoxin in 0.2 ml is the mixture that produces the same degree of local reaction as that produced by the injection into the same animals of the mixture of the Standard Preparation that contains in 0.2 ml the test dose (Lr/100) of the toxin and 0.01 Unit of antitoxin.

When at least four distinct tests are carried out by this method, the limits of error have been estimated to be between 90 per cent and 111 per cent.

Other tests. Complies with the tests stated under Antisera.

Storage. As stated under Antisera.

Labelling. The label states (1) the number of Units per ml; (2) the species of animal from which the preparation has been made; (3) the recommended dose (if space is inadequate, it may be stated in the instruction leaflet); (4) the name and proportion of any added preservative; (5) that the preparation, if liquid, should not be allowed to freeze; (6) that the preparation, if dried, should be used immediately after reconstitution in the stated quantity of the diluent supplied by the manufacturer.

Diphtheria and Tetanus Vaccine (Adsorbed)

Diphtheria and Tetanus Vaccine (Adsorbed) is a preparation of diphtheria formol toxoid and tetanus formol toxoid adsorbed on mineral carrier. The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

The specification for individual component used in formulation is referred in the text of individual monograph.

Production

General provisions

Bulk purified diphtheria and tetanus toxoids

The bulk purified diphtheria and tetanus toxoids are prepared as described in the monographs on Diphtheria vaccine (adsorbed) and Tetanus vaccine (adsorbed) and comply with the requirements prescribed therein.

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FINAL BULK VACICNE

The final bulk vaccine is prepared by adsorption of suitable quantities of bulk purified diphtheria toxoid and tetanus toxoid onto mineral carrier such as hydrated aluminium phosphate, aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Antimicrobial preservatives of the phenolic type must not be used.

Only final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

pH (2.4.24). 6.0 to 7.0.

Specific toxicity. Use 5 normal, healthy guinea-pigs weighing between 250 and 350 g which have been maintained for at least 1 week on a uniform, unrestricted diet, and have not been previously treated with any material that will interfere with the test. Weigh the animals separately and record their weights. Inject subcutaneously into each animal 5 times the dose stated on the label. Weigh all the animals at weekly intervals for 6 weeks. None of the animals shows any symptoms of diphtheria or tetanus toxaemia or dies from diphtheria within 42 days or loses weight at the end of the test. If more than one animal dies from non-specific causes or loses weight, repeat the test. If an animal dies or loses weight in the second test, the vaccine fails the test.

The specific toxicity test on the final bulk may be omitted for routine lot release once consistency of production has been established to the satisfaction of the NRA.

Assay

Diphtheria toxoid

Complies with the test as stated under Diphtheria Vaccine (Adsorbed).

Tetanus toxoid

Complies with the test as stated under Tetanus Vaccine (Adsorbed).

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk vaccine is filled and stored aseptically into sterile containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for specific toxicity and antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified toxoid or on the final bulk and it has been shown that the content in the final lot will not excced 0.2 g per litre, the test for free formaldehyde may be omitted on the final lot.

Identification

A. Diphtheria toxoid is identified by a suitable immuno-chemical method (2.2.14).

Dissolve in the vaccine under examination by adding sufficient *sodium citrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant liquid is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate or visible floccules.

B. Tetanus toxoid is identified by a suitable immuno-chemical method (2.2.14).

The clear supernatant liquid obtained during test A reacts with a suitable tetanus antitoxin, giving a precipitate or visible floccules.

Tests

Sterility (2.2.11). Complies with the test for sterility.

Aluminium (2.3.9). Not more than 1.25 mg per single human dose when hydrated aluminium phosphate or aluminium hydroxide is used as the adsorbent.

pH (2.4.24). The pH of the vaccine is within the range approved for the product (6.0 to 7.0).

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the quantity stated on the label.

Assay

Carry out one of the described methods for the assay of Diphtheria Vaccine (Adsorbed). (If already performed on final bulk the assay can be omitted on final lot).

Diphtheria component

a) Intradermal challenge method, b) Lethal challenge method, c) Antibody induction method, d) Validated serological assay in guinea-pigs or mice as approved by National Regulatory Authority.

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Tetanus component

Carry out one of the described methods for the assay of Tetanus Vaccine (Adsorbed) Viz a) Antibody induction method; b) Challenge method in guinea-pigs/mice; c) Validated serological assay in guinea-pigs or mice as approved by National Regulatory Authority.

Labelling. The label states (1) the human dose; (2) the minimum Lf units per single human dose or the minimum International Units per single human dose if potency test done by challenge method; (3) the name and the amount of the adsorbent and preservative; (4) that the vaccine must be shaken before use; (5) that the vaccine is not to be frozen.

Diphtheria and Tetanus Vaccine (Adsorbed) for Adults and Adolescents

Diphtheria and Tetanus Vaccine (Adsorbed) for Adults and Adolescents is a preparation of diphtheria formol toxoid and tetanus formol toxoid adsorbed on a mineral carrier. The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

Production

General provisions

Bulk purified diphtheria and tetanus toxoids

The bulk purified diphtheria and tetanus toxoids are prepared as described in the monographs on *Diphtheria vaccine (adsorbed)* and *Tetanus vaccine (adsorbed)* and comply with the requirements prescribed therein.

FINAL BULK VACCINE

The vaccine is prepared by adsorption of suitable quantities of bulk purified diphtheria toxoid and tetanus toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

pH (2.4.24). 6.0 to 7.0.

Specific toxicity

Inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing between 250 and 350 g, that have not previously been treated with any material that will interfere with the test. Weigh the animals separately and record their weights. Inject subcutaneously into each animal 5 times the dose stated on the label. Weigh all the animals at weekly intervals for 6 weeks. None of the animals shows any symptoms of diphtheria or tetanus toxaemia or dies from diphtheria within 42 days or loses weight at the end of the test. If more than one animal dies from non-specific causes or loses weight, repeat the test. If an animal dies or loses weight in the second test, the vaccine fails the test.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Sterility (2.2.11). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for free formaldehyde and antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Identification

A. Diphtheria toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine under examination sufficient *sodium citrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate. If a satisfactory result is not obtained with a vaccine adsorbed on aluminium hydroxide, carry out the test as follows.

Centrifuge 15 ml of the vaccine under examination and suspend the residue in 5 ml of a freshly prepared mixture of 1 volume of a 56 g/l solution of *sodium edetate* and 49 volumes of a 90 g/l solution of *disodium hydrogen phosphate*. Maintain at 37° for not less than 6 hours and centrifuge. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

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Tests

Aluminium (2.3.9). Maximum 1.25 mg per single human dose.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Complies with the test for sterility.

pH (2.4.24). 6.0 to 7.0.

Assay

Diphtheria component

Carry out the prescribed method for assay of Diphtheria Vaccine by lethal challenge method described in the assay of Diphtheria Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than 2 IU per single human dose.

Tetanus component

Carry out one of the prescribed methods for the assay as stated under Tetanus Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than 20 IU per single human dose.

Labelling. The label states (1) the human dose; (2) the minimum units/ International Units of each component per single human dose, if potency determined by challenge method; (3) the name and the amount of the adsorbent and preservative; (4) that the vaccine must be shaken before use; (5) that the vaccine is not to be frozen.

Diphtheria, Tetanus and Pertussis Vaccine (Adsorbed)

Diphtheria, Tetanus and Pertussis Vaccine (Adsorbed) is a preparation of diphtheria formol toxoid, tetanus formol toxoid adsorbed on mineral carrier and a suspension of killed *Bordetella pertussis* organisms. The formal toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively. The *Bordetella pertussis* suspension is prepared by growth of suitable strains in an appropriate medium, under controlled conditions.

The specification for individual component used in formulation is referred in the text of individual monograph.

Production

General Provisions

The production method must be validated to demonstrate that the product if tested, would comply with the tests for specific toxicity as described under monographs of Diphtheria Vaccine, Tetanus Vaccine (Adsorbed) and Pertussis Vaccine.

The bulk purified diphtheria and tetanus toxoids and inactivated *B. pertussis* suspension are prepared as described in the monograph on Diphtheria Vaccine (Adsorbed), Tetanus Vaccine (Adsorbed) and Pertussis Vaccine respectively and comply with the respective requirements.

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption of suitable quantities of bulk purified diphtheria toxoid and tetanus toxoid onto hydrated aluminium phosphate or aluminium hydroxide and admixture of an appropriate quantity of a suspension of inactivated *B. pertussis*. The *B. pertussis* concentration of the final bulk vaccine does not exceed that corresponding to an opacity of 20 IOU per single human dose. If two or more strains of *B. pertussis* are used, the composition of consecutive lots of the final bulk vaccine shall be consistent with respect to the proportion of each strain as measured in opacity units. Suitable antimicrobial preservatives may be added to the bulk vaccine. Antimicrobial preservatives particularly those of phenolic type which affect the antigenic activity must not be used.

Only final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not more than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk vaccine is filled and stored aseptically into sterile containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests, antimicrobial preservative and the Assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Identification

A. Diphtheria toxoid is identified by a suitable immunochemical method (2.2.14).

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Dissolve in the vaccine under examination by adding sufficient *sodium citrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant is obtained; reserve the precipitate for identification test C. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (2.2.14).

The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis component is identified by agglutination of the bacteria from the resuspended centrifugation residue (see identification test A; other suitable methods for separating the bacteria from the adsorbent may also be used) by antisera specific to *B. pertussis* or by the assay of the pertussis component.

Tests

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Specific toxicity

Diphtheria and tetanus components. Inject subcutaneously five times the single human dose stated on the label into each of five healthy guinea-pigs, each weighing between 250 and 350 g, that have not previously been treated with any material that will interfere with the test. The animals should be weighted every week and observations be made. None of the animals shows any symptoms of diphtheria or tetanus toxaemia or dies from diphtheria within 42 days or loses weight at the end of the test. If more than one animal dies from non-specific cause or loses weight, repeat the test. If an animal dies or loses weight in the second test, the vaccine fails the test.

Pertussis component. Use not less than 10 healthy mice each weighing between 14 and 16 g for the vaccine group and for the saline control. Use mice of the same sex or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 hours before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 ml, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 ml of a 0.9 per cent sterile solution of *sodium chloride*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the mice groups immediately before the injection and 72 hours and 7 days after the injection. The vaccine complies with the test if: (a) at the end of 72 hours the total mass of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of vaccinated mice should die during the test. The test may be repeated and the results of the tests combined.

The specific toxicity test for Tetanus, Diphtheria and Pertussis on the final bulk may be omitted for routine lot release once consistency of production has been established to the satisfaction of the National Regulatory Authority.

Aluminium (2.3.9). Not more than 1.25 mg per single human dose, when hydrated aluminium phosphate or aluminium hydroxide is used as the adsorbent.

pH (2.4.24). 6.0 to 7.0.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not more than115.0 per cent of the intended amount.

Assay

Diphtheria component

Carry out one of the methods for the assay as stated under Diphtheria Vaccine (Adsorbed).

Tetanus component

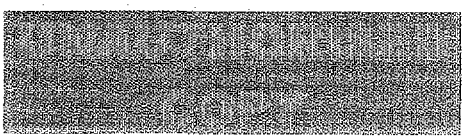
Carry out one of the methods for the assay as stated under Tetanus Vaccine (Adsorbed).

If test is carried out in Guinea-pigs, the lower confidence limit (P=0.95) of estimated potency is not less than 40 IU/Single human dose; if the test is carried in mice, the lower confidence limit (P=0.95) of estimated potency is not less than 60 IU/ Single human dose.

Pertussis component

Carry out the assay as stated under Pertussis Vaccine.

Labelling. The label states (1) in case done by challenge method the minimum number of International Units; if units (as applicable for each component) per single human dose; (2) In case done by antibody induction method the minimum number of International Units per single human dose of pertussis component and minimum number of Lf of diphtheria toxoid and tetanus toxoid; (3) the name and the amount of the adsorbent and preservative; (4) that the vaccine must be shaken before use; (5) that the vaccine is not to be frozen; (6) for vaccine contained in single-dose containers where the space is too small to accommodate the full name of the vaccine, the abbreviation 'DTP' may be used in the label and the container provided that the same code is also stated in the label on the package.



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Diphtheria, Tetanus, Pertussis (Whole Cell), Hepatitis B (rDNA) and *Haemophilus influenzae* Type b Conjugate Vaccine (Adsorbed)

Diphtheria, Tetanus, Pertussis (Whole cell), Hepatitis B (rDNA) and *Haemophilus influenzae* Type b Conjugate Vaccine (Adsorbed) is a combined vaccine composed of diphtheria formol toxoid containing not less than 1,500 Lf, (2.2.16) per mg of protein nitrogen, purified tetanus formol toxoid containing not less than 1,000 Lf, (2.2.16), per mg of protein nitrogen, hepatitis B surface antigen and *Haemophilus influenzae* Type b conjugated to suitable protein with a mineral adsorbent to which a suspension of killed *Bordetella pertussis* has been added. Mineral adsorbent is a suspension of hydrated aluminium hydroxide, aluminium phosphate or calcium phosphate, in saline solution or other appropriate solution isotonic with blood.

The formol toxoids are prepared from the toxin produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively, in suitable media. The toxins are converted to toxoids by treatment with formaldehyde solution by methods which avoid reversibility of the toxoids.

Hepatitis B surface antigen is a component protein of hepatitis B virus; the antigen is obtained by recombinant DNA technology.

The polysaccharide, polyribosyl ribitol phosphate, PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* Type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell dependent B-cell immune response to the polysaccharide.

The product may be presented with the haemophilus component in a separate container, the contents of which are mixed with the other components immediately before or during use.

The final product contains a suitable antimicrobial preservative. The antigenic properties of the vaccine are adversely affected by the presence of certain antimicrobial preservatives particularly those of the phenolic type and some of the quaternary ammonium type and must not be used.

Production

General provisions

The production method shall have been shown to yield consistently the vaccines comparable with the vaccine of proven clinical efficacy and safety in human.

If the vaccine is presented with the haemophilus component in a separate vial, as part of consistency studies the assays of the diphtheria, tetanus, pertussis and hepatitis B are carried out on a suitable number of batches of vaccine reconstituted as for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

The production method is validated to demonstrate that the product, if tested, would comply with the following test for specific toxicity of the diphtheria and tetanus component: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea pigs, each weighing between 250 and 350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria, toxemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal shows signs of or dies in the second test, the vaccine does not comply with the test.

The stability of the final lot and the relevant intermediates is evaluated using one or more indicator tests. For the haemophilus component, such tests may include determination of molecular size, determination of free PRP in the conjugate and kinetics of depolymerisation. Taking account of the results of the stability testing, release requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

Reference vaccine(s)

Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilized by a method that has been shown to have no effect on the assay procedure.

Production of the components

The production of the components complies with the requirements of the monographs on Diphtheria Vaccine (Adsorbed), Tetanus Vaccine (Adsorbed), Pertussis Vaccine, Hepatitis B Vaccine (rDNA) and *Haemophilus influenzae* Type b Conjugate Vaccine.

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FINAL BULK VACCINE

Vaccine with all components in the same container

The final bulk is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, bulk purified tetanus toxoid, bulk purified hepatitis B surface antigen onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, admixture of an appropriate quantity of a suspension of inactivated *B. pertussis* component and admixture of a suitable quantity of PRP conjugate; the resulting mixture is approximately isotonic with blood. The *B. pertussis* concentration of the final bulk vaccine does not exceed that corresponding to an opacity of 20 IU per single human dose. If 2 or more strains of *B. pertussis* are used, the composition of consecutive lots of the final bulk vaccine shall be consistent with respect to the proportion of each strain as measured in opacity units. Suitable antimicrobial preservatives may be added.

Vaccine with the haemophilus component in a separate container

The final bulk is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, bulk purified tetanus toxoid, bulk purified hepatitis B surface antigen onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, admixture of an appropriate quantity of a suspension of inactivated *B. pertussis* component and admixture of a suitable quantity of PRP conjugate; the resulting mixture is approximately isotonic with blood. The *B. pertussis* concentration of the final bulk vaccine does not exceed that corresponding to an opacity of 20 IU per single human dose. If 2 or more strains of *B. pertussis* are used, the composition of consecutive lots of the final bulk vaccine shall be consistent with respect to the proportion of each strain as measured in opacity units. The final bulk is filled separately. Suitable antimicrobial preservatives may be added. The final bulk of the haemophilus component is prepared by dilution of the bulk conjugate to the final concentration with a suitable diluent. A stabiliser may be added filled separately and lyophilisd.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85.0 per cent and not greater than 115.0 per cent of the intended content.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for specific toxicity of diphtheria toxoid, tetanus toxoid and pertussis component and antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot. Provided the content of free formaldehyde has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot. If an *in vivo* assay is used for the hepatitis B component, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Free PRP

Unbound PRP is determined after removal of the conjugate, for example by anion exchange, size exclusion or hydrophobic chromatography (2.4.16), ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

Osmolality (2.4.23). The osmolality of the vaccine is within the limits approved for the particular preparation.

pH (2.4.24). 6.0 to 7.0.

Description. Whitish turbid liquid in which the mineral carrier tends to settle down slowly on keeping.

Identification

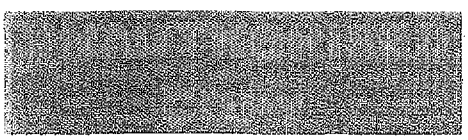
Tests A, B, C, D and E may be omitted if test F is carried out. Test F may be omitted if tests A, B, C, D and E are carried out.

A. *Diphtheria toxoid*. Dissolve sufficient *sodium citrate* in the vaccine under examination to give a 10 per cent w/v concentration. Maintain at 37° for about 16 hours and centrifuge. Reserve the residue for test C. The clear supernatant reacts with a suitable diphtheria antitoxin and yields a precipitate.

B. *Tetanus toxoid*. Tetanus toxoid is identified by suitable immunochemical method. The clear supernatant obtained as described in identification test A reacts with a suitable tetanus antitoxin to give a positive reaction, when tested by a suitable validated immunological method.

C. *Pertussis component*. To a suspension of the residue obtained in test A in *saline solution* add a suitable *Bordetella pertussis* antiserum; *agglutination* indicates presence of pertussis component.

D. *Hepatitis B surface antigen*. The suspension of the residue obtained in test A gives a positive reactions when tested by suitable *in-vitro* assay.



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E. *PRP*. The suspension of the residue obtained in the test A gives a positive reaction when tested by a suitable immunochemical method for PRP.

F. The vaccine confers an active immunity in mice and guinea-pigs when administered as directed in the test for Assay.

Tests

If the product is presented with the haemophilus component in a separate container; the tests for specific toxicity of diphtheria toxoid, tetanus toxoid and pertussis component, aluminium, free formaldehyde, antimicrobial preservative and sterility are carried out on the container with the diphtheria, tetanus, pertussis and hepatitis B components; the tests for PRP content, water (where applicable), sterility and pyrogens are carried out on the container with the haemophilus component.

If the haemophilus component is freeze-dried, some tests may be carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component under test.

Moisture content

The test should be performed on 1 vial per 1000 up to a maximum of 10 vials but on no less than 5 vials taken at random from throughout the final lot. The average residual moisture content should generally be no greater than 2.5 per cent and no vial should be found to have a residual moisture content of 3 per cent or greater.

PRP. Not less than 80.0 per cent and not more than 120.0 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (2.7.1), or phosphorus (2.7.1), by an immunochemical method (2.2.14) or by anion exchange liquid chromatography with pulsed amperometric detection.

Aluminium (2.3.9). Not more than 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and is not greater than 115 per cent of the quantity stated on the label.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Pyrogens (2.2.8). This test is carried out for *Haemophilus influenzae* Type b vaccine only if *Haemophilus influenzae* Type b vaccine is presented as separate lyophilized vial. The vaccine complies with the test for pyrogens. Inject per kg of the rabbit's mass a quantity of the vaccine equivalent to: 1 µg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria toxoid as carrier; 0.1 µg of PRP for a vaccine with tetanus toxoid as carrier protein; 0.025 µg of PRP for vaccine with OMP as carrier or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be within the limits approved by the National Regulatory Authority.

Specific toxicity

Diphtheria and tetanus components

Complies with the test as stated under Diphtheria and Tetanus Vaccine (Adsorbed).

Pertussis component

Complies with the test as stated under Diphtheria, Tetanus and Pertussis Vaccine (Adsorbed).

Assay

Diphtheria toxoid (adsorbed)

Complies with the test as stated under Diphtheria and Tetanus Vaccine (Adsorbed).

Tetanus component

Carry out one of the prescribed methods for the assay as stated under Tetanus Vaccine (Adsorbed).

If the test is carried out in guinea pigs, the lower confidence limit (P = 0.95) of the estimated potency is not less than 40 IU per single human dose; if the test is carried out in mice, the lower confidence limit (P = 0.95) of the estimated potency is not less than 60 IU per single human dose.

Pertussis vaccine

Complies with the test as stated under Diphtheria, Tetanus and Pertussis Vaccine (Adsorbed).

Hepatitis B surface antigen (adsorbed)

Complies with the test as stated under Hepatitis B Vaccine (Adsorbed).

Storage. When stored under the prescribed conditions the vaccine may be expected to retain potency for not less than 2 years from the date on which the potency test for the pertussis component was started.

Labelling. The label states (1) the human dose; (2) Diphtheria and Tetanus components; (a) in case done by challenge method, the minimum number of International Units (as applicable for each component) per single human dose; (b) in case done by antibody induction, the minimum Lf units per single human dose; (3) pertussis component – IU or IOU per single human dose; (4) hepatitis B component - µg HBsAg

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per single human dose; (5) haemophilus conjugate component - µg PRP per single human dose; (6) the type and nominal amount of carrier protein per single human dose; (7) the name and amount of adsorbent and added preservative; (8) that the vaccine must be shaken before use; (9) that the vaccine is not to be frozen.

Diphtheria, Tetanus, Pertussis (Whole Cell), Hepatitis B (rDNA), Poliomyelitis (Inactivated) and *Haemophilus influenzae* Type b Conjugate Vaccine (Adsorbed)

Diphtheria, Tetanus, Pertussis (Whole Cell), Hepatitis B (rDNA), Poliomyelitis Inactivated and *Haemophilus influenzae* Type b conjugate Vaccine (Adsorbed) is a combined vaccine composed of diphtheria toxoid containing not less than 1,500 Lf, (2.2.16) per mg of protein nitrogen, purified tetanus toxoid containing not less than 1,000 Lf, (2.2.16), per mg of protein nitrogen, hepatitis B surface antigen, suitable strains of human polioviruses grown in suitable cell cultures and inactivated by a suitable method and *Haemophilus influenzae Type* b conjugated to suitable protein with a mineral adsorbent to which a suspension of killed *Bordetella pertussis* has been added. Mineral adsorbent is a suspension of hydrated aluminum hydroxide, aluminum phosphate or calcium phosphate or any suitable adsorbent as approved by NRA, in saline solution or other appropriate solution isotonic with blood.

The purifiedtoxoids are prepared from the toxin produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively, in suitable media. The toxins are detoxified by methods which avoid reversibility of the toxoids.

The *Bordetella pertussis* suspension is prepared by growing suitable strains in an appropriate medium and inactivated under controlled conditions.

Hepatitis B surface antigen is a component protein of hepatitis B virus; the antigen is obtained by recombinant DNA technology.

The suitable strains of human Polioviruses are grown in suitable cell cultures and inactivated by validated method.

The polysaccharide, polyribosylribitol phosphate, PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* Type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell dependent B-cell immune response to the polysaccharide.

The final product may contain a suitable antimicrobial preservative.

Production

General provisions

The production method shall have been shown to yield consistently the vaccines comparable with the vaccine of proven clinical efficacy and safety in human.

The production method is validated to demonstrate that the product, if tested, would comply with the following test for specific toxicity of the diphtheria and tetanus component: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea pigs, each weighing between 250 and 350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria, toxemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once and even if more than 1 animal shows signs of or dies in the second test, the vaccine does not comply with the test.

As a measure of consistency of production, For IPV, determine D -Antigen content for inactivated human polioviruses by a suitable immunochemical method (2.2.14) using an appropriate reference preparation calibrated in D-antigen units.

The stability of the final lot and the relevant intermediates is evaluated using one or more indicator tests. For the haemophilus component, such tests may include determination of molecular size, determination of free PRP in the conjugate and kinetics of depolymerisation. Taking into account the results of the stability testing, release requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

Reference vaccine(s)

Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilized by a method that has been shown to have no effect on the assay procedure.

Production of the components

The production of the components complies with the requirements of the monographs on Diphtheria Vaccine

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(Adsorbed), Tetanus Vaccine (Adsorbed), Pertussis Vaccine (Whole cell), Hepatitis B Vaccine (rDNA), Poliomyelitis Vaccine (Inactivated) and *Haemophilus influenzae* Type b Conjugate Vaccine.

FINAL BULK VACCINE

The final bulk is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, bulk purified tetanus toxoid, bulk purified hepatitis B surface antigen onto a mineral carrier such as aluminum hydroxide or hydrated aluminum phosphate, admixture of an appropriate quantity of a suspension of inactivated *B.pertussis* component , suitable quantity of PRP conjugate and purified monovalent harvests of inactivated human polioviruses or a suitable quantity of a trivalent pool of such purified monovalent harvests aseptically under constant stirring; the resulting mixture is approximately isotonic with blood. The *B.pertussis* concentration of the final bulk vaccine does not exceed that corresponding to an opacity of 20 IU per single human dose. If 2 or more strains of *B.pertussis* are used, the composition of consecutive lots of the final bulk vaccine shall be consistent with respect to the proportion of each strain as measured in opacity units. Suitable antimicrobial preservatives may be added such as 2-phenoxyetanol, thiomersal etc

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable method. The amount is not less than 85.0 per cent and not greater than 115.0 per cent of the intended content.

Bovine Serum. If Bovine serum is added in the formulation, determine the poliomyelitis components by a suitable immunochemical method (2.2.14) during preparation of the final bulk vaccine, before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will not be more than 50 ng per single human dose. Test may be omitted if it is performed with satisfactory results on the trivalent pool of inactivated monovalent harvests.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for specific toxicity of diphtheria toxoid, tetanus toxoid and pertussis component and antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot. Provided the content of free formaldehyde has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2g/L, the test for free formaldehyde may be omitted on the final lot. If an in vivo assay is used for the hepatitis B component, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Free PRP

Unbound PRP is determined after removal of the conjugate, for example by anion exchange, size exclusion or hydrophobic chromatography (2.4.16), ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

Osmolality (2.4.23). The osmolality of the vaccine is within the limits approved for the particular preparation.

pH (2.4.24)The pH of the vaccine is within the limits approved for the particular preparation.

Description. Turbid liquid in which mineral carrier tends to settle down slowly on keeping and is free from foreign particles and floccules

Identification

Tests A, Band C may be omitted if test G is carried out. Test G may be omitted if test A, B and C, are carried out.

A. *Diphtheria toxoid*. Dissolve sufficient *sodium citrate* in the vaccine under examination to give a 10 per cent w/v concentration. Maintain at 37° for about 16 hours and centrifuge. Reserve the residue for test C. The clear supernatant reacts with a suitable diphtheria antitoxin and yields a precipitate.

B. *Tetanus toxoid*. Tetanus toxoid is identified by suitable immunochemical method. The clear supernatant obtained as described in identification test A reacts with a suitable tetanus antitoxin to give a positive reaction, when tested by a suitable validated immunological method.

C. *Pertussis component*. To a suspension of the residue obtained in test A in *saline solution* add a suitable *Bordetella pertussis* antiserum; *agglutination* indicates presence of pertussis component.

D. *Hepatitis B surface antigen*. The suspension of the residue obtained in test A gives a positive reactions when tested by suitable *in-vitro* assay.

E. Inactivated Polio Virus strains (IPV) (D-antigen) by a suitable immunochemical method using an appropriate reference preparation calibrated in D-antigen units.

F. *PRP*. The suspension of the residue obtained in the test A gives a positive reaction when tested by a suitable any other

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suitable andvalidated method as approved by NRA for identification of PRP. Alternately, formulation can be spinned and the supernatant can be used for identity.

G. The vaccine confers an active immunity in mice and guinea -pigs when administered as directed in the test for Assay for Diphtheria toxoid, Tetanus toxoid and Pertussis component.

Tests

PRP. Not less than 80.0 per cent and not more than 120.0 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (2.7.1), or phosphorus (2.7.1), by an immunochemical method (2.2.14) or by anion exchange liquid chromatography with pulsed amperometric detection.

Pyrogens (2.2.8). This test is carried out for *Haemophilus influenzae* Type b vaccine only if *Haemophilus influenza* Type b vaccine is presented as separate lyophilisd vial. The vaccine complies with the test for pyrogens. Inject per kg of the rabbit's mass a quantity of the vaccine equivalent to: 1 mg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria toxoid as carrier; 0.1 mg of PRP for a vaccine with tetanus toxoid as carrier protein; 0.025 mg of PRP for vaccine with OMP as carrier or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be within the limits approved by the National Regulatory authority.

Aluminum (2.3.9). Not more than 1.25 mg per single human dose, if aluminum hydroxide or hydrated aluminum phosphate is used as the adsorbent.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11)

Specific toxicity

Diphtheria and tetanus components

Complies with the test as stated under Diphtheria and Tetanus Vaccine (Adsorbed).

Pertussis component

Complies with the test as stated under Diphtheria, Tetanus and Pertussis vaccine (Adsorbed)

Assay

Diphtheria component

Complies with the test as stated under Diphtheria Vaccine (Adsorbed).

Tetanus component

Carry out one of the prescribed methods for the assay as stated under Tetanus Vaccine (Adsorbed). If the test is carried out in guinea pigs, the lower confidence limit (P = 0.95) of the estimated potency is not less than 40 IU per single human dose; if the test is carried out in mice, the lower confidence limit (P = 0.95) of the estimated potency is not less than 60 IU per single human dose.

Pertussis component

Complies with the test as stated under Diphtheria, Tetanus and Pertussis Vaccine (Adsorbed)

Hepatitis B surface antigen (Adsorbed)

Complies with the test as stated under Hepatitis B Vaccine (Adsorbed).

Poliomyelitis component

Complies with the test of D-antigen content as stated under Poliomyelitis Vaccine (Inactivated)

Labelling. The label states (1) the human dose; (2) Diphtheria and Tetanus components; (a) in case done by challenge method/ELISA, the minimum number of International Units (As applicable for each component) per single human dose; (b) in case done by antibody induction, the minimum Lf units per single human dose; (3) pertussis component – IU or IOU per single human dose; (4) hepatitis B component - µg HBsAg per single human dose; (5) Haemophilus conjugate component - µg PRP per single human dose; (6) the nominal amount of poliovirus of each type (1,2 and 3), expressed in units of D-antigen per single human dose; (7) the type and nominal amount of carrier protein conjugated per single human dose of PRP (8) the type of cells used for the production of the poliomyelitis component; (9) the name and amount of adsorbent and added preservative; (10) that the vaccine must be shaken before use; (11) that the vaccine is not to be frozen.

Diphtheria, Tetanus, Pertussis (Whole Cell) and Hepatitis B (rDNA) Vaccine (Adsorbed)

Diphtheria, Tetanus, Pertussis and Hepatitis B (rDNA) Vaccine (Adsorbed) is a combined vaccine composed of diphtheria formol toxoid containing not less than 1,500 Lf (2.2.16), per mg of protein nitrogen, purified tetanus formol toxoid containing not less than 1,000 Lf (2.2.16), per mg of protein nitrogen and hepatitis B surface antigen with a mineral adsorbent to which a suspension of killed *Bordetella pertussis* has been added. Mineral adsorbent is a suspension of

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hydrated aluminium hydroxide, aluminium phosphate or calcium phosphate in saline solution or other appropriate solution isotonic with blood.

The formol toxoids are prepared from the toxin produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively, in suitable media. The toxins are converted to toxoids by treatment with formaldehyde solution by methods, which avoid reversibility of the toxoids.

Hepatitis B surface antigen is a component protein of hepatitis B virus; the antigen is obtained by recombinant DNA technology.

The final product contains a suitable antimicrobial preservative. The antigenic properties of the vaccine are adversely affected by the presence of certain antimicrobial preservatives particularly those of the phenolic type and some of the quaternary ammonium type and must not be used.

Production

General provisions

The production method shall have been shown to yield consistently the vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The production method is validated to demonstrate that the product, if tested, would comply with the following test for specific toxicity of the diphtheria and tetanus component: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea pigs, each weighing between 250 and 350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria, toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal shows signs of or dies in the second test, the vaccine does not comply with the test.

The stability of the final lot and the relevant intermediates is evaluated using one or more indicator tests. Taking account of the results of the stability testing, release requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

Reference vaccine(s)

Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine.

Production of the components

The production of the components complies with the requirements of the monographs on Diphtheria Vaccine (Adsorbed), Tetanus Vaccine (Adsorbed), Pertussis Vaccine and Hepatitis B Vaccine (rDNA).

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, pertussis whole cell suspension and hepatitis B surface antigen onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. Suitable antimicrobial preservatives may be added. Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85.0 per cent and not greater than 115.0 per cent of the intended content.

pH (2.4.24). 6.0 to 7.0.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for specific toxicity of diphtheria toxoid, tetanus toxoid and pertussis component and antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot. Provided the content of free formaldehyde has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot. If an *in vivo* assay is used for the hepatitis B component, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Osmolality (2.4.23). The osmolality of the vaccine is within the limits approved for the particular preparation.

Description. Whitish turbid liquid in which the mineral carrier tends to settle down slowly on keeping.

Identification

Tests A, B, C and D may be omitted if test E is carried out. Test E may be omitted if tests A, B C and D are carried out.

A. *Diphtheria toxoid*. Dissolve sufficient *sodium citrate* in the vaccine under examination to give a 10 per cent w/v

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concentration. Maintain at 37° for about 16 hours and centrifuge. Reserve the residue for test C. The clear supernatant reacts with a suitable diphtheria antitoxin and yields a precipitate.

B. *Tetanus toxoid*. The clear supernatant obtained in test A reacts with a suitable tetanus antitoxin and yields a precipitate.

C. *Pertussis component*. To a suspension of the residue obtained in test A in *saline solution* add a suitable *B. pertussis* antiserum; agglutination indicates presence of pertussis component.

D. *Hepatitis B surface antigen*. The suspension of the residue obtained in test A gives a positive reactions when tested by suitable *in-vitro* assay.

E. The vaccine confers an active immunity in mice and guinea-pigs when administered as directed under Assay.

Tests

pH (2.4.24). 6.0 to 7.0.

Aluminium (2.3.9). Not more than 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Specific toxicity

Diphtheria and tetanus components

Complies with the test as stated under Diphtheria and Tetanus Vaccine (Adsorbed).

Pertussis component

Complies with the test as stated under Pertussis Vaccine (Adsorbed).

Assay

Diphtheria toxoid (adsorbed)

Complies with the test as stated under Diphtheria Vaccine (Adsorbed).

Tetanus component

Carry out one of the prescribed methods for the assay as stated under Tetanus Vaccine (Adsorbed).

If the test is carried out in guinea pigs, the lower confidence limit (P = 0.95) of the estimated potency is not less than 40 IU per single human dose; if the test is carried out in mice, the lower confidence limit (P = 0.95) of the estimated potency is not less than 60 IU per single human dose.

Pertussis vaccine

Complies with the test as stated under Pertussis Vaccine (Adsorbed).

Hepatitis B surface antigen (adsorbed)

Complies with the test as stated under Hepatitis B Vaccine (Adsorbed).

Storage. When stored under the prescribed conditions the vaccine may be expected to retain potency for not less than 2 years from the date on which the potency test for the *pertussis component* was started.

Labelling. The label states (1) the human dose (ml); (2) diphtheria and tetanus components, (a) in case done by challenge method, the minimum number of International Units (as applicable for each component) per single human dose and (b) in case done by antibody induction, the minimum Lf units per single human dose; (3) pertussis component - IU or IOU per single human dose; (4) hepatitis B component -µg HBsAg per single human dose; (5) the name and amount of adsorbent and added preservative; (6) that the vaccine must be shaken before use; (7) that the vaccine is not to be frozen.

Diphtheria, Tetanus, Pertussis (Whole Cell) and *Haemophilus influenzae* Type b Conjugate Vaccine (Adsorbed)

Diphtheria, Tetanus, Pertussis and *Haemophilus influenzae* type b Conjugate Vaccine (Adsorbed) is a combined vaccine composed of diphtheria formol toxoid containing not less than 1,500 Lf, (2.2.16) per mg of protein nitrogen, purified tetanus formol toxoid containing not less than 1,000 Lf, (2.2.16), per mg of protein nitrogen, and *Haemophilus influenzae* type b conjugated to suitable protein with a mineral adsorbent to which a suspension of killed *Bordetella pertussis* has been added. The mineral adsorbent is a suspension of hydrated aluminium hydroxide, aluminium phosphate or calcium phosphate, in saline solution or other appropriate solution isotonic with blood.

The formol toxoids are prepared from the toxin produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively in suitable media. The toxins are converted to toxoids by treatment with formaldehyde solution by methods which avoid reversibility of the toxoids.

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The polysaccharide, polyribosyl ribitol phosphate, PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* Type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell dependent B-cell immune response to the polysaccharide.

The product may be presented with the haemophilus component in a separate container, the contents of which are mixed with the other components immediately before or during use.

The final product contains a suitable antimicrobial preservative. The antigenic properties of the vaccine are adversely affected by the presence of certain antimicrobial preservatives particularly those of the phenolic type and some of the quaternary ammonium type must not be used.

Production

General provisions

The production method shall have been shown to yield consistently the vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

If the vaccine is presented with the haemophilus component in a separate vial, as part of consistency studies, the assays of the diphtheria, tetanus and pertussis are carried out on a suitable number of batches of vaccine reconstituted for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

The production method is validated to demonstrate that the product, if tested, would comply with the following test for specific toxicity of the diphtheria and tetanus component: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea pigs, each weighing between 250 and 350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria, toxemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal shows signs of or dies in the second test, the vaccine does not comply with the test.

The stability of the final lot and the relevant intermediates is evaluated using one or more indicator tests. For the haemophilus component, such tests may include determination of molecular size, determination of free PRP in the conjugate and kinetics of depolymerisation. Taking account of the results of the stability testing, release requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

Reference vaccine(s)

Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilized by a method that has been shown to have no effect on the assay procedure.

Production of the components

The production of the components complies with the requirements of the monographs on Diphtheria Vaccine (Adsorbed), Tetanus Vaccine (Adsorbed), Pertussis Vaccine (Whole Cell) and *Haemophilus influenzae* Type b Conjugate Vaccine.

FINAL BULK VACCINE

Vaccine with all components in the same container

The final bulk is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, bulk purified tetanus toxoid, onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, admixture of an appropriate quantity of a suspension of inactivated *B. pertussis* component and admixture of a suitable quantity of PRP conjugate; the resulting mixture is approximately isotonic with blood. The *B. pertussis* concentration of the final bulk vaccine does not exceed that corresponding to an opacity of 20 IU per single human dose. If 2 or more strains of *B. pertussis* are used, the composition of consecutive lots of the final bulk vaccine shall be consistent with respect to the proportion of each strain as measured in opacity units. Suitable antimicrobial preservatives may be added.

Vaccine with the haemophilus component in a separate container

The final bulk is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, bulk purified tetanus toxoid onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, admixture of an appropriate quantity of a suspension of inactivated *B. pertussis* component and admixture of a suitable quantity of PRP conjugate; the resulting mixture is approximately isotonic with blood. The *B. pertussis* concentration of the final bulk vaccine does not exceed that corresponding to opacity of 20 IU per single human dose. If 2

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or more strains of *B. pertussis* are used, the composition of consecutive lots of the final bulk vaccine shall be consistent with respect to the proportion of each strain as measured in opacity units. The final bulk is filled separately. Suitable antimicrobial preservatives may be added. The final bulk of the haemophilus component is prepared by dilution of the bulk conjugate to the final concentration with a suitable diluent. A stabilizer may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85.0 per cent and not greater than 115.0 percent of the intended content.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

Where the haemophilus component is in a separate container, the final bulk of the haemophilus component is freeze-dried. Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests specific toxicity of diphtheria toxoid, tetanus toxoid and pertussis component and antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot. Provided the content of free formaldehyde has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Free PRP. Unbound PRP is determined after removal of the conjugate, for example by anion exchange, size exclusion or hydrophobic chromatography (2.4.16), ultrafiltration or other validated methods. The amount of free PRP is not greater than approved for the particular product.

Osmolality (2.4.23). The osmolality of the vaccine is within the limits approved for the particular preparation.

pH (2.4.24). 6.0 to 7.0.

Description. Whitish turbid liquid in which the mineral carrier tends to settle down slowly on keeping.

Production

Identification

Tests A, B, C and D may be omitted if test E is carried out. Test E may be omitted if tests A, B, C and D are carried out.

A. *Diphtheria toxoid.* Dissolve sufficient *sodium citrate* in the vaccine under examination to give a 10 per cent w/v concentration. Maintain at 37° for about 16 hours and centrifuge. Reserve the residue for test C. The clear supernatant reacts with a suitable diphtheria antitoxin and yields a precipitate.

B. *Tetanus toxoid.* The clear supernatant obtained in test A reacts with a suitable tetanus antitoxin and yields a precipitate.

C. *Pertussis component.* To a suspension of the residue obtained in test A in *saline solution* add a suitable *Bordetella pertussis* antiserum; agglutination indicates presence of pertussis component.

D. *PRP.* The suspension of the residue obtained in test A gives a positive reaction when tested by a suitable immunochemical method for PRP.

E. The vaccine confers an active immunity in mice and guinea pigs when administered as directed in the test for Potency.

Tests

If the product is presented with the haemophilus component in a separate container; the tests for specific toxicity of diphtheria toxoid, tetanus toxoid and pertussis component, aluminium, free formaldehyde, antimicrobial preservative and sterility are carried out on the container with the diphtheria, tetanus and pertussis components; the tests for PRP content, water (where applicable), sterility and pyrogens are carried out on the container with the haemophilus component.

If the haemophilus component is freeze-dried, some tests may be carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component under test.

PRP. Not less than 80.0 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (2.7.1) or phosphorus (2.7.1), by an immunochemical method (2.2.14) or by anion exchange liquid chromatography with pulsed amperometric detection.

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Pyrogens (2.2.8). This test is carried out for *Haemophilus influenzae* Type b vaccine only if *Haemophilus influenzae*

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Type b vaccine is presented as separate lyophilisd vial. The vaccine complies with the test for pyrogens. Inject per kg of the rabbit's mass a quantity of the vaccine equivalent to: 1 mg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria toxoid as carrier; 0.1 mg of PRP for a vaccine with tetanus toxoid as carrier protein; 0.025 mg of PRP for vaccine with OMP as carrier or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be within the limits approved by the National Regulatory authority.

Specific toxicity

Diphtheria and tetanus components

Complies with the test as stated under Diphtheria and Tetanus Vaccine (Adsorbed).

Pertussis component

Complies with the test as stated under Diphtheria, Tetanus and Pertussis Vaccine (Adsorbed).

Assay

Diphtheria toxoid (adsorbed)

Complies with the test as stated under Diphtheria Vaccine (Adsorbed).

Tetanus component

Carry out one of the prescribed methods for the assay as stated under Tetanus Vaccine (Adsorbed).

If the test is carried out in guinea pigs, the lower confidence limit (P = 0.95) of the estimated potency is not less than 40 IU per single human dose; if the test is carried out in mice, the lower confidence limit (P = 0.95) of the estimated potency is not less than 60 IU per single human dose.

Pertussis vaccine

Complies with the test as stated under Pertussis Vaccine (Adsorbed).

Storage. When stored under the prescribed conditions the vaccine may be expected to retain potency for not less than 2 years from the date on which the potency test for the *pertussis component* was started.

Labelling. The label states (1) the human dose (ml); (2) Diphtheria and Tetanus components (a) in case done by challenge method the minimum number of international units (as applicable for each component) per single human dose; (b) in case done by antibody induction method, the minimum Lf units per single human dose; (3) pertussis component – IU or IOU per single human dose; (4) *Haemophilus influenzae* conjugate component - mg PRP per single human dose; (5) the type and nominal amount of carrier protein per single human dose; (6) the name and amount of adsorbent and added preservative; (7) that the vaccine must be shaken before use; (9) that the vaccine is not to be frozen.

Diphtheria Vaccine (Adsorbed)

Diphtheria Vaccine (Adsorbed) is a preparation of diphtheria formol toxoid with a mineral adsorbent. The formol toxoid is prepared from the toxin produced by the growth of *Corynebacterium diphtheriae*.

Production

General provisions

The maximum number of Lf units per single human dose of diphtheria vaccine (adsorbed) is 30.

Bulk purified toxoid

For the production of diphtheria toxin, from which toxoid is prepared, seed cultures are managed in a defined seed-lot system in which toxinogenicity is conserved and, where necessary, restored by deliberate reselection. A highly toxinogenic strain of *Corynebacterium diphtheriae* with known origin and history is grown in a suitable liquid medium. At the end of cultivation, the purity of each culture is tested and contaminated cultures are discarded. Toxin-containing culture medium is separated aseptically from the bacterial mass as soon as possible. The toxin content (Lf per ml) is checked to monitor consistency of production. Single harvests may be pooled to prepare the bulk purified toxoid. The toxin is purified to remove components likely to cause adverse reactions in humans. The purified toxin is detoxified with formaldehyde by a method that avoids destruction of the immunogenic potency of the toxoid and reversion of the toxoid to toxin, particularly on exposure to heat. Alternatively, purification may be carried out after detoxification.

Only bulk purified toxoid that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

Absence of toxin and irreversibility of toxoid

Inject subcutaneously into each of 5 guinea-pigs at least 500 Lf of the *bulk purified toxoid* in a volume of 1 ml, using the same buffer solution as for the final vaccine, without adsorbent. Animals that die shall be autopsied and examined for symptoms of diphtheria intoxication (red adrenals). The bulk purified toxoid shall pass the test for the absence of toxin, if no guinea-pig shows symptoms of specific intoxication within

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six weeks of injection and if at least 80 per cent of the animals survive the test period. The guinea-pigs shall not have been used previously for experimental purposes.

Alternatively, a cell-culture test system may be used; in this case, the sensitivity of the test shall have been demonstrated to be not less than that of the guinea-pig test, and the test procedures shall be approved by the National Regulatory Authority.

Each bulk purified toxoid shall be tested to ensure that reversion to toxicity cannot take place on storage. Reversion of toxicity is determined using diluted toxoid that has been stored at 34-37° for six weeks, along with diluted sample stored at 5 ± 3° for the same duration, as a control.

A group of 5 guinea pigs are given subcutaneous injection of the test sample or control sample, such that a total volume of 5 ml containing equivalent of 10 SHD should be used and if necessary at multiple sites. The animals are observed for 42 days for signs of ill health. No toxicity shall be detected.

Intradermal tests in guinea-pigs and cell-culture tests both are considered to be suitable.

Cell culture method

Using the same buffer solution as for the final vaccine, without adsorbent, prepare a solution of bulk purified toxoid at 100 Lf per ml. Divide the solution into 2 equal parts. Maintain 1 part at 5° ± 3° and the other at 37° for 6 weeks. Carry out a test in Vero cells for active diphtheria toxin using 50 µl per well of both samples. The sample should not contain antimicrobial preservatives and detoxifying agents should be determined to be below the concentration toxic to Vero cells. Non-specific toxicity may be eliminated by dialysis.

Use *freshly trypsinised Vero cells* at a suitable concentration, for example 2.5×10^5 per ml and a *reference diphtheria toxin* diluted in 100 Lf per ml *diphtheria toxoid*. A suitable *reference diphtheria toxin* will contain either not less than 100 LD_{50} per ml or 67 to 133 lr per 100 in 1 Lf and 25,000 to 50,000 minimal reacting doses for guinea-pig skin in 1 Lf (*diphtheria toxin RP* is suitable for use as the reference toxin). Dilute the toxin in 100 Lf per ml *diphtheria toxoid* to a suitable concentration, for example 2×10^{-4} Lf per ml. Prepare serial twofold dilutions of the diluted diphtheria toxin and use undiluted test samples (50 µl per well). Distribute them in the wells of a sterile tissue culture plate containing a medium suitable for Vero cells. To ascertain that any cytotoxic effect noted is specific to diphtheria toxin, prepare in parallel dilutions where the toxin is neutralised by a suitable concentration of *diphtheria antitoxin*, for example 100 IU per ml. Include control wells without toxoid or toxin and with non-toxic toxoid at 100 Lf per ml on each plate to verify normal cell growth. Add cell suspension to each well, seal the plates and incubate at 37° for 5 to 6 days. Cytotoxic effect is judged to be present where there is complete metabolic inhibition of the Vero cells, indicated by the pH indicator of the medium. Confirm cytopathic effect by microscopic examination or suitable staining such as *MTT dye*. The test is invalid if 5×10^{-5} Lf per ml of reference diphtheria toxin in 100 Lf per ml toxoid has no cytotoxic effect on Vero cells or if the cytotoxic effect of this amount of toxin is not neutralised in the wells containing *diphtheria antitoxin*. The bulk purified toxoid complies with the test if no toxicity neutralisable by antitoxin is found in either sample.

Antigenic purity. Not less than 1,500 Lf per mg of protein nitrogen.

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption of a suitable quantity of bulk purified toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Identification

Dissolve sufficient *sodium citrate* in the vaccine under examination to give a 10 per cent w/v concentration. Maintain at 37° for about 16 hours and centrifuge. The clear supernatant reacts with a suitable diphtheria antitoxin and yields a precipitate.

pH (2.4.24). 6.0 to 7.0.

Specific toxicity. Use 5 normal, healthy guinea-pigs weighing between 250 and 350 g, which have been maintained for at least 1 week on a uniform, unrestricted diet, and have not been previously treated with any material that will interfere with the test. Weigh the animals separately and record their weights. Inject subcutaneously into each animal 5 times the dose stated on the label. Weigh all the animals at weekly intervals for 6 weeks. None of the animals show signs of or dies from diphtheria toxaemia within 42 days or loses weight at the end of the test. If more than one animal dies from non-specific causes or loses weight, repeat the test. If an animal dies or loses weight in the second test, the vaccine fails the test.

Alternate test by injecting at least 20 Lf of purified toxoid, intradermally into rabbits or guinea-pigs and observe the injection sites for specific erythema may be used. Erythema with a diameter greater than 5 mm is typically considered to be positive.

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Potency. Determine by any of the methods of biological assay of adsorbed diphtheria vaccine described. The method chosen for the assay of adsorbed diphtheria vaccine depends on the intended purpose. Method A or method B is used:

1. during development of a vaccine, to assay batches produced to validate the production;
2. wherever revalidation is needed following a significant change in the manufacturing process.

Method A or B may also be used for the routine assay of the batches of vaccine, menthod C can be used where ever possible, except as specified under 1 and 2 above, after verification of the suitability of the method for the product.

Biological assay of adsorbed diphtheria vaccine

After establishing consistency/stability of product by any of the following multiple dilution assay, a single dilution assay based on serological methods or other principles may be used. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 30 IU (i.e. of the minimum potency of a single human dose), that elicits a minimal protective effect, and comparing its effect with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine ($P < 0.05$), the potency of the test vaccine is satisfactory. Single dilution assay is a qualitative approach and provides an assurance that the lower limit of the estimated potency is in excess of the minimum requirement. It does not give information on the dose response curves and their linearity, parallelism and significant slope. Where a single dilution is used, production and test consistency over time are monitored via suitable indicators and by carrying out a full multiple-dilution assay periodically as approved by National Regulatory Authority.

(A) Intradermal challenge method

The potency of adsorbed diphtheria vaccine is determined by comparing the dose necessary to protect guinea-pigs against the erythrogenic effects of a range of intradermal injections of diphtheria toxin with the dose of the Standard preparation of adsorbed diphtheria toxoid necessary to give the same protection. For this comparison, the Standard preparation of adsorbed diphtheria toxoid and a suitable preparation of diphtheria toxin, for use as a challenge toxin, are required.

Standard preparation

The Standard preparation is International standard of Diphtheria toxoid, adsorbed, or another suitable preparation the potency of which has been determined in relation to the International Standard.

Suggested method

Test animals. Use white guinea-pigs, weighing between 250 and 350 g, from the same stock. Distribute the guinea-pigs into no fewer than six equal groups; use groups containing a number of animals sufficient to obtain results that fulfill the requirements for a valid Assay prescribed below. The guinea-pigs are all of the same sex or the males and females are distributed equally among the groups. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardized, include five guinea-pigs as unvaccinated controls.

Selection of the challenge toxin. Select a preparation of diphtheria toxin containing 67 to133 Limes reaction's per 100 (Lr per 100) in Limes flocculationis (Lf) and 25,000 to 50,000 minimal reacting doses for guinea-pig skin in 1 Lf. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the activity for every assay.

Preparation of the challenge toxin solutions. Immediately prior to use, dilute the challenge toxin with a suitable diluent to obtain a challenge toxin solution containing about 512×10^{-4} Lf in 0.2 ml. Dilute a portion of this challenge toxin solution to give a series of five 4-fold dilutions.

Determination of potency of the vaccine. Prepare in *saline solution* dilutions of the vaccine under examination and of the Standard preparation such that, for each, the dilutions form a series differing by not more than 2.5 fold steps and in which the dilutions of intermediate concentration, when injected subcutaneously in 1.0 ml volumes into guinea-pigs, result in an intradermal score of approximately three when the animals are challenged. Allocate the dilutions, one to each of the groups of guinea-pigs, and inject subcutaneously 1.0 ml of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days shave both flanks of each guinea-pig and inject each animal intradermally with 0.2 ml of the challenge toxin solution and with 0.2 ml of each of the five dilutions thereof in such a way as to minimize interference between adjacent sites. If necessary, inject the unvaccinated control guinea-pigs with dilutions containing 8×10^{-5}, 4×10^{-5}, 2×10^{-5}, 1×10^{-5} and 5×10^{-6} Lf of the challenge toxin. Examine all the injection sites 48 hours after injection of the challenge toxin and record the incidence of specific diphtheria erythema. Record also the number of sites free from such reactions as the intradermal challenge score. Tabulate the intradermal challenge scores for all the animals receiving the same dilution of vaccine and use those data with a suitable transformation, such as $(score)^2$ or $\arcsin[(score/6)^2]$, to obtain an estimate of the relative potency for each of the test preparations by parallel-line quantitative analysis.

The test is not valid unless (a) for both the preparation under examination and the Standard preparation, the mean score

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obtained at the lowest dose level is more than three; (b) if applicable, the toxin dilution that contains $4x10^{-5}$ Lf gives a positive erythema in at least 80.0 per cent of the control guinea-pigs and the dilution that contains $2x10^{-5}$ Lf gives no reaction in at least 80 per cent of the guinea-pigs (if these criteria are not met a different toxin has to be selected); (c) the fiducial limits of the assay fall between 50.0 and 200.0 per cent of the estimated potency; (d) the statistical analysis shows no deviation from linearity and parallelism. The test may be repeated but when more than one test is performed the results of all valid tests must be combined in the estimate of potency.

The lower fiducial limit of error of the estimated potency is not less than 30 Units per dose.

(B) Lethal challenge method

Test animals. Use healthy, white or light-coloured guinea-pigs from the same stock, weighing between 250 and 350 g. Distribute them into six groups of sixteen; and four groups of four. The guinea-pigs should all be of the same sex or the males and females should be distributed equally between the six groups of sixteen.

Challenge toxin. Select a preparation of diphtheria toxin containing not less than 100 LD_{50} in 1.0 ml.

Preparation of the challenge toxin solutions. Immediately prior to use, prepare from the challenge toxin by dilution in *phosphate buffered saline pH 7.4*, or *normal saline* a challenge toxin containing approximately 100 LD_{50} in 1.0 ml. Dilute portions of this challenge toxin solution to 2 LD_{50}, 1 LD_{50} and ½ LD_{50} in the same solution

Determination of potency of the vaccine. Prepare in *saline solution* three dilutions of the vaccine under examination and three dilutions of the Standard preparation such that for each, the dilutions form a series differing by not more than 2.5 fold steps and in which the dilutions of intermediate concentration, when injected subcutaneously in 1.0 ml volumes into guinea-pigs, protect approximately 50 per cent of the animals from the lethal effects of the subcutaneous injection of the quantity of diphtheria toxin prescribed for this test. Allocate the six dilutions, one to each of the six groups of sixteen guinea-pigs, and inject subcutaneously 1.0 ml of each dilution into each guinea-pig in the groups to which that dilution is allocated. After 28 days inject subcutaneously into each animal in the six groups of sixteen, 1.0 ml of the challenge toxin solution. Allocate the challenge toxin solution and the three dilutions made from it, one to each of the four groups of four guinea-pigs and inject subcutaneously 1.0 ml of each toxin solution into each guinea-pig in the group to which that solution is allocated. Examine the guinea pigs twice in a day, remove dead animals and kill the animals showing definite signs of diphtheria. Count the number of surviving animals 5 days later and calculate the potency of the vaccine under examination relative to the potency of the Standard preparation on the basis of the number of animals that survive in each of the six groups of sixteen, using appropriate statistical methods.

The test is not valid unless (a) for the vaccine under examination and the Standard preparation the 50.0 per cent protective doses lie between the largest and smallest doses of the preparations given to the guinea-pigs; (b) the survivors among the four groups of guinea-pigs injected with the challenge toxin and its dilutions indicate that the challenge was approximately 100 LD_{50} and; (c) statistical analysis shows parallelism, linearity and a significant slope of the dose-response lines. The test may be repeated any number of times but when more than one test is performed the results of all valid tests must be combined in the estimate of potency.

Estimated potency should not be less than 30 IU per single human dose. If the lower limit of 95.0 per cent confidence interval of estimated potency is less than 30 IU per single human dose then the limits of the 95.0 per cent confidence interval should be within 50 to 200 of the estimated potency.

(C) Antibody induction method

Inject subcutaneously on each of two occasions separated by an interval of not more than 4 weeks, one-fiftieth of the stated human dose diluted to 1 ml with *saline solution*, into each of 10 normal, healthy guinea-pigs weighing between 250 and 350 g. Not earlier than 2 weeks and not later than 3 weeks after the second injection, collect the serum from each animal and determine the antitoxin content of the serum of each animal, as described under Diphtheria Antitoxin or any other method approved by National Regulatory Authority. The geometric mean of the antitoxin contents shall be not less than 2.0 Units per ml with reference to the Diphtheria antitoxin standard.

(D) Any other validated serological assay in guinea pigs or mice as approved by National Regulatory Authority

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under, Identification, Tests and Assay may be released for use. Provided the tests for specific toxicity, free formaldehyde and antimicrobial preservative and

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the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Identification

Diphtheria toxoid is identified by a suitable immunochemical method. The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine under examination sufficient *sodium citrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.

Tests

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the absorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the quantity stated on the label.

Sterility (2.2.11). Complies with the test for sterility.

pH (2.4.24). 6.0 to 7.0.

Assay

Carry out one of the prescribed methods for the assay of Diphtheria Vaccine (Adsorbed).

The lower confidence limit (P = 0.95) of the estimated potency is not less than 30 IU per single human dose.

Labelling. The label states (1) the human dose; (2) the minimum Lf units per single human dose or the minimum International Units per single human dose if potency test done by challenge method; (3) the name and the amount of the adsorbent and preservative; (4) that the vaccine must be shaken before use; (5) that the vaccine is not to be frozen.

Diphtheria Vaccine (Adsorbed) for Adults and Adolescents

Diphtheria Vaccine (Adsorbed) Reduced antigen content

Diphtheria Vaccine (Adsorbed) for Adults and Adolescents is a preparation of diphtheria formol toxoid adsorbed on mineral carrier. The formol toxoid is prepared from the toxin produced by the growth of *Corynebacterium diphtheriae*.

Production

General provisions

The maximum number of Lf units per single human dose of diphtheria vaccine (Adsorbed) for adult and adolescents is as approved by National Control Authority.

Bulk purified diphtheria toxoid

The bulk purified diphtheria toxoid is prepared as described in the monograph on Diphtheria vaccine (adsorbed) and comply with the requirements prescribed therein.

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption of a suitable quantity of bulk purified diphtheria toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Antimicrobial preservatives of the phenolic type must not be used.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Identification

Dissolve sufficient *sodium citrate* in the vaccine under examination to give a 10 per cent w/v concentration. Maintain at 37° for about 16 hours and centrifuge. The clear supernatant reacts with a suitable diphtheria antitoxin and yields a precipitate.

pH (2.4.24). 6.0 to 7.0.

Specific toxicity. Use 5 normal, healthy guinea-pigs weighing between 250 and 350 g, which have been maintained for at least 1 week on a uniform, unrestricted diet, and have not been previously treated with any material that will interfere with the test. Weigh the animals separately and record their weights. Inject subcutaneously into each animals 5 times the dose stated on the label. Weigh all the animals at weekly interval for 6 weeks. None of the animals shows signs of or dies from diphtheria toxaemia within 42 days or loses weight at the end of test. If more than one animal dies from non-specific causes or loses weight, repeat the test. If an animals dies or loses weight in the second test, the vaccine fails the test.

Assay

Carry out the prescribed method for assay of Diphtheria Vaccine by lethal challenge method described under the assay of Diphtheria Vaccine (Adsorbed).

The lower confidence limit (P=0.95) of the estimated potency is not less than 2 IU per single human dose.

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Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Carry out the test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk vaccine is filled, stored and distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under, Identification, Tests and Assay may be released for use. Provided the tests for specific toxicity, free formaldehyde, antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Identification

Diphtheria toxoid is identified by a suitable immunochemical method (2.2.14).

Dissolve sufficient sodium citrate in the vaccine under examination to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate or visible floccules.

Tests

pH (2.4.24). 6.0 to 7.0.

Aluminium (2.3.9). Not more than 1.25 mg per single human dose when aluminium hydroxide or hydrated aluminium phosphate is used as an adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115.0 per cent of the quantity stated on the label.

Sterility (2.2.11). Complies with the test for sterility.

Assay

Carry out the lethal challenge method described in the assay of Diphtheria Vaccine (Adsorbed) or a validated in vitro serological assay approved by the National Control Laboratory.

The lower confidence limit (P=0.95) of the estimated potency is not less than 2 IU per single human dose.

Labelling. The label states (1) the human dose; (2) the minimum Lf units per single human dose or the minimum International Units per single human dose if potency test done by challenge method; (3) the name and the amount of adsorbent and preservative; (4) that the vaccine must be shaken before use; (5) that the vaccine is not to be frozen.

Gas-Gangrene Antitoxin (Oedematiens)

Gas-gangrene Antitoxin (Novyi); Anti-gas-gangrene (Oedematiens) Serum

Gas-gangrene Antitoxin (Oedematiens) is a preparation containing the specific antitoxic globulins obtained by purification of hyperimmune serum of horses or other suitable animals and having the specific activity of neutralising the alpha toxin formed by *Clostridium oedematiens (C. novyi)*.

Gas-gangrene Antitoxin (Oedematiens) reconstituted where necessary as stated on the label complies with the following tests.

Gas-Gangrene Antitoxin (Oedematiens) has a potency of not less than 3750 Units per ml.

Description. A clear colourless or pale yellow liquid free from suspended particles or a freeze-dried, cream coloured powder or pellet for reconstitution with the diluent supplied by the manufacturer.

Identification

Specifically neutralises and renders the alpha toxin formed by *Cl. oedematiens* harmless to susceptible animals or may be identified by any other suitable *in-vitro* test.

Tests

Potency. Carry out the biological assay of gas-gangrene antitoxin (oedematiens).

Biological Assay of Gas-Gangrene Antitoxin (Oedematiens)

The potency of the gas-gangrene antitoxin (oedematiens) is determined by comparing the dose necessary to protect mice or other suitable animals against the lethal effects of gas-gangrene toxin (oedematiens) with the dose of the Standard Preparation of gas-gangrene antitoxin (*C. oedematiens*) necessary to give the same protection. For this purpose the Standard Preparation of gas-gangrene toxin (*C. oedematiens*) and a suitable preparation of gas-gangrene toxin (oedematien), for use as a test toxin, are required. The test dose of the toxin

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is determined in the relation to the Standard Preparation and the potency of the preparation under examination is then determined in relation to the Standard preparation using the test toxin.

Standard Preparation

The Standard Preparation is a dried hyperimmune horse serum or another suitable preparation of gas-gangrene antitoxin (oedematiens), the potency of which has been determined in relation to the International Standard. The Unit is the specific neutralising activity for gas-gangrene toxin (oedematiens) contained in such an amount of the Standard Preparation as the Ministry of Health and Family Welfare, Government of India may from time to time indicate as the quantity exactly equivalent to the Unit accepted for international use.

Suggested Method

Test animals. Use healthy mice having body weights such that the difference between the lightest and heaviest is not more than 5 g.

Test toxin. Prepare gas-gangrene toxin (oedematiens) by growing *C. oedematiens* in a liquid culture medium for about 5 days, filtering aseptically and precipitating with *ammonium sulphate*. The resulting precipitate, which contains the toxin, is collected, dried over *phosphorus pentoxide* at a pressure of 1.5 to 2.5 kPa, powdered and kept dry.

Selection of test toxin. Select toxin for use as the test toxin by determining the following quantities.

L+ dose — This is the smallest quantity of the toxin which, when mixed with 1 Unit of antitoxin and injected intramuscularly into mice, causes the death of the animals within 72 hours.

LD_{50} — This is the smallest quantity of toxin which, when injected intramuscularly into mice, causes the death of half the animals within 72 hours.

A suitable toxin is one which has an L+ dose in 0.5 mg or less and contains not less than 25 LD_{50} in an L+ dose.

Determination of test dose of toxin *(L+ dose).* Prepare a solution of the Standard Preparation with *saline solution* such that 1.0 ml contains 12.5 Units. Weigh accurately a quantity of the dried toxin and dissolve in *saline solution* so that 1.0 ml contains a precise known amount such as 10 mg. Prepare mixtures so that 2.0 ml of each mixture contains 0.8 ml of the solution of the Standard Preparation (10 Units) and one of a series of graded volumes of the solution of the test toxin. Dilute each mixture with *saline solution* to the same final volume (2.0 ml). Allow the mixtures to stand at room temperature, protected from light, for 60 minutes and, using six mice for each mixture, inject intramuscularly into each mouse a dose of 0.2 ml of each mixture. Observe the mice thereafter for 72 hours.

The test dose of toxin is the amount present in 0.2 ml of that mixture which contains the smallest amount of toxin sufficient to cause the death within 72 hours of all six mice injected with it.

Determination of potency of the antitoxin. Dilute the test toxin with *saline solution* so that 1.0 ml contains 12.5 times the test dose. Prepare mixtures such that 2.0 ml of each mixture contains 0.8 ml of the dilution of the toxin and one of a series of graded volumes of the preparation under examination. Prepare further mixtures such that 2.0 ml of each contains 0.8 ml of the dilution of the toxin and one of a series of graded volumes, of the Standard Preparation diluted with *saline solution* so that the central graded volume contains 10 Units. Dilute each mixture with *saline solution* to the same final volume (2.0 ml). Allow the mixtures to stand at room temperature, protected from light, for 60 minutes and then inject intramuscularly a dose of 0.2 ml of each mixture into each of six mice under the conditions described for determination of the test dose of toxin. Observe the mice for 72 hours. If none of the mice is killed, 0.2 ml of the mixture contains more than 1 Unit of antitoxin. If all the mice are killed, 0.2 ml of the mixture contains less than 1 Unit of antitoxin.

The mixture that contains the largest volume of the preparation under examination that fails to protect the mice from death contains 10 Units. The test is not valid unless all the mice injected with mixtures containing 0.8 ml or less of the solution of the Standard Preparation die and all those injected with mixtures containing more survive.

Other tests. Complies with the tests stated under Antisera.

Storage. Store protected from light at a temperature between 2° to 8°. Liquid preparations should not be allowed to freeze.

Labelling. The label states (1) the number of Units per ml, where appropriate; (2) the animal species from which the preparation has been made; (3) the recommended dose; (4) the name and concentration of any antimicrobial preservative added; (5) that in case of a liquid preparation, it should not to be allowed to freeze.

Gas-Gangrene Antitoxin (Perfringens)

Anti-gas-gangrene (Perfringens) Serum

Gas-gangrene Antitoxin (Perfringens) is a preparation containing the specific antitoxic globulins obtained by purification of hyperimmune serum of horses or other suitable animals and having the specific activity of neutralising the alpha toxin formed by *Clostridium perfringens*.

Gas-gangrene Antitoxin (Perfringens) reconstituted where necessary as stated on the label complies with the following tests.

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Gas-Gangrene Antitoxin (Perfringens) has a potency of not less than 1500 Units per ml.

Description. A clear, colourless or pale yellow liquid free from suspended particles or a freeze- dried, cream coloured powder or pellet for reconstitution with the diluent supplied by the manufacturer.

Identification

Specifically neutralises and renders the alpha toxin formed by *C. perfringens* harmless to susceptible animals or may be identified by any other suitable *in-vitro* test.

Tests

Potency. Carry out the biological assay of gas-gangrene antitoxin (perfringens).

Biological Assay of Gas-Gangrene Antitoxin (Perfringens)

The potency of the gas-gangrene antitoxin (perfringens) is determined by comparing the dose necessary to protect mice or other suitable animals against the lethal effects of fixed dose of the Standard Preparation of gas-gangrene toxin (perfringens, type A) with the dose of the Standard Preparation of gas-gangrene antitoxin (perfringens) (*C. perfringens* alpha antitoxin) necessary to give the same protection. For this purpose the Standard Preparation of gas-gangrene antitoxin (*C. perfringens* alpha antitoxin) and a suitable preparation of gas-gangrene toxin (perfringens, type A), for use as a test toxin, are required. The test dose of the toxin is determined in the relation to the Standard Preparation and the potency of the preparation under examination is then determined in relation to the Standard preparation using the test toxin.

Standard Preparation

The Standard Preparation is a dried 1:3 dilution of hyperimmune horse serum or another suitable preparation of gas-gangrene antitoxin (perfringens), the potency of which has been determined in relation to the International Standard. The Unit is the specific neutralising activity for gas-gangrene toxin (perfringens) contained in such an amount of the Standard Preparation as the Ministry of Health and Family Welfare, Government of India may from time to time indicate as the quantity exactly equivalent to the Unit accepted for international use.

Suggested Method

Test animals. Use healthy mice having body weights such that the difference between the lightest and heaviest is not more than 5 g.

Test toxin. Prepare gas-gangrene toxin (perfringens) by growing *C. perfringens*, type A, in a liquid culture medium for about 5 days, filtering aseptically and precipitating with *ammonium sulphate*. The resulting precipitate, which contains the toxin, is collected, dried over *phosphorus pentoxide* at a pressure of 1.5 to 2.5 kPa, powdered and kept dry.

Selection of test toxin. Select toxin for use as the test toxin by determining the following quantities.

L+ dose — This is the smallest quantity of the toxin which, when mixed with 1 Unit of antitoxin and injected intravenously into mice, causes the death of the animals within 48 hours.

LD_{50} — This is the smallest quantity of toxin which, when injected intravenously into mice, causes the death of half the animals within 48 hours.

A suitable toxin is one which has an L+ dose in 4 mg or less, and contains not less than 20 LD_{50} in an L+ dose.

Determination of test dose of toxin *(L+dose).* Prepare a solution of the Standard Preparation with *saline solution* such that 1.0 ml contains 5 Units. Weigh accurately a quantity of the dried toxin and dissolve it in *saline solution* so that 1.0 ml contains a precise known amount such as 10 mg. Prepare mixtures such that 5.0 ml of each mixture contains 2.0 ml of the solution of the Standard Preparation (10 Units) and one of a series of graded volumes of the solution of the toxin. Dilute each mixture with *saline solution* to the same final volume (5.0 ml). Allow the mixtures to stand at room temperature, protected from light, for 60 minutes and, using six mice for each mixture, inject intravenously into each mouse a dose of 0.5 ml of each mixture. Observe the mice thereafter for 48 hours.

The test dose of toxin is the amount present in 0.5 ml of that mixture which contains the smallest amount of toxin sufficient to cause the death within 48 hours of all six mice injected with it.

Determination of potency of the antitoxin. Dilute the test toxin with *saline solution* so that 1.0 ml contains five times the test dose. Prepare mixtures such that 5.0 ml of each mixture contains 2.0 ml of the dilution of the toxin and one of a series of graded volumes of the preparation under examination. Prepare further mixtures such that 5.0 ml of each contains 2.0 ml of the dilution of the toxin and one of a series of graded volumes of the Standard Preparation diluted with *saline solution* so that the central graded volume contains 10 Units. Dilute each mixture with *saline solution* to the same final volume (5.0 ml). Allow the mixtures to stand at room temperature, protected from light, for 60 minutes and then inject intravenously a dose of 0.5 ml of each mixture into each of six mice under the conditions described for determination of the test dose of toxin. Observe the mice for 48 hours. If none of the mice is killed, 0.5 ml of the mixture contains more than 1 Unit of antitoxin. If all mice are killed, 0.5 ml of the mixture contains less than 1 Unit of antitoxin.

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The mixture that contains the largest volume of the preparation under examination that fails to protect the mice from death contains 10 Units. The test is not valid unless all the mice injected with mixtures containing 2.0 ml or less of the solution of the Standard Preparation die and all those injected with mixtures containing more survive.

Other tests. Complies with the tests stated under Antisera.

Storage. Store protected from light at a temperature between 2° to 8°. Liquid preparations should not be allowed to freeze.

Labelling. The label states (1) the number of Units per ml, where appropriate; (2) the animal species from which the preparation has been made; (3) the recommended dose; (4) the name and concentration of any antimicrobial preservative added; (5) that in case of a liquid preparation, it should not be allowed to freeze.

Gas-Gangrene Antitoxin (Septicum)

Anti-gas-gangrene (Septicum) Serum

Gas-gangrene Antitoxin (Septicum) is a preparation containing the specific antitoxic globulins obtained by purification of hyperimmune serum of horses or other suitable animals and having the specific activity of neutralising the alpha toxin formed by *Clostridium septicum*.

Gas-gangrene Antitoxin (Septicum) reconstituted where necessary as stated on the label complies with the following tests.

Gas-gangrene Antitoxin (Septicum) has a potency of not less than 1500 Units per ml.

Description. A clear, colourless or pale yellow liquid free from suspended particles or a freeze-dried, cream coloured powder or pellet for reconstitution with the diluent supplied by the manufacturer.

Identification

Specifically neutralises and renders the alpha toxin formed by *C. septicum* harmless to susceptible animals or may be identified by any other suitable *in-vitro* test.

Tests

Potency. Carry out the biological assay of gas-gangrene antitoxin (septicum).

Biological Assay of Gas-Gangrene Antitoxin (Septicum)

The potency of the gas-gangrene antitoxin (septicum) is determined by comparing the dose necessary to protect mice or other suitable animals against the lethal effects of gas-gangrene toxin (septicum) with the dose of the Standard Preparation of gas-gangrene antitoxin (*C. septicum*) necessary to give the same protection. For this purpose the Standard Preparation of gas-gangrene antitoxin (*C. septicum*) and a suitable preparation of gas-gangrene toxin (septicum), for use as a test toxin, are required. The test dose of the toxin is determined in the relation to the Standard Preparation and the potency of the preparation under examination is then determined in relation to the Standard preparation using the test toxin.

Standard Preparation

The Standard Preparation is a dried 1:3 dilution of hyperimmune horse serum of gas-gangrene antitoxin (septicum) in *phosphate-buffered saline* or another suitable preparation the potency of which has been determined in relation to the International Standard. The Unit is the specific neutralising activity for gas-gangrene toxin (septicum) contained in such an amount of the Standard Preparation as the Ministry of Health and Family Welfare, Government of India may from time to time indicate as the quantity exactly equivalent to the Unit accepted for international use.

Suggested Method

Test animals. Use healthy mice having body weights such that the difference between the lightest and heaviest is not more than 5 g.

Test toxin. Prepare gas-gangrene toxin (septicum) by growing *C. septicum* (Vibrion septique) in a liquid culture medium for about 5 days, filtering aseptically and precipitating with *ammonium sulphate*. The resulting precipitate, which contains the toxin, is collected, dried over *phosphorus pentoxide* at a pressure of 1.5 to 2.5 kPa, powdered and kept dry.

Selection of test toxin. Select toxin for use as the test toxin by determining the following quantities.

L+ dose — This is the smallest quantity of toxin which, when mixed with 1 Unit of antitoxin and injected intravenously into mice, causes the death of the animals within 72 hours.

LD_{50} — This is the smallest quantity of the toxin which, when injected intravenously into mice, causes the death of half the animals within 72 hours.

A suitable toxin is one which has an L+ dose in 0.5 mg or less and contains not less than 25 LD_{50} in an L+ dose.

Determination of test dose of toxin *(L+dose).* Prepare a solution of the Standard Preparation in *saline solution* such that 1.0 ml contains 5 Units. Weigh accurately a quantity of the dried toxin and dissolve in *saline solution* so that 1.0 ml contains a precise known amount such as 20 mg. Prepare mixtures such that 5.0 ml of each mixture contains 2.0 ml of the solution of the Standard Preparation (10 Units) and one of a

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series of graded volumes of the solution of the toxin. Dilute each mixture with *saline solution* to the same final volume (5.0 ml). Allow the mixtures to stand at room temperature, protected from light, for 60 minutes and, using six mice for each mixture, inject intravenously into each mouse a dose of 0.5 ml of each mixture. Observe the mice thereafter for 72 hours.

The test dose of toxin is the amount present in 0.5 ml of that mixture which contains the smallest amount of toxin sufficient to cause the death within 72 hours of all six mice injected with it.

Determination of potency of the antitoxin. Dilute the test toxin with *saline solution* so that 1.0 ml contains five times the test dose. Prepare mixtures such that 5.0 ml of each mixture contains 2.0 ml of the dilution of the toxin and one of a series of graded volumes of the preparation under examination. Prepare further mixtures such that 5.0 ml of each contains 2.0 ml of the dilution of the toxin and one of a series of graded volumes of the Standard Preparation diluted with *saline solution* so that the central graded volume contains 10 Units. Dilute each mixture with *saline solution* to the same final volume (5.0 ml). Allow the mixtures to stand at room temperature, protected from light, for 60 minutes and then inject intravenously a dose of 0.5 ml of each mixture into each of six mice under the conditions described for determination of the test dose of toxin. Observe the mice for 48 hours. If none of the mice is killed, 0.5 ml of the mixture contains more than 1 Unit of antitoxin. If all the mice are killed, 0.5 ml of the mixture contains less than 1 Unit of antitoxin.

The mixture that contains the largest volume of the preparation under examination that fails to protect the mice from death contains 10 Units. The test is not valid unless all the mice injected with mixtures containing 2.0 ml or less of the solution of the Standard Preparation die and all those injected with mixtures containing more survive.

Other tests. Complies with the tests stated under Antisera.

Storage. Store protected from light at a temperature between 2° to 8°. Liquid preparations should not be allowed to freeze.

Labelling. The label states (1) the number of Units per ml, where appropriate; (2) the animal species from which the preparation has been made; (3) the recommended dose; (4) the name and concentration of any antimicrobial preservative added; (5) that in case of a liquid preparation, it should not to be allowed to freeze.

Mixed Gas-Gangrene Antitoxin

Mixed Gas-gangrene Antitoxin is prepared by mixing Gas-gangrene Antitoxin (Oedematiens), Gas-gangrene Anti-toxin (Perfringens) and Gas-gangrene Antitoxin (Septicum) in appropriate quantities.

Mixed Gas-gangrene Antitoxin has a potency of not less than 1000 Units per ml of Gas-gangrene Antitoxin (Oedematiens), not less than 1000 Units per ml of Gas-gangrene Antitoxin (Perfringens) and not less than 500 Units per ml of Gas-gangrene Antitoxin (Septicum).

Description. An almost colourless or very faintly yellow liquid, free from turbidity.

Identification

Specifically neutralises and renders the alpha-toxins formed by *C. oedematiens*, *C. perfringens* and *C. septicum* harmless to susceptible animals.

Tests

Potency. Carry out the biological assay for each component as described in the relevant individual monographs.

Other tests. Complies with the tests stated under Antisera.

Storage. Store protected from light at a temperature 2° to 8°. It should not be allowed to freeze. Liquid preparations should not be allowed to freeze.

Labelling. The label states the number of Units of each component per ml.

Haemophilus influenzae Type b Conjugate Vaccine

Haemophilus influenzae Type b Conjugate Vaccine is a liquid or freeze-dried preparation of a polysaccharide, derived from a suitable strain of *Haemophilus influenzae* Type b, covalently bound to a carrier protein. The polysaccharide, polyribosylribitol phosphate, referred to as PRP, is a linear copolymer composed of repeated units of 3-β-D-ribofuronosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, with a defined molecular size. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

Production

General provisions

The production method shall have been shown to yield consistently *Haemophilus influenzae* Type b conjugate vaccines of adequate safety and immunogenicity in humans. The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy of Vaccines. The stability of the final lot and relevant intermediates is evaluated using one or more indicator tests. Such tests may include determination of molecular size, determination of free PRP in the conjugate and the

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immunogenicity test on mice. Taking account of the results of the stability testing, release requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

SEED LOT

The strain of *Haemophilus influenzae* Type b used in preparing *Haemophilus influenzae* Type b conjugate vaccine shall be identified by a record of its history, including the source from which it was obtained and the tests made to determine the characteristics of the strain. The strain shall have been shown to be capable of producing Type b polysaccharide.

The production of PRP and of the carrier protein is based on defined seed lot systems. Master seed lot and working seed lot shall be properly characterized and defined. Cultures derived from the working seed shall have the same characteristics as of the master seed lot. The sample of culture of single harvests taken before killing shall be tested for contamination by examination of Gram-stained smears and by inoculation on suitable media.

Haemophilus influenzae Type b Polysaccharide (PRP)

Haemophilus influenzae Type b is grown in a liquid medium that does not contain high-molecular-weight polysaccharides; if any ingredient of the medium contains blood-group substances, the process shall be validated to demonstrate that after the purification step they are no longer detectable. The culture may be inactivated. PRP is separated from the culture liquid and purified by a suitable method. Volatile matter, including water, In the purified polysaccharide is determined by methods such as thermogravimetry, Karl Fischer or any other suitable method. All chemical analysis shall be based on the dry weight of the polysaccharide, in its salt form.

Only those pools of PRP that comply with the following requirements may be used in the preparation of the conjugate. The partially purified PRP shall be stored frozen at or below -20°.

Identification

The PRP is identified by an immunochemical method (2.2.14) or other suitable method (e.g. ^{1}H or ^{13}C NMR spectroscopy).

Molecular size. The percentage of PRP eluted before a given K_0 value or within a range of K_0 values, is determined by gel filtration or high performance size-exclusion chromatography (HPSEC) (2.4.16), either alone or in combination with light scattering and refractive index detectors (e.g. multiple angle laser light scattering i.e. MALLS) or any other suitable method. An acceptable value is established for the particular product and each batch of PRP must be shown to comply with this limit. Limits for currently approved products, using the indicated stationary phases, are shown for information in Tables 1 and 2. Where applicable, the molecular-size distribution is also determined after chemical modification of the polysaccharide.

A validated determination of the degree of polymerization or of the weight-average molecular weight and the dispersion of molecular masses may be used instead of the determination of molecular size distribution.

Ribose (2.7.1). Not less than 32.0 per cent, calculated with reference to the dried substance, as estimated by Bial reaction for pentose, using D-ribose as a standard or any other suitable assay.

Phosphorus (2.7.1). 6.8 per cent to 9.0 per cent, calculated with reference to the dried substance.

Protein (2.3.49). Not more than 1.0 per cent, calculated with reference to the dried substance. Use sufficient PRP to allow detection of 1 per cent of protein (e.g. a minimum of 1 mg of PRP).

Nucleic acid (2.7.1). Not more than 1.0 per cent, calculated with reference to the dried substance by spectroscopy or any other suitable method.

Bacterial endotoxins (2.2.3). Not more than 25 IU of endotoxin per microgram of PRP.

Residual reagents. Where applicable, tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established for the particular product and each batch of PRP must be shown to comply with this limit. Where validation studies have demonstrated removal of a residual reagent, the test on PRP may be omitted.

Carrier protein

The carrier protein is chosen in a way so that when the PRP is conjugated it is able to induce a T-cell-dependent immune response. Currently approved carrier proteins and coupling methods are listed for information in Table 1. The carrier proteins are produced by culture of suitable microorganisms; the bacterial purity of the culture is verified; the culture may be inactivated; the carrier protein is purified by a suitable method.

Only a carrier protein that complies with the following requirements may be used in preparation of the conjugate.

Identification

The carrier protein is identified by a suitable immunochemical method (2.2.14).

Sterility (2.2.11). Carry out the test for sterility using for each medium 10 ml or the equivalent of one hundred doses, whichever is less.

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Table 1 - Different carrier proteins and coupling methods of *Haemophilus influenzae* Type b Conjugate Vaccine for information

Polysaccharide		Carrier Protein			Conjugation	
Type of PRP	Nominal amount per dose	Type	Purity	Nominal amount per dose	Coupling method	Procedure
Polysaccharide (size reduced) K_0 60.0 per cent: 0.6-0.7	25 µg	Diphtheria toxoid	>1500 Lf per mg of protein nitrogen	18 µg	Cyanogen bromide activation of PRP	Activated diphtheria toxoid (D-AH$^+$), cyanogen bromide activated PRP
Polysaccharide: PRP ≥ 50.0 per cent ≤ K_0: 0.30	10 µg	Tetanus toxoid	>1500 Lf per mg of protein nitrogen	20 µg	Carbodiimide-mediated coupling	ADH-activated PRP (PRP-cov.-AH) + tetanus toxoid + EDAC
Polysaccharide (size reduced) Dp=15-35 or 10-35	10 µg	CRM 197 diphtheria protein	>90.0 per cent diphtheria protein	25µg	Reductive amination one-(step method) or *N*-hydroxy-succinimide activation	Direct coupling of PRP to CRM 197 (cyanoboro-hydride activated)
Polysaccharide (size-reduced) K_0 0.3 – 0.6	15 µg	Meningococcal group B outer membrane protein (OMP)	Outer membrane protein vesicles <8.0 per cent of lipopolysaccharide	125 or 250 µg	Thioether bond	PRP activation by CDI PRP-IM + BuA2 + BrAc = PRP-BuA2-BrAc + thioactivated OMP

Abbreviations:

ADH	=	adipic acid dihydrazide	Dp	=	degree of polymerization
BrAc	=	bromoacetyl chloride	EDAC	=	l-ethyl-3-(3-dimethylaminopropyl) carbodimide
BuA2	=	butane-1, 4-diamide	IM	=	imidazolium
CDI	=	carbonyl-di-imidazole	Mw	=	weight-average molecular weight.

Diphtheria toxoid. Diphtheria toxoid is produced as stated under Diphtheria Vaccine (Adsorbed) and complies with the requirements prescribed there for bulk purified toxoid.

Tetanus toxoid. Tetanus toxoid is produced as stated under Tetanus Vaccine (Adsorbed) and complies with the requirements prescribed there for bulk purified toxoid except that the antigenic purity is not less than 1500 Lf per mg of protein nitrogen.

Diphtheria protein CRM 197. Suitable tests are carried out, for validation or routinely, to demonstrate that the product is non-toxic. The protein obtained contains not less than 90.0 per cent of diphtheria CRM 197 protein, when prepared by liquid chromatography (2.4.14) or any other suitable method. The carrier protein shall be characterized by a suitable chemical or physicochemical method like SDS-PAGE, HPLC, isoelectric focusing, amino acid sequencing, circular dichroism, fluorescence spectroscopy, peptide mapping or mass spectroscopy, as appropriate.

OMP (Meningococcal group B outer membrane protein complex)

OMP complex of *Neisseria meningitidis* complies with the following requirements for lipopolysaccharide and pyrogens.

Lipopolysaccharide. Not more than 8.0 per cent of lipopolysaccharide, determined by a suitable method.

Pyrogens (2.2.8). Inject into each rabbit 0.25 µg of OMP per kg body weight, for determining the pyrogenic effect.

Bulk conjugate

PRP is chemically modified to enable conjugation; it is usually partly depolymerised either before or during this procedure. Reactive functional groups or spacers may be introduced into the carrier protein or PRP prior to conjugation. The conjugate is obtained by the covalent binding of PRP and carrier protein. Where applicable, unreacted but potentially reactogenic functional groups are made unreactive by means of capping

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agents; the conjugate is purified to remove reagents. Where validation studies have demonstrated removal of a residual reagent (eg. CN, Br etc.), the test on bulk conjugate may be omitted.

Only a bulk conjugate that complies with the following requirements may be used in preparation of the final bulk vaccine. For each test and for each particular product, limits of acceptance are established and each batch of conjugate must be shown to comply with these limits. Limits applied to currently approved products for some of these tests are listed for information in Table 2.

PRP. The PRP content is determined by assay of phosphorus (2.7.1) or by assay of ribose (2.7.1) or by an immunochemical method (2.2.14) or by any suitable method.

Protein (2.7.1). The protein content is determined by a suitable chemical method.

PRP to protein ratio. Determine the ratio by calculation.

Molecular size. Molecular-size distribution is determined by gel filtration or size-exclusion chromatography (2.4.16), using a gel matrix, appropriate to the expected size of the conjugate.

Free PRP. Unbound PRP is determined after removal of the conjugate, for example by size-exclusion or hydrophobic chromatography (2.4.16), ultra-filtration or other validated methods.

Free carrier protein. Free carrier protein is determined by a suitable method (which may include deriving the content by calculation from the results of other tests). The amount is within the limits approved for the particular product.

Unreacted functional groups. No unreacted functional groups are detectable in the bulk conjugate unless process validation has shown that unreacted functional groups detectable at this stage are removed during the subsequent manufacturing process (for example, owing to short half-life).

Residual reagents. Removal of residual reagents such as *cyanide*, EDAC (*ethyldimethylaminopropylcarbodimide*) and *phenol* is confirmed by suitable tests or by validation of the purification process.

Sterility (2.2.11). Carry out the tests for sterility using for each medium 10 ml or the equivalent of one hundred doses, whichever is less.

FINAL BULK VACCINE

An adjuvant, an antimicrobial preservative and a stabilizer may be added to the bulk conjugate before dilution to the final concentration with a suitable diluent.

Only a final bulk vaccine that complies with the following requirements may be used in preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

The final lots are filled in suitable containers, under stringent aseptic conditions.

Table 2 - Requirement on bulk conjugate for currently approved products

Test	Protein Carrier			
Specifications	*Diphtheria toxoid*	*Tetanus toxoid*	*CRM 197*	*OMP*
Free Polysaccharide (PRP)	<37.0 per cent	<20.0 per cent	<25.0 per cent	<15.0 per cent
Unbound protein	<5.0 per cent, where applicable	<1.0 per cent, where applicable	<1.0 per cent or <2.0 per cent, depending on the coupling method	Not applicable
PRP to protein ratio	1.25-1.75	0.30-0.55	0.3-0.7	0.05-0.1
Molecular size (K_0): Cross-linked agarose for chromatography R	95.0 per cent <0.75	60.0 per cent <0.2	50.0 per cent 0.3-0.6	85.0 per cent < 0.25
Cross-linked agarose for chromatography R1	0.6-0.7	85.0 per cent <0.5		

1. In the case of combination vaccines containing PRP-T conjugate, the specification for free PRP content should be as established for the particular product.
2. The specification for PRP-T ratio will vary within limits and approved based on the method used for the PRP estimation.

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Only a final lot that is satisfactory with respect to each of the requirements given under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative has been carried out on the final bulk vaccine, it may be omitted on the final lot.

Identification

The vaccine is identified by a suitable immunochemical method (2.2.14) for PRP.

Tests

Sterility (2.2.11). Complies with the test for sterility.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject per kg of the rabbit's mass a quantity of the vaccine equivalent to 1 µg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 µg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 µg of PRP for a vaccine with OMP as carrier or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be within limits approved by the National Regulatory Authority for the particular product. If any components of the vaccine prevents the determination of the endotoxin, a test for Pyrogen should be carried out.

pH (2.4.24). The pH of the vaccine, reconstituted if necessary, is within the range approved for the product.

Aluminium (2.3.9). Not more than 1.25 mg per single human dose. When aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and is not greater than 115.0 per cent of that stated on the label.

Water (2.3.43). Not more than 3.0 per cent.

PRP. Not less than 80.0 per cent and not greater than 120.0 per cent of the amount of PRP stated on the label as determined by ribose assay (2.7.1) or by phosphorus assay (2.7.1) or by an immunochemical method (2.2.14) or by any other suitable method like colorimetery or by anion exchange liquid chromatography (2.4.14) with pulsed amperometric detection.

Free PRP. Unbound PRP is determined after removal of the conjugate for example by size-exclusion or hydrophobic chromatography (2.4.16), ultra filtration or other validated methods.

Labelling. The label states (1) the number of micrograms of PRP per human dose; (2) the type and nominal amount of carrier protein per single human dose; (3) for vaccine contained in single-dose containers where the space is too small to accommodate the full name of the vaccine the abbreviation 'Hib' may be used in the label on the container provided that the same code is also stated in the label on the package.

Hepatitis A (Inactivated) and Hepatitis B (rDNA) Vaccine (Adsorbed)

Hepatitis A (Inactivated) and Hepatitis B (rDNA) Vaccine (Adsorbed) is a suspension consisting of a suitable strain of hepatitis A virus, grown in cell cultures and inactivated by a validated method, and of hepatitis B surface antigen (HBsAg), a component protein of hepatitis B virus obtained by recombinant DNA technology; the antigens are adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate.

Production

General provisions

The two components are prepared as described in the monographs on Hepatitis A Vaccine (Inactivated, Adsorbed) and Hepatitis B Vaccine (rDNA) and comply with the requirements prescribed therein. The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for antisera and vaccines.

Reference preparation

The reference preparation is part of a representative batch shown to be at least as immunogenic in animals as a batch that, in clinical studies in young, healthy adults, produces not less than 95.0 per cent seroconversion, corresponding to a level of neutralizing antibody recognized to be protective, after a full-course primary immunization. For hepatitis A, an antibody level not less than 20 mIU per ml determined by enzyme-linked immunosorbent assay is recognized as being protective. For hepatitis B, antibody level not less than 10 mIU per ml against HBsAg is recognized as being protective.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more inactivated harvests of hepatitis A virus and one or more batches of purified antigen of Hepatitis B (rDNA).

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be

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released for use. Provided that the tests for free formaldehyde (where applicable) and antimicrobial preservative content (where applicable) have been carried out on the final bulk vaccine with satisfactory results, they may be omitted on the final lot. If the assay of the hepatitis A and/or the hepatitis B component is carried out *in vivo*, then provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Identification

The vaccine is shown to contain hepatitis A virus antigen and hepatitis B surface antigen by suitable immunochemical methods (2.2.14), using specific antibodies or by the mouse immunogenicity tests described under assay.

Tests

Aluminium (2.3.9). Maximum 1.25 mg per single human dose if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Bacterial endotoxins (2.2.3). Less than 2 IU per human dose.

Assay

Hepatitis A component

Complies with the assay as stated under Inactivated Hepatitis A Vaccine (Adsorbed).

Hepatitis B component

Complies with the assay as stated under Hepatitis B Vaccine (rDNA).

Labelling. The label states (1) the amount of hepatitis A virus antigen and hepatitis B surface antigen per container; (2) the type of cells used for production of the vaccine; (3) the name and amount of the adsorbent used; (4) that the vaccine must be shaken before use; (5) that the vaccine must not be frozen.

Hepatitis B Vaccine (rDNA)

Hepatitis B Vaccine (rDNA) is a non-infectious preparation containing the purified major surface antigen of Hepatitis B virus (HBsAg). This preparation is white or almost white translucent liquid in which the mineral carrier tends to settle down slowly on keeping but is free from foreign particles/ floccules.

Production

General provisions

The antigen is manufactured by recombinant DNA technology by culturing genetically engineered yeast cells or other suitable cell lines which carry the gene that codes for major surface antigen of the Hepatitis-B virus as approved by the competent authority. Several physico-chemical steps are employed to purify the Hepatitis-B surface antigen (HBsAg). The vaccine may contain the product of the S gene (major protein), a combination of the S gene and pre-S2 gene products (middle protein) or a combination of S gene, the pre-S2 gene, and pre-S1 gene products (large protein). The purity of the antigen is determined by comparison with a reference preparation using liquid chromatography or other suitable methods such as SDS-PAGE with any suitable staining method. The purified antigen is finally adsorbed on aluminium hydroxide or aluminium phosphate.

The method used for production of the vaccine must have been shown to yield a product consistently complying with the requirements for immunogenicity and safety. It must also have been shown to induce specific, protective antibodies in human beings.

The production method must be validated to demonstrate that the product if tested, would comply with the tests for safety and efficacy.

Reference preparation. A part of a batch shown to be at least as immunogenic as a batch that was used in clinical studies and or a representative batch approved by National Regulatory Authority and determined by any suitable method. For hepatitis B, antibody level not less than 10 mIU per ml against HBsAg is recognized as being protective.

Characterisation of the substance

Development studies are carried out to characterize the antigen. The complete protein, lipid and carbohydrate structure of the antigen is established. The morphological characteristics of the antigen particles are established by electron microscopy. The buoyant density of the antigen particles is determined by a physico-chemical method, for example gradient centrifugation. The antigenic epitopes are characterized. The protein fraction of the antigen is characterized in terms of the primary structure (for example, by determination of the amino-acid composition, by partial amino-acid sequence analysis).

PROPAGATION AND HARVEST

Identity, microbial purity, plasmid retention and consistency of yield are determined at suitable production stages. If mammalian cells are used, tests for extraneous agents and mycoplasmas are performed in accordance with tests for extraneous agents in viral vaccines for human use.

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PURIFIED ANTIGEN

Only a purified antigen that complies with the following requirements may be used in the preparation of the final bulk.

Total protein (2.3.49). The total protein is determined by a validated method. The content is within the limits approved for the specific product.

Antigen content and identification. The quantity and specificity of HBsAg is determined in comparison with the International standard for HBsAg subtype ad or an in-house reference, by a suitable immunochemical method such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot (preferably using a monoclonal antibody directed against a protective epitope) or single radial diffusion. The antigen/protein ratio is within the limits approved for the specific product.

The molecular weight of the major band in a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions corresponds to the value expected from the gene sequence and possible glycosylation.

Antigenic purity. The purity of the antigen is determined by comparison with a reference preparation using liquid chromatography or other suitable methods such as SDS-PAGE with staining. A suitable method is sensitive enough to detect a potential contaminant at a concentration of 1.0 per cent of total protein. Not less than 95.0 per cent of the total protein consists of hepatitis B surface antigen.

Composition. The content of proteins, lipids, nucleic acids and carbohydrates is determined.

Host-cell and vector-derived DNA (2.2.15). If mammalian cells are used for production, not more than 10 ng of DNA in the quantity of purified antigen equivalent to a single human dose of vaccine. In case of yeast derived products Host cell DNA may be omitted for routine lot release upon demonstration that the purification process consistently eliminates the residual components from the monovalent bulks to the satisfaction of National Regulatory Authority.

Caesium. If a caesium salt is used during production, a test for residual caesium is carried out on the purified antigen. The content is within the limits approved for the specific product.

Sterility (2.2.11). The purified antigen complies with the test for sterility, carried out using 10 ml for each medium.

Additional tests on the purified antigen may be required depending on the production method used: for example, a test for residual animal serum where mammalian cells are used for production or tests for residual chemicals used during extraction and purification.

FINAL BULK VACCINE

An antimicrobial preservative and an adjuvant may be included in the vaccine.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde, antimicrobial preservative content and the assay in animals, where applicable, have been carried out on the final bulk vaccine with satisfactory results, they may be omitted on the final lot. The estimate of potency shall be expected in terms of µg/ml i.e not less than 20 µg per ml.

Identification

The assay or, where applicable, the electrophoretic profile, also serves to identify the vaccine.

Tests

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbant.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115.0 per cent of that stated on the label.

Sterility (2.2.11). Complies with the test for sterility.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject the equivalent of 1 human dose into each rabbit or a validated test for bacterial endotoxin (2.2.3) may be used instead of the test for pyrogens. If used, the content should be less than 30 EU per Single Human Dose.

Assay. The vaccine complies with the assay of Hepatitis-B Vaccine (rDNA) described below.

Potency. The upper fiducial limit (P = 0.95) of the estimated relative potency is not less than 1.0 when assay by Method A (Biological). The estimate of potency shall be expressed in terms of µg/ml when assay by Method B (*in vitro*).

Determine the potency either in animals (Method A) or by a validated *in vitro* procedure (Method B) described below:

Method A (Biological)

The potency of the vaccine under examination is determined in animals by comparing in given conditions its capacity to

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induce specific anti-HBsAg antibodies in mice or guinea-pigs with the same capacity as with the reference standard.

Inject intraperitoneally not less than three suitable dilutions of the vaccine under examination diluted with adjuvant used in the vaccine into groups of a suitable strain of mice, weighing between 15 and 20 g (about 5 weeks old), of either sex distributed randomly into several groups of mice. Healthy guinea pigs weighing between 300 and 350 g (about 7 weeks old) that have not been previously treated with any material that will interfere with the test will also be suitable for the test. Use animals of the same sex in the test. Inject similar groups of animals with the reference preparation of Hepatitis-B vaccine (rDNA). One group of control animals remains unvaccinated but is injected intraperitoneally with the same volume of the diluent alone. Anaesthetise and bleed the animals 28 to 42 days later, keeping the individual sera separate. Assay the individual sera for specific HBsAg antibody concentration by a suitable immunochemical method such as ELISA or RIA.

Calculate the result of the assay by standard statistical methods (5.7). From the distribution of reaction levels measured on all the sera in the unvaccinated (control group), the maximum reaction level that can be expected to occur in an unvaccinated animal for that particular assay is determined. Any response in the vaccinated animals that exceeds this level is by definition seroconversion. The percentage of animals showing seroconversion in each group is transformed (for example, probit transformation) and a parallel line model, using the log dose response curve, is applied to the data. The potency of the preparation under examination relative to the reference preparation is thus established. The test is not valid unless (a) for both the test and reference vaccine, the ED_{50} lies between the smallest and the largest doses given to animals; (b) the statistical analysis shows no deviation from linearity or parallelism; (c) the fiducial limits of the estimated relative potency fall between 33.0 and 300.0 per cent of the estimated potency.

Method B *(In vitro)*

The potency of the vaccine under examination is determined by an *in vitro* method that has been validated against the biological test.

Enzyme Linked Immunosorbent Assay (ELISA) using monoclonal antibodies specific for protection inducing epitopes of HBsAg have been shown to be suitable. Adequate number of dilutions and replicates of the vaccine under examination and the reference standard are employed in the assay. The data obtained is analyzed by a parallel-line model and may be suitably transformed for statistical evaluation. Commercially available kits for measuring HBsAg *in vitro* may be used provided they are validated to produce equally precise and accurate results.

The test is not valid unless (a) the statistical analysis shows no deviation from linearity or parallelism; (b) the fiducial limits of the estimated relative potency fall between 80.0 and 125.0 per cent of the estimated potency.

The acceptance criteria are approved for a given reference preparation by the National Regulatory Authority in the light of the validation data.

Labelling. The label states (a) the amount of HBsAg per dose; (b) the type of cells used for production of the vaccine; (c) the name and amount of the adjuvant; (d) that the vaccine must be shaken before use; (e) that the vaccine must not be frozen.

Inactivated Hepatitis A Vaccine (Adsorbed)

Hepatitis A Vaccine (Inactivated, Adsorbed) is a liquid preparation of a suitable strain of hepatitis A virus grown in cell cultures, inactivated by a validated method and adsorbed on a mineral carrier. The vaccine is an opalescent suspension.

The vaccine complies with the monograph on Vaccines.

Production

Production of the vaccine is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to yield consistently vaccines that comply with the requirements for immunogenicity, safety and stability.

The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy.

Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

Reference preparation. A part of a batch shown to be at least as immunogenic as a batch that, in clinical studies in young healthy adults, produced not less than 95.0 per cent seroconversion, corresponding to a level of neutralising antibody accepted to be protective, after a full-course of primary immunisation is used as a reference preparation. An antibody level of 20 mIU per ml determined by enzyme-linked immunosorbent assay is recognised as being protective.

Substrate for virus propagation

The virus is propagated in a human diploid cell line or in a continuous cell line approved by the competent authority.

SEED LOT

The strain of hepatitis A virus used to prepare the master seed lot shall be identified by historical records that include

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information on the origin of the strain and its subsequent manipulation.

Only a seed lot that complies with the following requirements may be used for virus propagation.

Description. A clear, colourless or light coloured liquid.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

Identification

Each master and working seed lot is identified as hepatitis A virus using specific antibodies.

Virus concentration. The virus concentration of each master and working seed lot is determined to monitor consistency of production.

Extraneous agents (2.7.3). The master and working seed lots comply with the requirements for seed lots for virus vaccines. In addition, if primary monkey cells have been used for isolation of the strain, measures are taken to ensure that the strain is not contaminated with simian viruses such as simian immunodeficiency virus and filoviruses.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are being handled. Animal serum (but not human serum) may be used in the cell culture media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator, such as phenol red and approved antibiotics at the lowest effective concentration. Not less than 500 ml of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells). Multiple harvests from the same production cell culture may be pooled and considered as a single harvest.

Only a single harvest that complies with the following requirements may be used in the preparation of the vaccine. When the determination of the ratio of virus concentration to antigen content has been carried out on a suitable number of single harvests to demonstrate consistency, it may subsequently be omitted as a routine test.

Identification

The test for antigen content also serves to identify the single harvest.

Bacterial and fungal contamination. The single harvest complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

Mycoplasmas (2.7.4). The single harvest complies with the test for mycoplasmas carried out using 1 ml for each medium.

Control cells. The control cells of the production cell culture comply with a test for identity and the requirements for extraneous agents.

Antigen content. Determine the hepatitis A antigen content by a suitable immunochemical method (2.2.14) to monitor production consistency; the content is within the limits approved for the particular product.

Ratio of virus concentration to antigen content. The consistency of the ratio of the concentration of infectious virus, as determined by a suitable cell culture method, to antigen content is established by validation on a suitable number of single harvests.

PURIFICATION AND PURIFIED HARVEST

The harvest, which may be a pool of several single harvests, is purified by validated methods. If continuous cell lines are used for production, the purification process shall have been shown to reduce consistently the level of host-cell DNA. Only a purified harvest that complies with the following requirements may be used in the preparation of the inactivated harvest.

Virus concentration. The concentration of infective virus in the purified harvest is determined by a suitable cell culture method to monitor production consistency and as a starting point for monitoring the inactivation curve.

Antigen total protein ratio. Determine the hepatitis A virus antigen content by a suitable immunochemical method (2.2.14). Determine the total protein by a validated method. The ratio of hepatitis A virus antigen content to total protein content is within the limits approved for the particular product.

Bovine serum albumin. Not more than 50 ng in the equivalent of a single human dose, determined by a suitable immunochemical method (2.2.14). Where appropriate in view of the manufacturing process, other suitable protein markers may be used to demonstrate effective purification.

Residual host-cell DNA (2.2.15). If a continuous cell line is used for virus propagation, the content of residual host-cell DNA, determined using a suitable method is not greater than 10 ng per single human dose.

Residual chemicals. If chemical substances are used during the purification process, tests for these substances are carried out on the purified harvest (or on the inactivated harvest), unless validation of the process has demonstrated total clearance. The concentration must not exceed the limits approved for the particular product.

INACTIVATION AND INACTIVATED HARVEST

Several purified harvests may be pooled before inactivation. In order to avoid interference with the inactivation process, virus aggregation must be prevented or aggregates must be

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removed immediately before and/or during the inactivation process. The virus suspension is inactivated by a validated method; the method shall have been shown to be consistently capable of inactivating hepatitis A virus without destroying the antigenic and immunogenic activity; as part of the validation studies, an inactivation curve is plotted representing residual live virus concentration measured on at least three occasions (for example, on days 0, 1 and 2 of the inactivation process). If formaldehyde is used for inactivation, the presence of excess free formaldehyde is verified at the end of the inactivation process.

Only an inactivated harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Inactivation. Carry out an amplification test for residual infectious hepatitis A virus by inoculating a quantity of the inactivated harvest equivalent to 5 per cent of the batch or if the harvest contains the equivalent of 30,000 doses or more, not less than 1,500 doses of vaccine into cell cultures of the same type as those used for production of the vaccine and incubating the cells for at least 28 days. Make two passages and at the end of incubation carry out a test of suitable sensitivity for residual infectious virus. No evidence of hepatitis A virus multiplication is found in the samples taken at the end of the inactivation process. Use infective virus inocula concurrently as positive controls to demonstrate cellular susceptibility and absence of interference.

Sterility (2.2.11). The inactivated viral harvest complies with the test for sterility, carried out using 10 ml for each medium.

Bacterial endotoxins (2.2.3). Not more than 2 IU of endotoxin in the equivalent of a single human dose.

Antigen content. Determine the hepatitis A virus antigen content by a suitable immunochemical method (2.2.14).

Residual chemicals. As stated under Purification and Purified Harvest.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more inactivated harvests. Approved adjuvants, stabilisers and antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile containers. The containers are then closed so as to avoid contamination.

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde (where applicable) and antimicrobial preservative content (where applicable) and the assay have been carried out on the final bulk vaccine with satisfactory results, these tests may be omitted on the final lot. If the assay is carried out using mice or other animals, then provided it has been carried with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Identification

The vaccine is shown to contain hepatitis A virus antigen by a suitable immunochemical method using specific antibodies or by the mouse immunogenicity test described under Assay.

Tests

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if hydrated aluminium phosphate or aluminium hydroxide is used as the adsorbent.

Free formaldehyde (2.3.20). When formaldehyde has been used for inactivation, the vaccine complies with the test for free formaldehyde as stated under General Requirements for Vaccines for Human Use.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Assay

The vaccine complies with the assay of Hepatitis A vaccine.

The assay is carried out either *in vivo*, by comparing in given condition the capacity to induce specific antibodies in mice with the same capacity of a reference preparation or *in vitro* by an immunochemical determination of antigen content (2.2.14).

In vivo assay

The test on mice shown below is given as an example of a method that has been found suitable for a given vaccine; other validated methods may also be used.

Selection and distribution of the test animals. Healthy mice from the same stock, about 5 weeks old and from a strain

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shown to be suitable should be used in the test. Use animals of the same sex. Distribute the animals in at least seven equal groups of a number suitable for the requirements of the assay.

Determination of potency of the vaccine under examination. Using a 0.9 per cent w/v solution of *sodium chloride* containing the aluminium adjuvant used for the vaccine, prepare at least three dilutions of the vaccine under examination and matching dilutions of the reference preparation. Allocate the dilutions one to each of the groups of animals and inject subcutaneously not more than 0.5 ml of each dilution into each animal in the group to which that dilution is allocated. Maintain a group of unvaccinated controls, injected subcutaneously with the same volume of diluent. After 28 to 32 days, anaesthetise and bleed all animals, keeping the individual sera separate. Assay the individual sera for specific antibodies against hepatitis A virus by a suitable immunochemical method (2.2.14).

Calculations. Carry out the calculations by the usual statistical methods (5.7) for an assay with a quantal response.

From the distribution of reaction levels measured on all the sera in the unvaccinated group, determine the maximum reaction level that can be expected to occur in an unvaccinated animal for that particular assay. Any response in vaccinated animals that exceeds this level is by definition a seroconversion.

Make a suitable transformation of the percentage of animals showing seroconversion in each group (for example, a probit transformation) and analyse the data according to a parallel-line log dose-response model. Determine the potency of the test preparation relative to the reference preparation.

Validity conditions. The test is not valid unless (a) for both the test and the reference vaccine, the ED_{50} lies between the smallest and the largest doses given to the animals; (b) the statistical analysis shows no significant deviation from linearity or parallelism; (c) the fiducial limits of the estimated relative potency fall between 33.0 and 300.0 per cent of the estimated potency.

Potency. The upper fiducial limit (P = 0.95) of the estimated relative potency is not less than 1.0.

In vitro *assay*

Carry out an immunochemical determination of antigen content (2.2.14) with acceptance criteria validated against the *in vivo* test. The acceptance criteria are approved for a given reference preparation by the National Regulatory Authority in the light of the validation data.

Labelling. The label states (1) the biological origin of the cells and; (2) the adjuvant used for the preparation of the vaccine.

Inactivated Hepatitis B Vaccine

Inactivated Hepatitis B Vaccine is a non-infectious inactivated liquid preparation derived from the surface antigen of Hepatitis B virus (HbsAg). This preparation is white or almost white translucent liquid in which the mineral carrier tends to settle down slowly on keeping but is free from foreign particles/ floccules.

Production

The antigen is harvested and purified from the plasma of human carriers of Hepatitis B virus. The surface antigen contains all the three antigen species (S, Pre-S1, Pre-S2). The individual donor plasma is shown by sensitive tests to be seronegative for HIV-I and HIV-2 and for HCV. The plasma pool is tested for freedom from adventitious viruses and blood borne transmissible pathogens by appropriate methods. The purified antigen is further inactivated by a validated method, usually with formalin or any other inactivating agent, to render the hepatitis B virus harmless. The preparation is also tested for the residual HBV DNA using a sensitive test approved by the competent authority and the level is shown to be less than 1 pg HBV DNA per 50 doses.

The method used for production of the vaccine must have been shown to yield a product consistently complying with the requirements of immunogenicity, safety and stability. The production method must also be validated to demonstrate that the product, if tested, would comply with the tests for safety and efficacy.

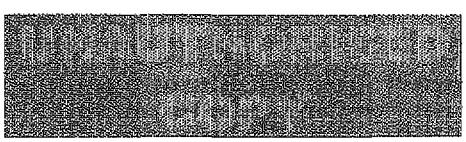
Reference preparation. A part of a batch shown to be at least as immunogenic as a batch that produced in clinical studies in young healthy adults not less than 95.0 per cent seroconversion, corresponding to a level of neutralizing antibody accepted to be protective (HbsAg antibody titre not less than 10 mIU per ml after a full course of primary immunization determined by Enzyme-Linked Immunosorbent Assay (ELISA) is used as a reference preparation.

Characterisation of the substance

Development studies are carried out to characterize the antigen. The complete protein, lipid and carbohydrate structure of the antigen is established. The morphological characteristics of the antigen particles are established by electron microscopy. The buoyant density of the antigen particles is determined by a physico-chemical method (2.4.29), for example gradient centrifugation. The antigenic epitopes are characterized. The protein fraction of the antigen is characterized in terms of the primary structure (for example, by determination of the amino-acid composition, by partial amino-acid sequence analysis).

PROPAGATION AND HARVEST

Identity, microbial purity, plasmid retention and consistency of yield are determined at suitable production stages.



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Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

PURIFIED ANTIGEN

Only a purified antigen that complies with the following requirements may be used in the preparation of the final bulk.

Total protein (2.3.49). The total protein is determined by a validated method. The content is within the limits approved for the specific product.

Antigen content and identification. The quantity and specificity of HBsAg is determined in comparison with the International standard for HBsAg subtype *ad* or an in-house reference, by a suitable immunochemical method such as radio immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot (preferably using a monoclonal antibody directed against a protective epitope) or single radial diffusion. The antigen/protein ratio is within the limits approved for the specific product.

The molecular weight of the major band in a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions corresponds to the value expected from the gene sequence and possible glycosylation.

Antigenic purity. The purity of the antigen is determined by comparison with a reference preparation using liquid chromatography or other suitable methods such as SDS-PAGE with staining. A suitable method is sensitive enough to detect a potential contaminant at a concentration of 1.0 per cent of total protein. Not less than 95.0 per cent of the total protein consists of hepatitis B surface antigen.

Composition. The content of proteins, lipids, nucleic acids and carbohydrates is determined.

Bacterial and fungal contamination. The purified antigen complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

Additional tests on the purified antigen may be required depending on the production method used.

FINAL BULK VACCINE

An antimicrobial preservative and an adjuvant may be included in the vaccine.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the 85.0 per cent and not greater than 115.0 per cent of that stated on the label.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde, antimicrobial preservative content and the assay in animals, where applicable, have been carried out on the final bulk vaccine with satisfactory results, they may be omitted on the final lot.

Identification

The assay or, where applicable, the electrophoretic profile, also serves to identify the vaccine.

Tests

Aluminium (2.3.9). When hydrated aluminium phosphate or aluminium hydroxide is used as the adsorbent, the vaccine complies with the test prescribed in the monograph on Vaccines.

Test for inactivating agent. The concentration of any inactivating agent remaining in the final vaccine shall be determined by methods approved by the competent authority. The concentration shall not exceed a specified upper limit.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject the equivalent of one human dose into each rabbit.

A validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens.

Assay

The upper fiducial limit ($P = 0.95$) of the estimated relative potency is not less than 1.0.

Determine the potency by method A (Biological) as described under Hepatitis-B vaccine (rDNA).

Labelling. The label states (1) the amount of HBsAg per dose; (2) the name and amount of inactivating agent; (3) the name and amount of the adjuvant; (4) that the vaccine must be shaken before use; (5) that the vaccine must not be frozen.

Inactivated Influenza Vaccine (Split Virion)

Influenza Vaccine (Split Virion, Inactivated) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or

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B, or a mixture of strains of the two types grown individually in eggs derived from specific pathogen free flock or cell cultures, inactivated and treated so that the integrity of the virus particles has been disrupted without diminishing the antigenic properties of the haemagglutinin and neuraminidase antigens. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount.

Production

The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy.

Choice of vaccine strain

The World Health Organisation reviews the world epidemiological situation and if necessary recommends new strains corresponding to prevailing epidemiological evidence which should be complied with.

The origin and passage history of virus strains shall be approved by the National Regulatory Authority.

Substrate for virus propagation

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens or in suitable cell cultures, such as chick-embryo fibroblasts or chick kidney cells obtained from chicken flocks free from specified pathogens. For production, the virus of each strain is grown in the allantoic cavity of eggs derived from specific pathogen free flocks.

SEED LOT

The production of vaccine is based on a seed-lot system. Working seed lots represent not more than fifteen passages from the approved reassorted virus or the approved virus isolate. The final vaccine represents one passage from the working seed lot. The haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

Bacterial and fungal contamination: Carry out the test for sterility (2.2.11) using 10 ml for each sterility medium.

Mycoplasmas (2.7.4). Carry out the test for mycoplasmas using 10 ml.

PROPAGATION AND HARVEST

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form a monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest. At no stage in the production, penicillin or streptomycin is used.

Mycoplasma (2.7.4).Complies with the test for absence of mycoplasma.

MONOVALENT POOLED HARVEST

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on three consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process should cause minimum alteration of the haemagglutinin and neuraminidase antigens. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at a temperature of 5 ± 3°. If formaldehyde solution is used, the concentration does not exceed 0.2 g/l of formaldehyde at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent v/v at any time during inactivation.

Before or after the inactivation procedure, the monovalent pooled harvest is concentrated and purified by high-speed centrifugation or other suitable method and the virus particles are disrupted into component subunits by the use of approved procedures. For each new strain, a validation test is carried out to show that the monovalent bulk consists predominantly of disrupted virus particles.

Only a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Haemagglutinin antigen. Determine the content of haemagglutinin antigen by an immunodiffusion test (2.2.14), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it. Carry out the test at 20° to 25°.

For some vaccines, the physical form of the haemagglutinin particles prevents quantitative determination by immunodiffusion after inactivation of the virus. For these vaccines, a determination of haemagglutinin antigen is made on the monovalent pooled harvest before inactivation. The production process is validated to demonstrate suitable conservation of haemagglutinin antigen and a suitable tracer is used for formulation, for example, protein content.

Neuraminidase antigen. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods (2.2.14) on the first three monovalent pooled harvests from each working seed lot.

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Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml for each sterility medium.

Viral inactivation. Carry out as described below under Tests.

Chemicals used for disruption. Tests are carried out on the monovalent pooled harvest for the chemicals used for disruption, the limits being approved by the National Regulatory Authority.

FINAL BULK VACCINE

Appropriate quantities of the monovalent pooled harvests are blended to make the final bulk vaccine.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml for each sterility medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Tests and Assay may be released for use. Provided that the test for viral inactivation has been performed with satisfactory results on each monovalent pooled harvest and that the tests for free formaldehyde, ovalbumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Description. The vaccine is a slightly opalescent liquid.

Identification

The assay serves to confirm the antigenic specificity of the vaccine.

Tests

Viral inactivation. Inoculate 0.2 ml of the vaccine into the allantoic cavity of each of ten fertilised eggs and incubate at 33° to 37° for 3 days. The test is not valid unless at least eight of the ten embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further ten fertilised eggs and incubate at 33° to 37° for 3 days. The test is not valid unless at least eight of the ten embryoes survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

Total protein (2.3.49). Not more than six times the total haemagglutinin content of the vaccine as determined in the assay, but in any case, not more than 100 µg of protein per virus strain per human dose and not more than a total of 300 µg of protein per human dose.

Ovalbumin. Not more than 1 µg of ovalbumin per human dose, determined by a suitable technique using a suitable reference preparation of ovalbumin.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Bacterial endotoxins (2.2.3). Not more than 100 IU of endotoxin per human dose.

Assay

Determine the content of haemagglutinin antigen by an immunodiffusion test (2.2.14), by comparison with an appropriate haemagglutinin antigen reference preparation. Carry out the test at 20° to 25°. The confidence interval (P = 0.95) of the assay is not greater than 80.0 per cent to 125.0 per cent of the estimated content. The lower confidence limit (P = 0.95) of the estimate of haemagglutinin antigen content is not less than 80.0 per cent of the amount stated on the label for each strain.

For some vaccines, quantitative determination of haemagglutinin antigen with respect to available reference preparations is not possible. An immunological identification of the haemagglutinin antigen and a semi-quantitative determination are carried out instead by suitable methods.

Labelling. The label complies with the requirements stated under Vaccines and also states (a) that the vaccine has been prepared on eggs; (b) the strain or strains of influenza virus used to prepare the vaccine; (c) the method of inactivation; (d) the haemagglutinin content in µg per virus strain per dose; (e) the season during which the vaccine is intended to protect.

Inactivated Influenza Vaccine (Surface Antigen)

Influenza Vaccine (Surface Antigen, Inactivated) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the two types grown individually in eggs derived from specific pathogen free flocks or cell cultures, inactivated and treated so that the preparation

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consists predominantly of haemagglutinin and neuraminidase antigens, without diminishing the antigenic properties of these antigens. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount.

Production

The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy.

Choice of vaccine strain

The World Health Organisation reviews the world epidemiological situation annually and if necessary recommends new strains corresponding to prevailing epidemiological evidence.

The origin and passage history of virus strains shall be approved by the competent authority.

Substrate for virus propagation

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens or in suitable cell cultures, such as chick-embryo fibroblasts or chick kidney cells obtained from chicken flocks free from specified pathogens. For production, the virus of each strain is grown in the allantoic cavity of eggs derived from SPF flocks.

SEED LOT

The production of vaccine is based on a seed-lot system. Working seed lots represent not more than fifteen passages from the approved reassorted virus or the approved virus isolate. The final vaccine represents one passage from the working seed lot. The haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml for each medium.

Mycoplasmas (2.7.4). Carry out the test for mycoplasmas using 10 ml.

PROPAGATION AND HARVEST

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form a monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest. At no stage in the production penicillin or streptomycin is used.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

MONOVALENT POOLED HARVEST

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on three consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process should cause minimum alteration of the haemagglutinin and neuraminidase antigens. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at a temperature of 5 ± 3°. If formaldehyde solution is used, the concentration does not exceed 0.2 g/l of formaldehyde at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent v/v at any time during inactivation.

Before or after the inactivation process, the monovalent pooled harvest is concentrated and purified by high-speed centrifugation or other suitable method. Virus particles are disrupted into component subunits by approved procedures and further purified so that the monovalent bulk consists mainly of haemagglutinin and neuraminidase antigens.

Only a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Haemagglutinin antigen. Determine the content of haemagglutinin antigen by an immunodiffusion test (2.2.14), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it. Carry out the test at 20° to 25°.

Neuraminidase antigen. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods (2.2.14) on the first three monovalent pooled harvests from each working seed lot.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml for each medium.

Viral inactivation. Carry out the test described below under Tests.

Purity. The purity of the monovalent pooled harvest is examined by polyacrylamide gel electrophoresis or by other approved techniques. Mainly haemagglutinin and neuraminidase antigens shall be present.

Chemicals used for disruption and purification. Tests are carried out on the monovalent pooled harvest for the chemicals used for disruption and purification, the limits being approved by the competent authority.

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FINAL BULK VACCINE

Appropriate quantities of the monovalent pooled harvests are blended to make the final bulk vaccine.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for viral inactivation has been performed with satisfactory results on each monovalent pooled harvest and that the tests for free formaldehyde, ovalbumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Description. The vaccine is a clear liquid.

Identification

The assay serves to confirm the antigenic specificity of the vaccine.

Tests

Viral inactivation. Inoculate 0.2 ml of the vaccine into the allantoic cavity of each of ten fertilised eggs and incubate at 33° to 37° for 3 days. The test is not valid unless at least eight of the ten embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further ten fertilised eggs and incubate at 33° to 37° for 3 days. The test is not valid unless at least eight of the ten embryos survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

Total protein (2.3.49). Not more than 40 µg of protein other than haemagglutinin per virus strain per human dose and not more than a total of 120 µg of protein other than haemagglutinin per human dose.

Ovalbumin. Not more than 1 µg of ovalbumin per human dose, determined by a suitable technique using a suitable reference preparation of ovalbumin.

Free formaldehyde (2.3.20). Complies with the test for free formaldehyde as stated under General Requirements for Vaccines for Human Use.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Bacterial endotoxins (2.2.3). Not more than 100 IU of endotoxin per human dose.

Assay

Determine the content haemagglutinin antigen by an immunodiffusion test (2.2.14), by comparison with an appropriate haemagglutinin antigen reference preparation. Carry out the test at 20° to 25°. The confidence interval (P = 0.95) of the assay is not greater than 80.0 per cent to 125.0 per cent of the estimated content. The lower confidence limit (P = 0.95) of the estimate of haemagglutinin antigen content is not less than 80.0 per cent of the amount stated on the label for each strain.

Labelling. The label states (1) that the vaccine has been prepared on eggs; (2) the strain or strains of influenza virus used to prepare the vaccine; (3) the method of inactivation; (4) the haemagglutinin content in micrograms per virus strain per dose; (5) the season during which the vaccine is intended to protect.

Inactivated Influenza Vaccine (Whole Virion)

Influenza Vaccine (Whole Virion, Inactivated) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the two types grown individually in eggs derived from specific pathogen free flocks or cell culture and inactivated in such a manner that their antigenic properties are retained. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount.

Production

The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy.

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Choice of vaccine strain

The World Health Organisation reviews the world epidemiological situation annually and, if necessary, recommends new strains corresponding to prevailing epidemiological evidence.

The origin and passage history of virus strains shall be approved by the competent authority.

Substrate for virus propagation

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens or in suitable cell cultures, such as chick-embryo fibroblasts or chick kidney cells obtained from chicken flocks free from specified pathogens .For production, the virus of each strain is grown in the allantoic cavity of eggs derived from specific pathogen free flocks.

SEED LOT

The production of vaccine is based on a seed-lot system. Working seed lots represent not more than fifteen passages from the approved reassorted virus or the approved virus isolate. The final vaccine represents one passage from the working seed lot. The haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml for each medium.

Mycoplasmas (2.7.4). Carry out the test for mycoplasmas using 10 ml.

PROPAGATION AND HARVEST

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form a monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest. At no stage in the production is penicillin or streptomycin used.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

MONOVALENT POOLED HARVEST

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on three consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process should cause minimum alteration of the haemagglutinin and neuraminidase antigens. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at a temperature of 5±3°. If formaldehyde solution is used, the concentration does not exceed 0.2 g/l of formaldehyde at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent v/v at any time during inactivation.

Before or after the inactivation process, the monovalent pooled harvest is concentrated and purified by high-speed centrifugation or other suitable method.

Only a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Haemagglutinin antigen. Determine the content of haemagglutinin antigen by an immunodiffusion test (2.2.14), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it. Carry out the test at 20° to 25°.

Neuraminidase antigen. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods (2.2.14) on the first three monovalent pooled harvests from each working seed lot.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml for each medium.

Viral inactivation. Carry out the test described below under Tests.

FINAL BULK VACCINE

Appropriate quantities of the monovalent pooled harvests are blended to make the final bulk vaccine.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml for each sterility medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Tests and Assay may be

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released for use. Provided that the test for viral inactivation has been performed with satisfactory results on each monovalent pooled harvest and that the tests for free formaldehyde, ovalbumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Description. The vaccine is a slightly opalescent liquid.

Identification

The assay serves to confirm the antigenic specificity of the vaccine.

Tests

Viral inactivation. Inoculate 0.2 ml of the vaccine into the allantoic cavity of each of ten fertilised eggs and incubate at 33° to 37° for 3 days. The test is not valid unless at least eight of the ten embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further ten fertilised eggs and incubate at 33° to 37° for 3 days. The test is not valid unless at least eight of the ten embryos survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

Total protein (2.3.49). Not more than six times the total haemagglutinin content of the vaccine as determined in the assay, but in any case, not more than 100 µg of protein per virus strain per human dose and not more than a total of 300 µg of protein per human dose.

Ovalbumin. Not more than 1 µg of ovalbumin per human dose, determined by a suitable technique using a suitable reference preparation of ovalbumin.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and is not greater than 115.0 per cent of the quantity stated on the label.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Bacterial endotoxins (2.2.3). Not more than 100 IU of endotoxin per human dose.

Assay

Determine the content of haemagglutinin antigen by an immunodiffusion test (2.2.14), by comparison with an appropriate haemagglutinin antigen reference preparation. Carry out the test at 20° to 25°. The confidence interval (P = 0.95) of the assay is not greater than 80.0 per cent to 125.0 per cent of the estimated content. The lower confidence limit(P = 0.95) of the estimate of haemagglutinin antigen content is not less than 80.0 per cent of the amount stated on the label for each strain.

Labelling. The label states (1) that the vaccine has been prepared on eggs; (2) the strain or strains of influenza virus used to prepare the vaccine; (3) the method of inactivation; (4) the haemagglutinin content in micrograms per virus strain per dose; (5) the season during which the vaccine is intended to protect.

Influenza Vaccine (Human, Live Attenuated)

Influenza Vaccine (Human, Live attenuated), for intranasal administration, is a freeze dried or a liquid preparation of a suitable attenuated strain or strains of influenza virus, type A or B, or a mixture of strains of the two types grown individually in embryonated hen's egg or cell culture. The reconstituted freeze dried vaccine, or the liquid preparation is a slightly opalescent liquid that may be coloured due to presence of stabilizers and/or indicator.

Production

The production of vaccine is based on a virus seed lot system and if the virus is propagated in cell culture, a cell bank system. The virus in the final vaccine shall have undergone no more passage from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

The production method is validated to demonstrate that the product would comply with the test for quality, safety and efficacy.

Choice of vaccine strain

Manufacturer should use reassortant prepared using National Regulatory Authority approved master donor virus and WHO recommended strain(s) for the relevant influenza season.

The World Health Organization reviews the world epidemiological situation and if necessary recommends new strains corresponding to prevailing epidemiological evidence, which should be complied with.

The origin and passage history of virus strains shall be approved by the National Regulatory Authority.

Reference preparation. A suitable reference preparation that is representative of each type and sub-type is established for use in tests to determine virus concentration.

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Substrate for virus propagation

The influenza virus seed to be used in the production of vaccine is propagated in specific pathogen free (SPF) embryonated hen's eggs or in suitable characterized cell cultures approved by National Regulatory Authority. The seed lot is prepared using specific pathogen free (SPF) embryonated hen's eggs.

SEED LOT

The influenza vaccine strain (master donor virus MDV and WHO recommended strain) used in the production of influenza vaccine shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are prepared in large quantities and stored at temperatures below -20° if freeze-dried, or below -60° if not freeze-dried.

Only a working seed lot that complies with the following requirements shall be used in the preparation of monovalent pooled harvest.

Identification

The haemagglutinin and neuraminidase antigens of working seed lots of each component strain should match with WHO recommended strain(s) of Influenza virus for the vaccine using an immunochemical methods (2.2.14) or by validated molecular identity test approved by National Regulatory Authority.

Attenuation markers. The key genetic and phenotypic characteristics markers related to attenuation of each component strain of the vaccine shall be demonstrated by suitable methods approved by National Regulatory Authority.

Virus concentration. The virus concentration of the working seed lots shall be determined using any suitable method approved by National Regulatory Authority.

Sterility (2.2.11). Carry out the test for sterility using 10 ml for each medium.

Mycoplasma (2.7.4). Carry out the test for mycoplasma using 10 ml for each medium.

Extraneous agents (2.7.3). Working seed lot complies with the test for extraneous agents test.

PROPAGATION AND HARVEST

During production using embryonated hen's eggs an antimicrobial agent may be added to the inoculum. However beta-lactam antimicrobial agents should not be used at any stage in the manufacture of the vaccine.

For cell culture vaccine suitable animal serum may be used in the growth medium. However, human serum should not be used. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. If animal serum is used in the growth medium for the cell cultures, the serum shall be removed from the cell cultures either before or after inoculation with the seed virus. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics (excluding beta-lactam antimicrobial agents) at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production.

After virus inoculation, eggs or cultures for vaccine production shall be incubated under controlled temperature conditions. The egg allantoic fluids or culture medium derived from cell culture should be harvested to prepare a monovalent single harvest.

Mycoplasma (2.7.4).Complies with the test for absence of mycoplasma.

MONOVALENT VIRUS POOL

A number of single harvests of the same virus strain may be combined to produce a monovalent virus pool. The monovalent virus pool should be clarified and may be purified by suitable method. Only a clarified monovalent virus pool that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Tests of control eggs. A portion (2 per cent or at least 20 eggs, whichever of the quantities is greater) of each batch of the eggs used for vaccine virus propagation is held as un-inoculated control eggs.

These control eggs are incubated for the same time and temperature as the inoculated eggs. At the time of harvesting the virus from the inoculated eggs, allantoic fluids are also taken from the un-inoculated control eggs and examined for the existence of haemagglutinating agents. A sample of 0.2 ml of allantoic fluid taken from each control egg should be tested for haemagglutinating agents using guinea pig red blood cells both directly and after one passage of the control allantoic fluid through embryonated hen's eggs and should be negative for heamagglutination.

For the test to be valid, not more than 20 per cent of the control eggs should be discarded for non-specific reasons.

Test of control cells. For influenza vaccine prepared in cell culture, testing for the presence of haemadsorbing viruses should include at least 5 per cent of total volume or 500 ml of cell suspension whichever is greater. Testing shall be conducted at the end of 14 days observation period from date of inoculation or at the time the virus is last harvested from the production substrate, whichever comes later.

Examine not less than 25 per cent of the control cultures for the presence of haemadsorbing viruses using guinea pig red blood cells. If guinea pig red blood cells have been stored, they shall have been stored at 2° to 8° for not more than

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7 days. Read half of the cultures after incubation for 30 minutes at 2° to 8° and the other half after incubation at 20° to 25° for 30 minutes. No evidence of haemadsorbing agent should be found.

For the test to be valid, not more than 20 per cent of the control cell cultures should be discarded for non-specific reasons.

The control cells of the production cell culture should comply with a test for identification and with the requirements for extraneous agents (2.7.3).

Potency. The influenza virus content in the clarified monovalent virus pool should be determined by titration in embryonated hen's eggs or cell culture. The number of infectious doses of vaccine should be expressed in terms of egg infectivity dose 50 per cent (EID_{50}), tissue culture infectivity dose 50 per cent ($TCID_{50}$) or Focus forming unit (FFU) or equivalent methods dependent on the replication of virus.

Attenuation markers

Retention of key genetic and phenotypic characteristic markers related to attenuation of each strain of influenza vaccine virus should be demonstrated for the clarified monovalent virus pool by suitable methods approved by National Regulatory Authority.

Identification

Each clarified monovalent virus pool lot shall be identified as containing live attenuated influenza virus of the appropriate strain by suitable methods approved by National Regulatory Authority.

Sterility (2.2.11). Carry out the test for sterility using 10 ml for each medium.

Mycoplasma (2.7.4). Carry out the test for mycoplasma by suitable method.

Extraneous agents (2.7.3). Complies with the test for extraneous agents.

Avian leucosis virus (ALV). For virus grown in embryonated eggs, a pool of harvest shall be tested for avian leucosis virus by suitable method.

FINAL BULK VACCINE

Final bulks are prepared by mixing and diluting clarified monovalent virus pools of the relevant strains. Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Sterility (2.2.11). Carry out the test for sterility using 10 ml of bulk for each medium.

Residual host cell DNA (2.2.15). The content of residual host-cell DNA, determined using a suitable method, should not be greater than 10 ng per single human dose if viruses grown in continuous cell culture for production. This test may be omitted with the agreement of the National Regulatory Authority, if the manufacturing process is validated for residual DNA.

FINAL LOT

For each component a minimum virus concentration for release of the product is established such as to ensure, in the light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with following requirements may be released for use.

Identification

Each final lot shall be identified as containing live attenuated influenza viruses of the appropriate strains for the types and subtypes by suitable methods.

Tests

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and is not greater than 115.0 per cent of the quantity stated on the label.

Total protein, Ovalbumin and Endotoxins. The manufacturers must establish these parameters suitable for the production to ensure consistency of the vaccine's composition and its clinical performance. The limits shall be approved by the National Regulatory Authority.

Sterility (2.2.11). Carry out the test for sterility using 10 ml for each medium.

Water (2.3.43). Not more than 3.0 per cent, of moisture content by thermogravimetery, Karl Fischer or any other suitable method.

Potency. Mix the vaccine with sufficient quantities of anti-bodies specific for any two strains of Influenza viruses. Titrate the non-neutralized strain for infective titer, at least in triplicate using a minimum of 8 cultures or eggs per dilution. Use appropriate virus reference preparation to validate each assay.

Estimation of individual virus titer can also be performed by any other suitable method.

The stated amount of Influenza virus of each strain or strains per human dose is in accordance to the established titer in clinical trials and shall be approved by the National Regulatory Authority.

Labelling. The label states that (1) the vaccine has been prepared on embryonated hen's eggs or cell culture; (2) the

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strain or strains of Influenza virus used to prepare the vaccine; (3) the infectivity titer per human dose of the vaccine expressed as EID_{50} or $TCID_{50}$ or FFU or equivalent suitable methods; (4) the season during which the vaccine is intended to protect; (5) Expiry Date (Should not exceed one year from the date of manufacturing).

Japanese Encephalitis Vaccine (Human)

Japanese Encephalitis Vaccine for human use is a liquid or freeze dried preparation of Japanese encephalitis virus grown in approved substrate and inactivated by a validated method.

Production

General provisions

The vaccine is produced on the basis of virus seed lot system. The production method shall have been shown to yield consistently the vaccines that comply with the tests for immunogenicity, safety and stability. The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy.

Substrate for virus propagation

The virus is propagated in an approved cell substrate like a Vero cell line.

SEED LOT

The strain of Japanese encephalitis virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

The National Regulatory Authority shall determine the acceptable number of passages from the master virus seed lot to produce working virus seed lots.

Only a working seed lot that complies with the following tests may be used for virus propagation.

Identification

Each working seed lot is identified as Japanese encephalitis virus using specific antibodies by an approved method.

Virus concentration. The virus concentration of each working seed lot is determined by a cell culture method using immunofluorescence or any other approved method.

Extraneous agents (2.7.3). The working seed lot complies with the tests for the virus seed lots.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

PROPAGATION AND HARVEST

a) Mouse brain vaccine

The vaccine is prepared by using a seed-lot system. An approved strain of virus is grown by inoculating intracerebrally into healthy mice. Virus harvests are pooled, concentrated and inactivated by addition of formalin or any other suitable inactivating agent. It may contain a suitable preservative. The vaccine may be issued in single or multidose containers.

b) Cell culture vaccine

All processing of the cell bank and subsequent cell cultures are done under aseptic conditions in an area where no other cells are handled. Approved animal (but not human) serum may be used in the media, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum; the media may contain human albumin. Serum proteins, if present are reduced to an acceptable level by suitable method of purification. Serum and trypsin used in the preparation of cell suspension and media are shown to be free from infectious extraneous agents. The cell culture media may contain a pH indicator such as phenol red. Not less than 500 ml of the cell cultures employed for vaccine production are set aside as uninfected cell cultures (control cells). The virus suspension is harvested on one or more occasions during incubation. Multiple harvests from the same production cell culture may be pooled and considered as a single harvest.

Virus harvests that comply with the tests given under Identification and Virus concentration are pooled in the preparation of the inactivated viral harvest.

Control cells. The control cells of the production cell culture from which the single harvest is derived should comply with the test for identification and with the tests for extraneous agents (2.7.3).

Purification and inactivation

The virus harvest may be concentrated and/or purified by suitable methods; the virus harvest is inactivated by a validated method at a fixed, well defined stage of the process which may be before, during or after any concentration or purification. The method shall have been shown to be capable of inactivating Japanese encephalitis virus without destruction of the immunogenic activity. If formalin is used, the concentration shall at no time exceed 1:2000.

Only an inactivated viral suspension that complies with the following tests may be used in the preparation of the final bulk vaccine.

Inactivation. Inactivation is confirmed by carrying out an amplification test for residual infectious Japanese encephalitis virus. Inoculate a quantity of inactivated viral suspension equivalent to not less than 25 doses into cell cultures of the

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same type as those used for production of the vaccine. Make a passage after 7 days. Maintain the cultures for a further period of 14 days and then examine the cell cultures for Japanese encephalitis virus using an immunofluorescence test. No Japanese encephalitis virus is detected. Alternatively, 5 ml of each culture fluid is pooled on day 14 and 21 and 0.03 ml is inoculated intracerebrally into each of the 10 mice weighing between 12 and 15 g. The mice are observed for 14 days for symptoms caused by Japanese encephalitis virus, and mice showing symptoms of Japanese encephalitis virus are sacrificed and virus presence is confirmed by immunofluorescence test. No Japanese encephalitis virus shall be detected.

Residual host-cell DNA (2.2.15). The content of residual host-cell DNA, determined using a suitable method, should not be greater than 10 ng per single human dose if cells are used in the production.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more inactivated viral suspensions. An approved stabilizer may be added to maintain the activity of the product. Thiomersal can be used as preservative.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Formaldehyde (2.3.20). Not more than 0.01 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile containers. The containers are then sealed so as to prevent contamination.

Only a final lot that complies with each of the tests given under Identification, Tests and Assay may be released for use. Provided that the test for inactivation has been carried out with satisfactory results on the inactivated virus suspension and the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, these tests may be omitted on the final lot.

Identification

The vaccine is shown to contain Japanese encephalitis virus antigen by a suitable immunochemical method using specific antibodies, alternatively, the Assay also serves to identify the vaccine.

Tests

Complies with the test for Inactivation under final Purification and Inactivation.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Bacterial endotoxins (2.2.3). Less than 25 IU per single human dose.

Bovine serum albumin (for cell culture vaccine). Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.2.14).

Residual host-cell DNA (for continuous cell line vaccines) (2.2.15). Not more than 10 ng per single human dose.

Free formaldehyde (2.3.20). Not more than 0.01 per cent w/v.

Antimicrobial preservative. Determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and is not greater than 115.0 per cent of that stated on the label.

Biological assay

Potency of Japanese encephalitis virus vaccine is determined by titrating the neutralizing antibodies produced in the immunized mice by plaque reduction method or serum neutralization test (SNT) using appropriate cell culture.

Standard preparation

The Standard preparation is a freeze-dried Japanese encephalitis virus vaccine the potency of which has been determined in relation to a suitable Japanese encephalitis reference vaccine as approved by the National Regulatory Authority.

Suggested method

Preparation of challenge virus suspension

The approved challenge virus strain is stored in freeze-dried form or in liquid form stored below -70°. Prepare a working pool of the challenge virus strain by inoculating intracerebrally 0.03 ml of 100 fold dilution of the standard strain in Hanks' balanced salt solution containing 5 per cent calf serum into a suitable number of 2 days old suckling mice. Sacrifice the animals after they show characteristic symptoms of encephalitis and become moribund. Harvest their brains aseptically, wash them in chilled sterile saline solution to remove blood clots. Homogenize the brains with Hanks' balanced salt solution containing 5 per cent calf serum to make a 10 per cent emulsion. Centrifuge the emulsion at 2000 g for 30 minutes. Dilute the supernatant with Hanks'

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balanced salt solution containing 5 per cent calf serum so as to contain about 200 Plaque-Forming Units (PFU) of the virus per 0.4 ml.

Determination of potency

Prepare appropriate dilutions of the vaccine under examination and of the Standard Preparation in a suitable medium. Inject intraperitoneally in two doses of 0.5 ml each at 7 days interval into at least 20 mice of 4 weeks of age. Bleed each mouse 7 days after the second injection, pool the separated serum from each group and inactivate the sera by heating at 56° for 30 minutes. The inactivated sera may be stored at -20°, if necessary.

Dilute the sera appropriately, e.g. 1:40, 1:160, 1:640 etc., mix with an equal volume of the challenge virus suspension and incubate at 37° for 90 minutes for neutralization. Inoculate the mixture into cell cultures and overlay the infected cells with 1 per cent agar. After incubation for an appropriate time (about 48 hours), stain the cells and count the number of plaques formed on the cultures to obtain the plaque reduction rates for the vaccine under examination and the Standard preparation. Calculate the neutralizing antibody titres for each group using standard statistical methods (5.7). The test is not valid unless (a) the mean number of plaques obtained with the Standard preparation is between 100 and 150 per dish and (b) the potency of the vaccine under examination is not less than that of the Standard preparation.

Labelling. The label states (1) the biological origin of the cells used for the preparation of the vaccine; (2) the strain of virus used.

Japanese Encephalitis Live Vaccine (Human)

Japanese Encephalitis live vaccine for human is a freeze dried preparation of Japanese Encephalitis Virus grown in an approved cell substrates by a validated method.

Production

General Provisions

The vaccine strains and the production method should show consistency in yield comparable with the vaccines of acceptable clinical safety, immunogenicity and efficacy in the target population. Production of the vaccine is based on virus seed-lot system and cell line shall be used according to cell-bank system. Unless otherwise justified and authorized, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine in clinical studies to be satisfactory with respect to safety and efficacy.

If purification steps are present, the reduction of selected process-related impurities such as residual host cell proteins, endotoxin, bovine serum, antibiotics is monitored to establish consistency of the purification process. For residual cellular-host cell DNA quantity of DNA and size distribution shall be evaluated during assessment of manufacturing consistency and process validation. Special care may be taken to exclude possible extraneous agents of animal origin material (such as serum, trypsin, growth factors, human serum albumin etc) used in the vaccine preparation; such contaminants shall be monitored using suitable validated methods.

Substrate for virus propagation

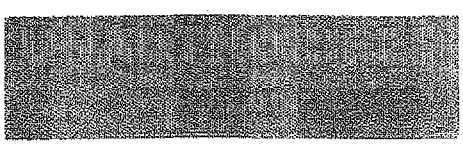
The production of vaccine is based on a virus seed lot system. Cell lines are used according to cell bank system. If primary hamster cells (PHK) are used it should be ensured that the retrovirus are absent in each batch of cell substrate used in production. In addition, the principle of reducing the risk of adventitious agents entering the manufacturing process should be encouraged by using specific pathogen-free colony that is monitored regularly as a source material for preparation of PHK cells.

If Vero cells are used for production process the passage level shall not exceed 150 numbers including passage level used for commercial production. If other than Vero, cell lines are used it should be demonstrated that the passage level(s) used in production is not tumerogenic. The purification process has to be validated by appropriate methods, including spiking studies to demonstrate its capability to remove DNA to an acceptable level. In addition batch to batch consistency needs to be demonstrated.

Control on animals

Healthy Syrian hamsters stocks of closed colony aged between 10 and 14 days shall be used as the source of kidneys for cell culture. The animals should be tested to ensure freedom from specified pathogens and antibodies to those pathogens Hantaan virus, Kilham rat virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse hepatitis virus, mouse poliovirus, pneumonia virus of mice, respiratory enteric orphan (REO) virus 3, Sendai virus (Parainfluenza virus 1), Simian virus 5 and Toolans H-a virus. Tests for production of hamster, mouse and rat antibodies should also be performed.

In addition, the colony should be screened for pathogenic bacteria, including mycobacteria, fungi and mycoplasmas. At the time of harvest of the kidneys, the animals should be examined for the presence of any abnormalities. If any kidney abnormalities or other evidence of pathology is found, those animals should not be used for JE vaccine production. Each group of control cultures derived from a single group of animals used to produce a single virus harvest should remain identifiable as such until all testing, especially for adventitious agents, is completed.



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The reduction and elimination of raw materials derived from animals and human from the manufacturing process is encouraged where feasible. If animal derived raw materials are used in cell culture medium example Insulin or transferrin, validation of the production process for the elimination of viruses can substitute for virus detection tests.

Serum of bovine origin should come from the countries or herds certified to be free of TSE/bovine spongiform encephalopathy. Human serum shall not be used. If human albumin, used, it should be tested by PCR to demonstrate freedom from human immunodeficiency virus (HIV), Hepatitis B and Hepatitis C virus nucleic acids.

Trypsin used for preparing cell cultures should be tested and found free from cultivable bacteria, fungi, mycoplasmas and infectious viruses, especially bovine or porcine parvoviruses, as appropriate.

SEED LOT

The strains of JE virus used in the production shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. The production of vaccine should be based on the virus seed lot system that includes a master seed and a working seed. The virus master and working seed lots used to produce JE vaccine (Live) should be shown to be safe i.e. stable through neuro attenuation and immunogenic. Only a seed lot that complies with the following tests may be used for virus propagation.

The virus working seed lot should be limited to three passages in PHK cells beyond the master seed lot. If the virus working seed is in liquid form, it should be stored at -60° or below. If the virus working seed is in freeze–dried form, it should be stored at -20° or below.

Identification

The master and working seed lots are identified as JE virus by serum neutralization in cell culture, using specific antibodies or by immunochemical methods or by any suitable appropriate sensitive methods like PCR.

Virus concentration. The virus concentration of each working seed lot is determined by a cell culture method using immunofluoresence or plaque assay or any other approved method.

Extraneous agents (2.7.3). The working seed lot complies with tests for the virus seed lots.

Neurovirulence (2.7.5). The master / working seed lot complies with the test for neurovirulence of live virus vaccine. *Macaca and Cercopithecus* monkeys susceptible to JE virus suitable for the test. The neurovirulence test shall be carried out in Kunming strain of Swiss mice.

Test for reversion in suckling mice. Both master seed and working seed should be tested for reversion to virulence in suckling mice.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) Each virus master seed lot should comply with the test for sterility.

Mycoplasmas contamination (2.7.4). Carry out the test for mycoplasmas. Each virus master seed lot should be free from mycoplasma contamination.

PROPAGATION AND HARVEST

All the processing of the cell bank and subsequent cell cultures are done under aseptic conditions in an area where no other cells are handled. Approved animal serum may be used in media, but the final medium for maintaining cell growth during virus multiplication shall not contain animal serum. Not less than 5 per cent cell cultures or not less than 500 ml of the cell cultures employed for vaccine production are set aside as uninfected cell cultures. The virus suspension is harvested on one or more occasions during incubation. Multiple harvests from the same production cell culture passage levels may be pooled and considered as a single harvest.

Penicillin and other beta-lactam antibiotics should not be used at any stage of manufacture. Minimal concentrations of suitable antibiotics such as kanamycin may be used.

Individual Viral harvests that comply with the following tests shall be pooled in the preparation of pooled viral harvest.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Test for virus Content. The virus content of each single harvest should be tested using an infectivity assay. Alternative assays, such as those to determine the CCID50, may be used.

Test for Identity. The assay serves to confirm the antigenic specificity of the vaccine. A test for identity should be performed if not done on the virus pool. It is not necessary to perform a test for genetic identity on single virus harvests.

Test for adventitious agents. If the single virus harvests are not pooled on the same day, a test for adventitious agents should be performed on each single virus harvest. The results shall comply as per Extraneous agents (2.7.3).

Tests for retroviruses. Samples from the filtered virus pool should be examined for the presence of retroviruses by an assay for reverse transcriptase. No indication of the presence of retrovirus is found.

Residual host cell DNA (2.2.15). The content of residual host cell DNA, determined using a suitable method, should not be greater than 10ng per single human dose.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

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FINAL BULK

If more than one individual viral harvest meeting their specifications may be pooled and diluted to manufacture the final bulk. Only the final bulk vaccine that complies with the following requirements may be used in the preparation of final lot.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

Test for neurovirulence in mice. Each final bulk should be tested for neurovirulence in mice as described in seed lot section. The test may be performed before or after the addition of other substances, such as stabilisers.

FINAL LOT

A minimum virus concentration for release of the product is established so as to ensure, in the light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the tests for minimum virus concentration for release, with the following requirements for thermal stability and with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted from the final lot.

Identification

Final lots are identified as JE virus by serum neutralization in cell culture, using specific antibodies or by immunochemical methods or by any suitable appropriate sensitive methods like PCR.

Tests

pH (2.4.24). When freeze dried vaccine is dissolved by the approved diluent, the pH value should be within the range of values as approved by National Regulatory Authority.

Water (2.3.43). Not more than 3.0 per cent, determined by Karl Fischer, semi-micro determination of water or by any suitable validated method.

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.2.11).

Bacterial Endotoxins (2.2.3). Less than 25 EU per single human dose.

Bovine serum albumin. Not more than 50ng per single human dose, determined by a suitable immunochemical method (2.2.14). Alternatively, this test may be performed on the clarified virus pool or on the final bulk.

Residual host cell DNA (2.2.15). The content of residual host cell DNA, determined using a suitable method, should not be greater than 10ng per single human dose.

Antimicrobial preservative. Determine the antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and is not more than 115.0 per cent of that stated on the label.

Test for residual antibiotics. If any antibiotics were added during production, the content of the residual antibiotics should be determined, and this should be within the approved limits.

Assay

The virus content in each of at least three containers selected at random from the final lot should be determined individually against a reference preparation of a live Japanese Encephalitis vaccine approved by National Regulatory Authority. The virus content should be determined after reconstitution of the freeze dried product. The potency value should be within the range of values found for vaccine lots shown to be clinically safe and effective.

Thermo stability test

Each final lot shall be tested for thermo stability. Samples shall be incubated at 37° for 7 days and the loss of titre in cell assay should not be more than 1.0 log.

Labelling. The label states (1) the biological origin of the cells used for the preparation of the vaccine; (2) the Virus strain used; (3) Recommended storage condition.

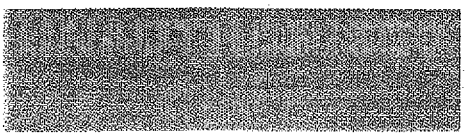
Japanese Encephalitis Vaccine Inactivated (Adsorbed, Human)

Japanese Encephalitis vaccine-adsorbed for human use is a liquid preparation of Japanese Encephalitis virus grown in approved substrate and inactivated by a validated method.

Production

General provisions

The vaccine strains and the production method should show consistency in yield comparable with the vaccines of acceptable clinical safety, immunogenicity and efficacy in the target population. Production of the vaccine is based on virus seed-lot system and cell line shall be used according to cell-bank system. Unless otherwise justified and authorized, the virus in the final vaccine shall have undergone no more passages from the master seed lot or working seed lot than were used to prepare the vaccine in clinical studies to be satisfactory with respect to safety and efficacy.



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If purification steps are present, the reduction of selected process-related impurities such as residual host cell proteins, endotoxins, mycoplasmas, bovine serum, antibiotics is monitored to establish consistency of the purification process. If the consistency of production has been validated, the monitoring programme could be conducted based on a reduced frequency of testing, but not on a batch-to-batch basis with the agreement of the National Regulatory Authority. For residual cellular-host cell DNA quantity of DNA and size distribution shall be evaluated during assessment of manufacturing consistency and process validation. Special care may be taken to exclude possible extraneous agents of animal origin material (such as serum trypsin, growth factors, human serum albumin etc) used in the vaccine preparation, endogenous and exogenous viruses; such contaminants shall be monitored using suitable validated methods.

Substrate for virus propagation

The virus is propagated in an approved cell substrate like a Vero cell line. If a primary cell culture (e.g. hamster kidney cells) are used, it should be approved by National Regulatory Authority. Requirements for primary culture remain same as described in JE Live attenuated vaccine.

SEED LOT

The strain of Japanese encephalitis virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation, a quantity of virus of uniform composition processed at one time and distributed in to a number of containers.

The National Regulatory Authority shall determine the acceptable number of passages from the master virus seed lot to produce working virus seed lots.

Only a working seed lot that complies with the following tests may be used for virus propagation.

Identification

Each working seed lot is identified as Japanese encephalitis virus using PCR with the 95 per cent homology with published sequence or master seed lot sequence.

Virus concentration. The virus concentration of each working seed lot is determined by a cell culture method using immunofluoresence or plaque assay or any other approved method.

Extraneous agents (2.7.3).The working seed lot complies with the tests for the virus seed lots.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml of each medium.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

PROPAGATION AND HARVEST

Cell culture vaccine

All processing of the cell bank and subsequent cell cultures are done under aseptic conditions in an area where no other cells are handled. Approved animal (but not human serum) serum free from TSE and BSE may be used in the media, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum proteins if present are reduced to an acceptable level by suitable method of purification. Serum and trypsin used in the preparation of cell suspension and media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator such as phenol red at a concentration of 0.002 per cent. Not less than 500 ml or 5 per cent of the cell suspension employed for vaccine production are set aside as uninfected cell cultures (control cells). The virus suspension is harvested on one or more occasions during incubation. Multiple harvests from the same production cell culture may be pooled and considered as a single harvest. Each virus suspension harvested remains identifiable as a single harvest and is kept separate from other harvests until the results of all the tests described below have been obtained.

Only viral harvests that comply with the following tests may be used in the preparation of the purified viral material.

Control cells. The control cells of the production cell culture from which the single harvest is derived should comply with the test for extraneous agents (2.7.3).

Identification

Each harvest is identified as Japanese encephalitis virus using specific antibodies by an approved validated method.

Virus concentration. The virus concentration of each harvest is determined by a cell culture method using immuno-fluoresence or plaque assay or any other approved method.

Bacterial endotoxins (2.2.3). Less than 5 IU per ml

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml of each medium.

Mycoplasma (2.7.4).Complies with the test for absence of mycoplasma.

Mycobacteria (2.7.14). Complies with the test for absence of mycobacteria.

PURIFICATION

The virus harvest is concentrated and purified by suitable methods; the virus harvest is inactivated by a validated method at a fixed, well defined stage of process which may be after concentration and purification.

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Only the purified viral suspension that complies with the following tests may be used in the preparation of the inactivated viral material.

Identification

Purified viral suspension is identified as Japanese encephalitis virus using specific antibodies by an approved method.

Virus concentration. The virus concentration of each individual harvest is determined by suitable cell line using plaque assay or any other suitable validated method.

Total protein (2.3.49). The total protein concentration of purified viral suspension is determined by a validated colorimetric method or any other approved validated method. The content is within the limits approved for the specific product.

Residual host cell protein. The content of residual host cell protein, determined using a suitable validated method should not be greater than 1 µg per single human dose.

INACTIVATION

The method shall have been shown to be capable of inactivating Japanese encephalitis virus without destruction of the immunogenic activity. If formalin is used, the concentration shall at no time exceed 1:2000.

Only an inactivated viral suspension that complies with the following tests may be used in the preparation of the final bulk vaccine.

Identification

Inactivated viral suspension is identified as Japanese encephalitis virus using specific antibodies by an approved method.

Inactivation. Inactivation is confirmed by carrying out a test for residual infectious Japanese encephalitis virus. Inoculate a quantity of inactivated viral suspension equivalent to not less than 25 doses into cell cultures of the same type as those used for production of the vaccine and should not show any Japanese encephalitis virus plaque formation at the end of incubation. The validated method used for the study should have a LOD to detect 1pfu (or equivalent units)/ml of Japanese encephalitis virus. No cytopathic changes shall be detected during the incubation period. At the end of the incubation period the culture supernatant shall be collected and 0.03 ml of the collected supernatant fluid shall be inoculated per mice in a group of 10 mice of approx 4 week old. Animals should be observed for the period of 14 days. The inactivated bulk passes the test if the sample shown to be free from residual live virus.

Total protein (2.3.49). The total protein concentration of inactivated viral suspension is determined by validated colorimetric method or any other approved validated method. The content is within the limits approved for the specific product.

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.2.14).

Residual host-cell DNA (2.2.15). Not more than 10 ng per single human dose if cells are used in production.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) by membrane filtration using 20 ml of bulk for each sterility medium.

Formaldehyde (2.3.20). Not more than 0.01 per cent w/v.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more inactivated viral suspensions. An approved adjuvant may be added to maintain the activity of the product. Thiomersal may be used as preservative at a concentration as approved by National Regulatory Authority.

Only the final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Formaldehyde (2.3.20). Not more than 0.01 per cent w/v.

Antimicrobial preservative: Wherever applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not more than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

Degree of Adsorption. Not less than 75 per cent. This test may be omitted upon demonstration of process consistency.

Bacterial endotoxins (2.2.3). Less than 25 IU per single human dose.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile containers. The containers are then sealed so as to prevent contamination.

Only a final lot that complies with each of the tests given under Identification, Tests and Assay may be released for use.

Identification

The vaccine is shown to contain Japanese encephalitis virus antigen by a suitable immunochemical method using specific antibodies, alternatively, the biological assay also serves to identify the vaccine.

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Tests

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Bacterial endotoxins (2.2.3). Less than 25 IU per single human dose.

Aluminium content (2.3.9). When aluminium compounds are used, the content of aluminium should not be more than 1.25 mg per single human dose.

Degree of adsorption. Not less than 75 per cent.

Osmolality. Osmolality of vaccine should be between 250-350 mOsmol/Kg

Pyrogen (2.2.8). Summed response of 3 rabbits should not exceed 1.4° or a validated test for bacterial endotoxin (2.2.3) may be used instead of the test for pyrogens. If used, the content should be less than 25 IU per Single Human Dose.

Free Formaldehyde (2.3.20). Not more than 0.01 per cent w/v.

Biological assay

Potency of Japanese encephalitis virus vaccine is determined by titrating the neutralizing antibodies produced in the immunized mice by plaque reduction method or serum neutralization test using appropriate cell culture. Neutralization antibody titres should be calculated as 50 per cent plaque-reduction neutralization titre. The test procedure used, including the reference vaccine, should be approved by the national regulatory authority

Standard preparation

The Standard preparation is a liquid Japanese encephalitis virus vaccine the potency of which has been determined in relation to the suitable Japanese encephalitis reference vaccine as approved by National Regulatory Authority.

Determination of potency

Prepare the dilution of the vaccine under examination in 0.1 per cent Alum. Dilute the vaccine appropriately, e.g. 5 ng, 20 ng, 50 ng, 100 ng and 0.1per cent Alum as control. Inject subcutaneously in two doses of 0.1ml each at 28 day interval into at least 10 mice of 4 weeks of age. Bleed each mouse 14 days after the second injection; inactivate the serum from each mice by heating at 56° for 30 minutes. The inactivated sera may be stored at -20° or below, if necessary.

Mix the sera with equal volume of the challenge virus suspension at 600 pfu/ml and incubate at 36° for 30 minutes in water bath for neutralization. Inoculate the mixture into cell cultures and overlay the infected cells with 0.9 per cent agar. After incubation for an appropriate time (about 72 hours), stain the cells with second agarose overlay with 1 per cent agar and neutral red. After appropriate time (about 18-24 hours) count the number of plaques formed on the cultures to obtain the plaque reduction rates for the vaccine under examination. Calculate the neutralizing antibody titres for each group using standard statistical methods e.g. SPSS. The test is not valid unless (a) the mean number of plaques obtained with the vaccine under examination is between 30-80 plaques per well and (b) the potency of the vaccine under examination is not less than 90 ng ID_{50}.

Labelling. The label states (1) the biological origin of the cells used for the preparation of the vaccine; (2) the strain of the virus used; (3) nominal amount of the vaccine antigen contained in single human dose and number of doses, if the product is presented as multiple dose container; (4) name and maximum quantity of antibiotic present in the vaccine, if any; (5) name and concentration of antimicrobial preservative added; (6) name and concentration of adjuvant used in the vaccine preparation; (7) recommended storage temperature; (8) expiry date; (9) route of administration.

Measles and Rubella Vaccine (Live)

Measles and Rubella Vaccine (Live) is a freeze-dried preparation of suitable attenuated strains of measles virus and rubella virus grown in suitable cell cultures.

The vaccine is reconstituted immediately before use to give a clear liquid that may be coloured owing to the presence of a pH indicator.

Production

General provisions

The two components are prepared as described in the monographs on Measles vaccine (live) and Rubella vaccine (live) and comply with the tests prescribed therein.

The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy.

FINAL BULK VACCINE

Virus harvests for each component are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate. Suitable quantities of the pooled harvest for each component are mixed.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

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FINAL LOT

For each component, a minimum virus concentration for release of the product is established such as to ensure, in the light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the tests for minimum virus concentration of each component for release, with the following test for thermal stability and with each of the tests given below under Identification and Tests and Assay may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Thermal stability. Maintain samples of the final lot of freeze-dried vaccine in the dry state at 37° for 7 days. Determine the virus concentration as described under Assay in parallel for the vaccine held at 37° for 7 days and for vaccine stored at 2° to 8°. For each component, the virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

Identification

When the vaccine reconstituted as stated on the label is mixed with antibodies specific for measles virus and rubella virus, it is no longer able to infect cell cultures susceptible to these viruses. When the vaccine reconstituted as stated on the label is mixed with quantities of specific antibodies sufficient to neutralize any one viral components, the second viral component infects susceptible cell cultures.

Tests

Water (2.3.43). Not more than 3.0 per cent, determined by Karl Fischer, semi-micro determination of water or by any suitable validated method.

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.2.11).

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.2.14).

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

Assay

A. Mix the vaccine with a sufficient quantity of antibodies specific for rubella virus. Titrate the vaccine for infective measles virus at least in triplicate, using at leasteight cell cultures for each dilution 0.5 $\log_{10}$ step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated measles virus concentration is not less than that stated on the label; the minimum measles virus concentration stated on thelabel is not less than 1 $\times 10^3$ $CC1D_{50}$ per single human dose or 3.0 $LOG_{10}CCID_{50}$ per single human dose. The assay is not valid if the confidence limits (P = 0.95) of the logarithm of the virus concentration are greater than ± 0.3.

Measles vaccine (Live) RS is suitable for use as a reference preparation.

B. Titrate the vaccine for infective rubella virus at least in triplicate, using at least eight cell cultures for each 0.5 $\log_{10}$ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated rubella virus concentration is not less than that stated on the label; the minimum rubella virus concentration stated on the label are not less than 1×10^3 $CCID_{50}$ per single human dose or 3.0 $LOG_{10}CCID_{50}$ per single human dose. The assay is not valid if the confidence limits (P = 0.95) of the logarithm of the virus concentration are greater than ± 0.3.

Rubella vaccine (Live) RS is suitable for use as a reference preparation.

Labelling. The label states (1) the strains of virus used in the preparation of the vaccine; (2) the type and origin of the cells used for the preparation of the vaccine; (3) the minimum virus concentration for each component of the vaccine; (4) the time within which the vaccine must be used after reconstitution.

Measles Vaccine (Live)

Measles Vaccine (Live) is a freeze-dried preparation of a suitable attenuated strain of measles virus. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

Production

General provisions

The production of vaccine is based on a virus seed-lot system and, if the virus is propagated in human diploid cells, a cell-bank system. Unless otherwise justified and authorized, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to abnormal toxicity and efficacy; even with authorized exceptions, the number of passages beyond the level used for clinical studies shall not exceed five.

The production method is validated to demonstrate that the product, if tested, would comply with the tests for abnormal toxicity and efficacy.

Substrate for virus propagation

The virus is propagated in human diploid cells or in cultures of chick embryo cells derived from a chicken flock free from specified pathogens.

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SEED LOT

The strain of measles virus used in the production of measles vaccine shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are prepared in large quantities and stored at temperatures below -20° if freeze-dried, or below -60° if not freeze-dried.

Only a seed lot that complies with the following tests may be used for virus propagation.

Identification

The master and working seed lots are identified as measles virus by serum neutralization in cell culture, using specific antibodies.

Virus concentration. The virus concentration of the master and working seed lots is determined to monitor consistency of production.

Extraneous agents (2.7.3). The working seed lot complies with the tests for seed lots.

Neurovirulence (2.7.5). The master/working seed lot complies with the test for neurovirulence of live virus vaccines. *Macaca* and *Cercopithecus* monkeys susceptible to measles virus are suitable for the test.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled. Suitable animal (but not human) serum may be used in the growth medium, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. Not less than 500 ml of the production cell culture is set aside as uninfected cell cultures (control cells). The viral suspensions are harvested at a time appropriate to the strain of virus being used.

Only a single harvest that complies with the following tests may be used in the preparation of the final bulk vaccine.

Identification

The single harvest contains virus that is identified as measles virus by serum neutralisation in cell culture, using specific antibody.

Virus concentration. The virus concentration in the single harvest is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

Extraneous agents (2.7.3). The single harvest or virus pool complies with the test for extraneous agents.

Control cells. If human diploid cells are used for production, the control cells comply with the test for identification and extraneous agents.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

FINAL BULK VACCINE

Virus harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabilizer may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. The final bulk vaccine complies with the test for sterility (2.2.11) carried out using 10 ml for each medium.

FINAL LOT

A minimum virus concentration for release of the product is established so as to ensure, in the light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the tests for minimum virus concentration for release, with the following requirement for thermal stability and with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Thermal stability. Maintain samples of the final lot of freeze-dried vaccine in the dry state at 37° for 7 days. Determine the virus concentration as described under Assay in parallel for the vaccine held at 37° for 7 days and for vaccine stored at 2° to 8°. The virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

Identification

When the vaccine reconstituted as stated on the label is mixed with specific measles antibodies, it is no longer able to infect susceptible cell cultures.

Tests

Water (2.3.43). Not more than 3.0 per cent, determined by Karl Fischer, semi-micro determination of water or by any suitable validated method.

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Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.2.11).

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.2.14).

Assay

Titrate the vaccine for infective measles virus at least in triplicate, using at leastfive cell cultures for each dilution 0.5 $\log_{10}$ step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated virus concentration is not less than that stated on the label; the minimum virus concentration stated on thelabel is not less than 1×10^3 $CC1D_{50}$ per single human dose or 3.0 $LOG_{10}CCID_{50}$ per single human dose. The assay is not valid if the confidence limits (P = 0.95) of the logarithm of the virus concentration are greater than ± 0.3.concentration are greater than ± 0.3.

Measles vaccine (Live) RS is suitable for use as a reference preparation.

Labelling. The label states (1) the strain of virus used for the preparation of the vaccine; (2) the type and origin of the cells used for the preparation of the vaccine; (3) the minimum virus concentration; (4) the time within which the vaccine must be used after reconstitution.

Measles, Mumps and Rubella Vaccine (Live)

Measles, Mumps and Rubella Vaccine (Live) is a freeze-dried preparation of suitable attenuated strains of measles virus, mumps virus and rubella virus grown in suitable cell cultures.

The vaccine is reconstituted immediately before use to give a clear liquid that may be coloured owing to the presence of a pH indicator.

Production

General provisions

The three components are prepared as described in the monographs on Measles Vaccine (Live), Mumps Vaccine (Live) and Rubella Vaccine (Live) and comply with the tests prescribed therein.

The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy.

FINAL BULK VACCINE

Virus harvests for each component are pooled and clarified to remove cells. A suitable stabilizer may be added and the pooled harvests diluted as appropriate. Suitable quantities of the pooled harvest for each component are mixed.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. Carry out the test for sterility (2.2.11) using 10 ml for each medium.

FINAL LOT

For each component, a minimum virus concentration for release of the product is established such as to ensure, in the light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the tests for minimum virus concentration of each component for release, with the following requirement for thermal stability and with each of the requirements given below under Identification and Tests may be released for use. Provided that the test for bovine serum albumin and ovalbumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Thermal stability. Maintain samples of the final lot of freeze-dried vaccine in the dry state at 37 ± 1° for 7 days. Determine the virus concentration as described under Assay in parallel for the vaccine held at 37 ± 1° for 7 days and for vaccine stored at 2° to 8°. The virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

Identification

When the vaccine reconstituted as stated on the label is mixed with antibodies specific for measles virus, mumps virus and rubella virus, it is no longer able to infect cell cultures susceptible to these viruses. When the vaccine reconstituted as stated on the label is mixed with quantities of specific antibodies sufficient to neutralize any two viral components, the third viral component infects susceptible cell cultures. Any appropriate validated method, such as PCR, approved by National Regulatory Authority may be used.

Tests

Water (2.3.43). Not more than 3.0 per cent, determined by Karl Fischer, semi-micro determination of water or by any suitable validated method.

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.2.11).

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.2.14).

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Ovalbumin. If the mumps component is produced in chick embryos, the vaccine contains not more than 1 µg of ovalbumin per single human dose, determined by a suitable immunochemical method (2.2.14).

Assay

The cell lines and/or neutralizing antisera are chosen to ensure that each component is assayed without interference for the other two components.

Titrate the vaccine for infective measles, mumps and rubella virus, using at least 3 separate vials of sample and one vial of the appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titer is established on a historical basis by each laboratory. Calculate the individual virus concentration for each vial of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations of each component, using the usual statistical method.

A. Mix the vaccine with a sufficient quantity of antibodies specific for mumps virus and rubella virus. Titrate the vaccine for infective measles virus using suitable number of wells for each dilution 0.5 $\log_{10}$ step or by a method of equal precision. For each dilution of sample and reference standard inoculate 0.1 ml and seed with 0.1 ml of suitable cell culture/cell line. The plates are incubated at the optimal growth temperature for the tested measles virus strain. The presence or absence of a cytopathic effect is recorded after five days, with the final reading at 10 days. The titre is calculated as a multiple of the median cell culture infective dose ($CCID_{50}$) by an appropriate method. If the potency is below the expected value, the assay can be repeated and the geometric mean of all assays calculated. The assay is not valid if:

— the confidence interval (P = 0.95) of the estimated virus concentration of the reference preparation for the three replicates combined is greater than ± 0.3 $\log_{10}$ $CCID_{50}$;

— the virus concentration of the reference preparation is greater than 0.5 $\log_{10}$ $CCID_{50}$ from the established value.

The estimated measles virus concentration is not less than that stated on the label; the minimum measles virus concentration stated on thelabel is not less than 1 x10^3 $CC1D_{50}$ per single human dose or 3.0 $LOG_{10}CCID_{50}$ per single human dose. The assay is not valid if the confidence limits (P = 0.95) of the logarithm of the virus concentration are greater than ±0.3.

Measles vaccine (Live) RS is suitable for use as a reference preparation.

B. Mix the vaccine with a sufficient quantity of antibodies specific for measles virus and rubella virus. Titrate the vaccine for infective mumps virus using at least eight to twelve wells for each dilution 0.5 $\log_{10}$ step or by a method of equal precision. For each dilution of sample and reference standard inoculate 0.1 ml and seed with 0.1 ml of suitable cell culture/cell line. The plates are inclubated at the optimal growth temperature for the tested Mumps virus strain. The presence or absence of a cytopathic effect is recorded after four days, with the final reading at 10 days. The titre is calculated as a multiple of the median cell culture infective dose ($CCID_{50}$) by an appropriate method. If the potency is below the expected value, the assay can be repeated and the geometric mean of all assays calculated. The assay is not valid if:

— the confidence interval (P = 0.95) of the estimated virus concentration of the reference preparation for the three replicates combined is greater than ± 0.3 $\log_{10}$ $CCID_{50}$;

— the virus concentration of the reference preparation is greater than 0.5 $\log_{10}$ $CCID_{50}$ from the established value.

The estimated mumps virus concentration is not less than that stated on the label; the minimum mumps virus concentration stated on the label is not less than 1 x 10^3 CCID50 per single human dose or 3.0 $LOG_{10}CCID_{50}$ per single human dose. The assay is not valid if the confidence limits (P = 0.95) of the logarithm of the virus concentration are greater than ±0.3.

Mumps vaccine (Live) RS is suitable for use as a reference preparation.

C. Mix the vaccine with a sufficient quantity of antibodies specific for mumps virus and measles virus. Titrate the vaccine for infective rubella virus at least in triplicate, using at least eight to twelve wells for each dilution 0.5 $\log_{10}$ step or by a method of equal precision. For each dilution of sample and reference standard inoculate 0.1 ml and seed with 0.1 ml of suitable cell culture/cell line. The plates are inclubated at the optimal growth temperature for the tested Rubella virus strain. The presence or absence of a cytopathic effect is recorded after four days, with the final reading at 10 days. The titre is calculated as a multiple of the median cell culture infective dose ($CCID_{50}$) by an appropriate method. If the potency is below the expected value, the assay can be repeated and the geometric mean of all assays calculated. The assay is not valid if:

— the confidence interval (P = 0.95) of the estimated virus concentration of the reference preparation for the three replicates combined is greater than ± 0.3 $\log_{10}$ $CCID_{50}$;

— the virus concentration of the reference preparation is greater than 0.5 $\log_{10}$ $CCID_{50}$ from the established value.

The estimated rubella virus concentration is not less than that stated on the label; the minimum rubella virus concentration stated on the label is not less than 1 x 10^3 CCID50 per single human dose or 3.0 $LOG_{10}CCID_{50}$ per single human dose. The assay is not valid if the confidence limits (P = 0.95) of the logarithm of the virus concentration are greater than ± 0.3.

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Rubella vaccine (Live) RS is suitable for use as a reference preparation.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However the vaccine must comply if tested.

Labelling. The label states (1) the strains of virus used in the preparation of the vaccine; (2) the type and origin of the cells used for the preparation of the vaccine; (3) the minimum virus concentration for each component of the vaccine; (4) the time within which the vaccine must be used after reconstitution.

Group A Meningococcal Conjugate Vaccine

Meningococcal A Conjugate Vaccine

Group A Meningococcal Conjugate Vaccine is a liquid or freeze-dried preparation of a polysaccharide derived from a suitable strain of *Neisseria meningitidis* type A, covalently bound to a carrier protein.

The polysaccharide, a linear copolymer, consists of partly O-acetylated repeating units of N-acetylmannosamine-6-phosphate, with a defined molecular size. The carrier protein, when conjugated to Meningitis A polysaccharide is capable of inducing a T-cell dependent B cell immune response to the polysaccharide.

Production

General provisions

Neisseria meningitidis group A is a Biosafety class 2 pathogen and represents a particular hazard to human health through infection by the respiratory route. The organism should be handled under appropriate conditions for this class of pathogen. Personnel employed in the production and control facilities should be adequately trained and appropriate protective measures including vaccination should be implemented.

The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy. The stability of the final lots and relevant intermediates is evaluated using one or more indicator tests. Such tests may include determination of molecular size, determination of free polysaccharide in the conjugate and the immunogenicity test in suitable animal model (mice). Taking account of the results of stability testing, release requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

SEED LOT

The strain of type A *Neisseria meningitidis* used for the seed lots shall be identified by historical records that include information on their origin and by their biochemical, serological, physicochemical or molecular characteristics. The strain shall have been shown to be capable of producing the Meningococcal A polysaccharide.

The production of Meningococcal A purified polysaccharide and of the carrier protein is based on the defined seed lot systems. Master seed lot and working seed lot shall have been properly characterized and defined. Cultures derived from the working seed shall have the same characteristics as of the master seed lot. The sample culture of single harvests taken before killing shall be tested for contamination by examination of Gram stained smears and by inoculating on suitable media.

Master seed lot. A quantity of live bacterial suspension of *N. meningitidis* derived from the original strain has been processed as a single lot and is of uniform composition. It is used for the preparation of the working seed lots. Master seed lots shall be maintained in liquid form, frozen at or below -45° or can be freeze-dried and stored at or below 4°.

Working seed lot. A quantity of live bacterial suspension of *N. meningitidis* derived from the master seed lot by growing the organisms and maintaining them in aliquots as liquid form frozen at or below -45° or can be freeze-dried and stored at or below 4°. When a new working seed lot is prepared, the capsular polysaccharide should be identified by appropriate methods such as Nuclear Magnetic resonance (NMR) spectroscopy or serological assays.

The strain should have the following characteristics:

a) Pure culture
b) Colonies obtained from the culture are round, uniform in shape and smooth with a mucous, opalescent, greyish appearance
c) Gram staining reveals characteristic Gram-negative diplococci in 'coffee-bean' arrangement
d) The oxidase test is positive
e) The culture utilizes glucose and maltose
f) Suspensions of the culture agglutinate with Meningococcal A specific antisera.

PROPAGATION AND HARVEST

The working seed lot is cultured in a liquid medium that does not contain high molecular weight polysaccharide, blood group substances and free from substances precipitated by *cetrimonium bromide (hexadecyltrimethylammonium bromide)*. If any ingredient of the medium contains blood group substances, the process shall be validated to demonstrate that after the purification step they are no longer

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detectable. The inoculum may undergo one or more subcultures in liquid medium before being used for inoculating the final medium. Samples of the culture from single harvests should be taken before killing and be examined for microbial contamination. The purity of the culture should be verified by suitable methods, which should include inoculation on to appropriate culture media. If any contamination is found, the culture or any product derived from it should be discarded. The killing process should be validated. The culture is inactivated, centrifuged and the polysaccharide precipitated from the supernatant by addition of *cetrimonium bromide*. The precipitate obtained is harvested and may be stored at -20° awaiting further purification.

PURIFIED POLYSACCHARIDE

The polysaccharides are purified, after dissociation of the complex polysaccharide and cetrimonium bromide, using suitable procedures to remove successively nucleic acids, proteins and lipopolysaccharides. The purified polysaccharide is to be stored as a lyophilised or dried powder or stored frozen at or below -20°. In case the purified polysaccharide is stored frozen, an aliquot of the purified polysaccharide should be lyophilized for testing purposes. Prepare a solution containing about 5 mg of dry polysaccharide per millilitre. The dry weight should be corrected for moisture content.

Only purified polysaccharides that comply with following requirements may be used in the preparation of conjugate bulk vaccine.

Identification and serological specificity

The identity and purity of purified polysaccharide shall be confirmed. In case where other serotypes are produced in the same manufacturing site it shall be shown that there is not more than 1per cent of group heterologous *N. meningitidis* polysaccharide. Identitiy and serological specificity are determined by suitable immunochemical methods (2.2.14) and/ or ^{1}H or ^{13}CNMR spectroscopy.

Moisture Content

If the purified polysaccharide is to be stored as a lyophilised or otherwise dried powder, the moisture content should be determined by suitable methods approved by the National Regulatory Authority and shown to be within agreed limits.

Polysaccharide content (Phosphorus)

The quantity of the purified polysaccharide can be estimated by the determination of the phosphorus content. The phosphorus content should be not less than 8.0 per cent of the dry weight of the isolated product. Methods such as Chemical assay or high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) or conductivity detection (HPAEC-CD), may be used to define the quantitative composition of the purified polysaccharide.

Protein (2.7.1). Not more than 10 mg of protein per gram of purified polysaccharide for group A.

Nucleic acid (2.7.1). Not more than 10 mg of nucleic acids per gram of purified polysaccharide for group A, determined by using ultraviolet spectroscopy (2.4.7), on the assumption that the absorbance of a 10gl^{-1} nucleic acid solution contained in a cell of 1 cm path length at 260 nm is 200 or by any other suitable and validated methods.

Bacterial endotoxin (2.2.3). Not more than 100 Endotoxin units per µg of purified polysaccharide group A.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject each of the rabbit with 1ml per kg body weight of solution containing 0.025 µg of purified polysaccharide per ml. The purified polysaccharide complies with the test for pyrogens. This test can be omitted if bacterial endotoxin test is validated for group A.

O-Acetyl groups (2.7.1). Not less than 2 mmol of O-acetyl groups per gram of purified polysaccharide group A. O-acetyl content is determined by a colorimetric assay, ^{1}H NMR or high-performance anion-exchange chromatography with conductivity detection.

Molecular size distribution. Examine by gel filtration or high performance size exclusion chromatography (HPSEC) (2.4.16), using agarose for chromatography or cross-linked agarose for chromatography either alone or in combination with light scattering and refractive index detectors (eg. Multiple angle laser light scattering (i.e MALLS) or any other suitable method. Use a column 30 cm long and 10 mm in internal diameter equilibrated with a solvent having an ionic strength of 0.2 mol per kg and a pH of 7.0 to 7.5.

At least 65 per cent of group A purified polysaccharide is eluted before a distribution coefficient (K_D) of 0.50 is reached.

Calcium. If a calcium salt is used during purification, a determination of calcium is carried out on the purified polysaccharide; the content is within the limits approved for the product.

Residual reagents. Where applicable, tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established and each batch of purified polysaccharide must be shown to comply with this limit. Where validation studies have demonstrated removal of residual reagent, the test on each batch may be omitted.

ACTIVATED SACCHARIDE

Polysaccharide preparations may be partially depolymerised either before or during the chemical modification to yield the activated saccharide.

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Chemical activation. Several methods for the chemical activation of polysaccharides prior to conjugation may be satisfactory. The chosen method should be approved by the National Regulatory Authority. Polysaccharide may be oxidised with periodate and the periodate activated polysaccharide attached to the carrier protein directly by reductive amination, or through a secondary linker. Alternatively, the polysaccharide can be randomly activated by cyanogen bromide, or a chemically similar reagent, and a bifunctional linker added, which then allows the polysaccharide to be attached to the carrier protein directly, or through a secondary linker. Size-reduced polysaccharides may be generated by reaction with acid and the derived oligosaccharide attached to the carrier protein directly or through a secondary linker, or by other methods.

Extent of activation of the saccharide. The manufacturer should demonstrate control of the degree of activation of the saccharide by an assay of each batch of the saccharide if applicable. Colorimetric, chromatographic assays and NMR spectroscopy have been used to determine the degree of activation of the saccharide.

Molecular size distribution. The average size distribution (degree of polymerization) of the processed saccharide should be measured by a suitable method and should be within defined limits. The size should be specified for each type of conjugate vaccine with appropriate limits for consistency, as the size may affect the reproducibility of the conjugation process. The molecular size may be determined by HPSEC either alone or in combination with light scattering and refractive index detectors such as Multiple angle laser light scattering (MALLS). Other suitable methods include gel filtration and ion exchange chromatography.

Carrier protein

The carrier protein is chosen in a way so that when the polysaccharide is conjugated it is able to induce a T-cell-dependent immune response. The carrier proteins are produced by culture of suitable microorganisms; the bacterial purity of the culture is verified; the culture may be inactivated; the carrier protein is purified by a suitable method.

Only a carrier protein that complies with the following requirements may be used in preparation of the conjugate.

Identification

The carrier protein is identified by a suitable immunochemical method (2.2.14).

Sterility (2.2.11). Complies the test for sterility using for each medium 10 ml or the equivalent of one hundred doses, whichever is less.

Diphtheria toxoid. Diphtheria toxoid is produced as stated under Diphtheria vaccine (Adsorbed) and complies with the requirements prescribed there for bulk purified toxoid.

Tetanus toxoid. Tetanus toxoid is produced as stated under Tetanus vaccine (Adsorbed) and complies with the requirements prescribed there for bulk purified toxoid except that the antigenic purity is not less than 1500 Lf per mg of protein nitrogen.

Diphtheria protein CRM 197. Suitable tests are carried out, for validation or routinely, to demonstrate that the product is non-toxic. The protein obtained contains not less than 90.0 per cent of diphtheria CRM197 protein, when tested by liquid chromatography (2.4.14) or any other suitable method. The carrier protein shall be characterized by a suitable chemical or physicochemical method like SDS-PAGE, HPLC, isoelectric focussing, amino acid sequencing, circular dichroism, fluorescence spectroscopy, peptide mapping or mass spectroscopy, as appropriate.

OMP (Meningococcal group B outer membrane protein complex)

OMP complex of *Neisseria meningitidis* complies with the following requirement for lipopolysaccharide and pyrogens.

Lipopolysaccharide. Not more than 8.0 per cent of lipopolysaccharide, determined by a suitable method.

Pyrogens(2.2.8). Inject into each rabbit 0.25 µg of OMP per kg body weight, for determining the pyrogenic effect.

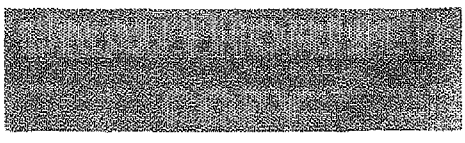
Bulk conjugate

Meningococcal A purified polysaccharide is chemically modified to enable conjugation; it is usually partly depolymerised either before or during this procedure. Reactive functional groups or spacers may be introduced into the carrier protein or polysaccharide prior to conjugation. The conjugate is obtained by the covalent binding of polysaccharide and carrier protein. Where applicable, unreacted but potentially reactogenic functional groups are made unreactive by means of capping agents; the conjugate is purified to remove reagents. Where validation studies have demonstrated removal of a residual reagent, the test on bulk conjugate may be omitted.

Only a bulk conjugate that complies with the following requirements may be used in preparation of the final bulk vaccine.

Residual reagents. Removal of residual reagents such as *cyanide, EDAC (ethyldimethylaminopropylcarbodimide)* and others depending on the conjugation chemistry is confirmed by suitable tests or by validation of the purification process. The residuals are process-specific and can be quantified by use of colorimetric and chromatographic assays. Techniques such as NMR spectroscopy may also be applied.

Group A meningococcal saccharide content. The group A meningococcal saccharide content is determined by assay of phosphorus (2.7.1) or sugar content by use of colorimetric or HPAEC-PAD assays.



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Free PRP. Unbound PRP is determined after removal of the conjugate, for example by size exclusion chromatography (2.4.16), ultra- filtration or other validated methods.

Free carrier protein. Free carrier protein is determined by a suitable method (which may include deriving the content by calculation from the results of other tests). The amount is within the limits approved for the particular product.

Protein content (2.7.1). The protein content in the bulk conjugate should be determined by means of an appropriate validated assay and comply with limits for the particular product.

Saccharide to protein ratio

The ratio of saccharide to carrier protein for each batch of the bulk conjugate should be determined as a marker of the consistency of the conjugation chemistry and as one measure of potency. For each conjugate, the ratio should be within the range approved by the National Regulatory Authority and should be consistent with vaccines shown to be effective in clinical trials.

The ratio can be determined either by independent measurement of the amounts of protein and saccharide present or by methods which give a direct measure of the ratio. Methods include ^1H nuclear magnetic resonance spectroscopy or the use of HPSEC with dual monitoring (e.g. refractive index and UV, for total material and protein content respectively). The other methods may be used with agreement of the National Regulatory Authority.

Molecular size distribution. Molecular size distribution is determined by gel filtration or size exclusion chromatography (2.4.16), using a gel matrix, appropriate to the expected size of the conjugate.

Sterility (2.2.11). Complies the tests for sterility using for each medium 10 ml or equivalent of one hundred doses, whichever is less.

FINAL BULK VACCINE

An adjuvant, an antimicrobial preservative and a stabilizer may be added to the bulk conjugate before dilution to the final concentration with a suitable diluent.

Only a final bulk vaccine that complies with the following requirement may be used in preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Complies test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

The final lots are filled in suitable containers, under stringent aseptic conditions.

Only a final lot that is satisfactory with respect to each of the requirements given under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative has been carried out on the final bulk vaccine, it may be omitted on the final lot.

Identification

The vaccine is identified by a suitable immunochemical method (2.2.14) for purified polysaccharide group A and the carrier protein.

Tests

Sterility (2.2.11). Complies with the test for sterility.

Group A meningococcal saccharide. Not less than 80.0 per cent and not greater than 120.0 per cent of the amount of polysacchadirde stated on the label as determined by a phosphorus assay (2.7.1) or immunochemical method (2.2.14) or by any other suitable method like colorimetry or by HPAEC-PAD.

Immunological assays such as rate nephelometry or ELISA inhibition may also be used.

Unbound (Free) polysaccharide. Unbound polysaccharide is determined after removal of the conjugate for example by acid precipitation on ice, precipitation with carrier protein-specific antibodies, ultracentrifugation, gel filtration and ultrafiltration. The amount of unbound saccharide can be determined by specific chemical or immunochemical method (2.2.14) or by HPAEC after hydrolysis.

Molecular size distribution. Molecular size distribution is determined by gel filtration or size exclusion chromatography (2.4.16) using a gel matrix, appropriate to the expected size of the conjugate.

Water (2.3.43). Not more than 3.0 per cent.

Pyrogen (2.2.8). Complies with the test for pyrogens. Inject a quantity of the vaccine equivalent to 0.025 µg of polysaccharide per kg of the rabbit's mass.

Or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. Endotoxin content or pyrogenic activity should be consistent with levels found to be acceptable in vaccine lots used in clinical trials and approved by the National Regulatory Authority.

Aluminium (2.3.9). Not more than 1.25 mg per single human dose. When aluminium compounds are used as an adjuvant.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical

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method. The content is not less than the amount shown to be efficacious and is not greater than 115.0 per cent of that stated on the label.

pH (2.4.24). The pH of the vaccine, reconstituted if necessary, is within the range approved for the product.

Labelling. The label states (1) the number of micrograms of group A meningococcal saccharide per human dose; (2) the type and nominal amount of carrier protein per single human dose; (3) the name and address of the manufacturer; (4) the recommended storage temperature and the expiry date if kept at that temperature; (5) in case of freeze-dried preparation, the direction for reconstitution and the time within which the vaccine must be used after reconstitution; (6) the adjuvant used for the preparation of the vaccine; (7) name and amount of diluents for reconstitution, in case of freeze-dried preparation.

Meningococcal Polysaccharide Vaccine

Meningococcal Polysaccharide Vaccine is a freeze-dried preparation of one or more purified capsular polysaccharides obtained from one or more suitable strains of *Neisseria meningitidis* group A, group C, group Y and group W135 that are capable of consistently producing polysaccharides known to be safe and effective in man.

N. meningitidis group A polysaccharide consists of partly O-acetylated repeating units of N-acetylmannosamine, linked with 1α→6 phosphodiester bonds.

N. meningitidis group C polysaccharide consists of partly O-acetylated repeating units of sialic acid, linked with 2α→9 glycosidic bonds.

N. meningitidis group Y polysaccharide consists of partly O-acetylated alternating units of sialic acid and D-glucose, linked with 2α→6 and 1α→4 glycosidic bonds.

N. meningitidis group W135 polysaccharide consists of partly O-acetylated alternating units of sialic acid and D-galactose, linked with 2α→6 and 1α→4 glycosidic bonds.

Production

General provisions

Production of the meningococcal polysaccharides is based on a well defined seed-lot system. The method of production shall have been shown to yield consistently meningococcal polysaccharide vaccines of satisfactory immunogenicity and safety for man.

The production method is validated to demonstrate that the product, if tested, would comply with the test of abnormal toxicity for antisera and vaccines.

SEED LOT

The strains of *N. meningitidis* used for the master seed lots shall be identified by historical records that include information on their origin and by their biochemical, serological, physicochemical or molecular characteristics. Cultures from the working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot. The strains have the following characteristics:

a) Colonies obtained from a culture are round, uniform in shape and smooth with a mucous, opalescent, greyish appearance.

b) Gram staining reveals characteristic Gram-negative diplococci in 'coffee-bean' arrangement.

c) The oxidase test is positive.

d) The culture utilizes glucose and maltose.

e) Suspensions of the culture agglutinate with specific antisera of known titre.

PROPAGATION AND HARVEST

The working seed lots are cultured on solid media that do not contain blood-group substances or ingredients of mammalian origin. The inoculum may undergo one or more subcultures in liquid medium before being used for inoculating the final medium. The liquid media used and the final medium are semisynthetic and free from substances precipitated by *cetrimonium bromide (hexadecyltrimethylammonium bromide)* and do not contain blood-group substances or high-molecular-mass polysaccharides. The bacterial purity of the culture is verified by microscopic examination of Gram-stained smears and by inoculation into appropriate media. The cultures are centrifuged and the polysaccharides precipitated from the supernatant by addition of *cetrimonium bromide*. The precipitate obtained is harvested and may be stored at or below -20° awaiting further purification.

PURIFIED POLYSACCHARIDES

The polysaccharides are purified, after dissociation of the complex of *polysaccharide and cetrimonium bromide*, using suitable procedures to remove successively nucleic acids, proteins and lipopolysaccharides. The purification step consists of *ethanol* precipitation of the *polysaccharides* or purification with *chloroform* and *n-butanol* or by cold *phenol* treatment, which are then dried and stored at or below -20°. The loss on drying is determined by thermogravimetry, Karl Fischer or any other suitable method and the value is used to calculate the results of the other chemical tests with reference to the dried substance.

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Only purified polysaccharides that tested comply with the following requirements may be used in the preparation of the final bulk vaccine.

Protein (2.7.1). Not more than 10 mg of protein per gram of purified polysaccharide for group A and C organisms and less than 50 mg of protein per gram of polysaccharide for group Y and W135 calculated using bovine plasma albumin as a reference or other methods approved by National Regulatory Authority.

Nucleic acids (2.7.1). Not more than 10 mg of nucleic acids per gram of purified polysaccharide, calculated with reference to the dried substance.

O-Acetyl groups (2.7.1). Not less than 2 mmol of O-acetyl groups per gram of purified polysaccharide for group A, not less than 1.5 mmol per gram of polysaccharide for group C, not less than 0.3 mmol per gram of polysaccharide for groups Y and W135, all calculated with reference to the dried substance.

Phosphorus (2.7.1). Not less than 80 mg of phosphorus per gram of group A purified polysaccharide, calculated with reference to the dried substance.

Sialic acid (2.7.1). Not less than 800 mg of sialic acid per gram of group C polysaccharide and not less than 560 mg of sialic acid per gram of purified polysaccharide for groups Y and W135, all calculated with reference to the dried substance. Use the following reference solutions:

Group C polysaccharide. A 150 mg/l solution of *N-acetylneuraminic acid.*

Group Y polysaccharide. A solution containing 95 mg/l of *N-acetylneuraminic acid* and 55 mg/l of *glucose.*

Group W135 polysaccharide. A solution containing 95 mg/l of *N-acetylneuraminic acid* and 55 mg/l of *galactose.*

Calcium. If a calcium salt is used during purification, determination of calcium is carried out on the purified polysaccharide by a suitable method; the content is within the limits approved for the product.

Molecular size. Examine by gel filtration or high performance size-exclusion chromatography (HPSEC) (2.4.16), using agarose for chromatography or cross-linked agarose for chromatography either alone or in combination with light scattering and refractive index detector (e.g. multiple angle LASER light scattering, MALLS) or any other suitable method. Use a column 0.9 m x 15 mm equilibrated with a solvent having an ionic strength of 0.2 mol/kg and a pH of 7.0 to 7.5. Apply to the column about 2.5 mg of polysaccharide in a volume of about 1.5 ml and elute at about 20 ml/h. Collect fractions of about 2.5 ml and determine the content of polysaccharide by a suitable method.

At least 65.0 per cent of group A polysaccharide, 75.0 per cent of group C polysaccharide, 80.0 per cent of group Y polysaccharide and 80.0 per cent of group W135 polysaccharide is eluted before a distribution coefficient (K_d) of 0.50 is reached. In addition, the percentages eluted before this distribution coefficient are within the limits approved for the particular product.

Identification and serological specificity

The identity and serological specificity are determined by a suitable immunochemical method (2.2.14). Identity and purity of each polysaccharide shall be confirmed; it shall be shown that there is not more than 1.0 per cent m/m of group-heterologous *N. meningitidis* polysaccharide.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject each of the rabbit with 1ml per kg body weight of solution containing

a) 0.025 µg of polysaccharide for a monovalent vaccine,
b) 0.050 µg of polysaccharide for a bivalent vaccine,
c) 0.075 µg of polysaccharide for a trivalent vaccine,
d) 0.100 µg of polysaccharide for a tetravalent vaccine.

FINAL BULK VACCINE

One or more purified polysaccharides of one or more *N. meningitidis* groups are dissolved in a suitable solvent that may contain a stabilizer.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile containers. The containers are then closed so as to avoid contamination. Only a final lot that is satisfactory with respect to each of the requirements prescribed below under Identification, Tests and Assay may be released for use.

Identification

Carry out an identification test for each polysaccharide present in the vaccine by a suitable immunochemical method (2.2.14).

Tests

Sterility (2.2.11). Complies with the test for sterility.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject each of the rabbit with 1ml per kg body weight of solution containing

a) 0.025 µg of polysaccharide for a monovalent vaccine,
b) 0.050 µg of polysaccharide for a bivalent vaccine,

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c) 0.075 µg of polysaccharide for a trivalent vaccine,

d) 0.100 µg of polysaccharide for a tetravalent vaccine.

or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be within the limits approved by the National Regulatory Authority.

Water (2.3.43). Not more than 3.0 per cent, of moisture content by thermogravimetery, Karl Fischer or any other suitable method.

Molecular size. Examine by gel filtration or size-exclusion chromatography (2.4.16). Use a column about 0.9 m long and 16 mm in internal diameter equilibrated with a solvent having an ionic strength of 0.2 mol/kg and a pH of 7.0 to 7.5. Apply to the column about 2.5 mg of each polysaccharide in a volume of about 1.5 ml and elute at about 20 ml/h. Collect fractions of about 2.5 ml and determine the content of polysaccharide by a suitable method.

Use cross-linked agarose for chromatography and apply a suitable immunochemical method (2.2.14) to establish the elution pattern of the different polysaccharide(s). The vaccine complies with the test if:

a) 65.0 per cent of Group A polysaccharide is eluted before K_0 of 0.50,

b) 75.0 per cent of Group C polysaccharide is eluted before K_0 of 0.50,

c) 80.0 per cent of Group Y and W135 polysaccharide is eluted before K_0 of 0.50.

For a tetravalent vaccine (group A + group C + group Y and group W135), use cross linked agarose for chromatography R1 and apply a suitable immunochemical method (2.2.14) to establish the elution pattern of the different polysaccharides. The vaccine complies with the test if K_0 for the principal peak is

a) not greater than 0.70 for group A and group C polysaccharide,

b) not greater than 0.57 for group Y polysaccharide,

c) not greater than 0.68 for group W135 polysaccharide.

Assay

Carry out an assay as stated under each polysaccharide present in the vaccine.

For a divalent vaccine (group A + group C), use measurement of phosphorus (2.7.1) to determine the content of polysaccharide A and measurement of sialic acid (2.7.1) to determine the content of polysaccharide C. To determine sialic acid, use as reference solution a 150 mg/l solution of *N-acetylneuraminic acid*.

For a tetravalent vaccine (group A + group C + group Y + group W135) a suitable immunochemical method (2.2.14) is used with a reference preparation of purified polysaccharide for each group.

The vaccine contains not less than 70.0 per cent and not more than 130.0 per cent of the quantity of each polysaccharide stated on the label.

Labelling. The label states (1) the group or groups of polysaccharides (A, C, Y or W135) present in the vaccine; (2) the number of µg of polysaccharide per human dose.

Meningococcal Polysaccharide A and C Vaccine

Meningococcal Polysaccharide Vaccine is a freeze-dried preparation of purified capsular polysaccharides obtained from suitable strains of *Neisseria meningitidis* group A and group C that are capable of consistently producing polysaccharides known to be safe and effective in man.

N. meningitidis group A polysaccharide consists of partly *O*-acetylated repeating units of *N*-acetylmannosamine, linked with $1\alpha \rightarrow 6$ phosphodiester bonds.

N. meningitidis group C polysaccharide consists of partly *O*-acetylated repeating units of sialic acid, linked with $2\alpha \rightarrow 9$ glycosidic bonds.

Production

General provisions

Production of the meningococcal polysaccharides is based on a well defined seed-lot system. The production method shall have been shown to yield consistently meningococcal polysaccharide vaccines of satisfactory immunogenicity and safety for man.

The production method is validated to demonstrate that the product, if tested, would comply with the test of abnormal toxicity for antisera and vaccines.

SEED LOT

The strains of *N. meningitidis* used for the master seed lots shall be identified by historical records that include information on their origin and by their biochemical, serological, physiochemical or molecular characteristics.

Cultures from the working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot.

The strains have the following characteristics:

a) Colonies obtained from a culture are round, uniform in shape and smooth with a mucous, opalescent, greyish appearance.

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b) Gram staining reveals characteristic Gram-negative diplococci in "coffee-bean" arrangement.

c) The oxidase test is positive.

d) The culture utilizes glucose and maltose.

e) Suspensions of the culture agglutinate with specific antisera.

PROPAGATION AND HARVEST

The working seed lots are cultured on solid media that do not contain blood-group substances or ingredients of mammalian origin. The inoculum may undergo one or more subcultures in liquid medium before being used for inoculating the final medium. The liquid media used and the final medium are semisynthetic and free from substances precipitated by *cetrimonium bromide (hexadecyltrimethylammonium bromide*) and do not contain blood-group substances or high-molecular-mass polysaccharides.

Purity of bacterial strains used for the seed lots is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera."

The cultures are centrifuged and the polysaccharides precipitated from the supernatant by addition of *cetrimonium bromide*. The precipitate obtained is harvested and may be stored at or below - 20° awaiting further purification.

PURIFIED POLYSACCHARIDES

The polysaccharides are purified, after dissociation of the complex of *polysaccharide and cetrimonium bromide*, using suitable procedures to remove successively nucleic acids, proteins and lipopolysaccharides.

The purification step consists of *ethanol* precipitation of the *polysaccharides* or purification with *chloroform* and *n-butanol* or by cold *phenol* treatment, which are then dried and stored at or below - 20°. The purified polysaccharide may also be stored as frozen liquid at or below - 20°. If the purified polysaccharide is stored as a frozen liquid, the tests must be conducted on an aliquot that has been dried or lyophilised. The loss on drying is determined by thermogravimetry, Karl Fischer or any other suitable method and the value is used to calculate the results of the other chemical tests with reference to the dried substance.

Only purified polysaccharides that tested comply with the following requirements may be used in the preparation of the final bulk vaccine.

Identification and serological specificity

The identity and serological specificity are determined by a suitable immunochemical method (2.2.14) or by other suitable methods (eg NMR spectroscopy). Identity and purity of each polysaccharide shall be confirmed; it shall be shown that there is not more than 1.0 per cent *m/m* of group-heterologous *N. meningitidis* polysaccharide.

Moisture content. The moisture content is used to calculate the results of other chemical tests with reference to the dried substance. If the purified polysaccharide is stored as a frozen liquid, this applied to the aliquot that has been dried.

Protein (2.7.1). Not more that 1.0 percent by weight, calculated with reference to the dried substance of purified polysaccharide for group A and C organisms, calculated with reference to the dried substance using bovine albumin as a reference or other methods approved by National Regulatory Authority. Use sufficient polysaccharide to allow detection of 1 per cent protein.

Nucleic acids. Not more that 1.0 percent by weight, calculated with reference to the dried substance of purified polysaccharide for group A and C organisms, determined by using ultraviolet spectroscopy, on the assumption that the absorbance of a $10 gl^{-1}$ nucleic acid solution contained in a cell of 1 cm path length at 260 nm is 200 or by any other suitable and validated methods.

O-Acetyl groups (2.7.1). Not less than 2 mmol of *O*-acetyl groups per gram of purified polysaccharide for group A and not less than 1.5 mmol per gram of polysaccharide for group C, calculated with reference to the dried substance. O-acetyl content is determined by a colorimetric assay, ^1H NMR or high-performance anion-exchange chromatography with conductivity detection.

Phosphorus (2.7.1). Not less than 80 mg of phosphorus per gram of group A purified polysaccharide, calculated with reference to the dried substance.

Sialic acid (2.7.1). Not less than 800 mg of sialic acid per gram of group C polysaccharide, calculated with reference to the dried substance. Use the following reference solutions:

Group C polysaccharide. A 0.0150 per cent solution of *N-acetylneuraminic acid.*

Calcium or sodium content. If a calcium or sodium salt is used during purification, a determination is carried out on the purified polysaccharide; the content is within the limits approved for the product.

Molecular size. Examine by gel filtration or high performance size-exclusion chromatography (HPSEC) (2.4.16), either alone or in combination with light scattering and refractive index detector (e.g. multiple angle LASER light scattering MALLS) or any other suitable method. Use a column either 90 cm long and 16 mm diameter 30 cm or long and 10 mm diameter equilibrated with a solvent having an ionic strength of 0.2 mol per kg and a pH of 7.0 to 7.5. Apply to 90 cm long and 16 mm diameter column about 2.5 mg of polysaccharide in a volume

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of about 1.5 ml and elute at about 20 ml per hour or apply to 30 cm long and 10 mm diameter column about 2.0 mg of polysaccharide in a volume of 100 to 200 µl and elute at 0.5 to 1.0 ml per minute.

At least 65.0 per cent of group A polysaccharide, 75.0 per cent of group C polysaccharide is eluted before a distribution coefficient (K_0) of 0.50 is reached. In addition, the percentages eluted before this distribution coefficient are within the limits approved for the particular product.

Pyrogens (2.2.8). Complies with the test for pyrogens.

A validated test for bacterial endotoxins may be used instead of the test for pyrogens.

If used, the limit should be less than 100 EU per single human dose per microgram of Meningococcal group A and group C polysaccharide.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v, if used for inactivation process.

Residual reagents. Where applicable, tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established and each batch of purified polysaccharide must be shown to comply with this limit. Where validation studies have demonstrated removal of residual reagent, the test on each batch may be omitted.

FINAL BULK VACCINE

One or more purified polysaccharides of *N. meningitidis* groups A and C are dissolved in a suitable solvent that may contain a stabiliser.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Sterility (2.2.11). Complies the test for sterility using 10 ml of bulk for each sterility medium.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a chemical method or any other suitable method. The content is not less that 85 per cent and not greater than 115 per cent of the intended amount.

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbant.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile containers. The containers are then closed so as to avoid contamination.

Only a final lot that is satisfactory with respect to each of the requirements prescribed below under Identification, Tests and Assay may be released for use.

Identification

Carry out an identification test for polysaccharide A and C by a suitable immunochemical method (2.2.14) or by any other suitable validated method.

Tests

Sterility (2.2.11). Complies with the test for sterility.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject each of the rabbit with 1 ml per kg body weight of a solution containing 0.050 µg of polysaccharide.

A validated test for bacterial endotoxins may be used instead of the test for pyrogens.

If used, the limit should be less than 25 IU per single human dose.

pH (2.4.24). Within the range approved for the product.

Osmolality (2.4.23). The osmolality of the vaccine is within the limits approved for the particular preparation.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by chemical method or any other suitable method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

Water (2.3.43). Not more than 3.0 per cent, of moisture content as determined by thermogravimetry, Karl Fischer or any other suitable method.

Molecular size. Examine by gel filtration or size-exclusion chromatography (2.4.16). Use a column about 90 cm long and 16 mm in internal diameter equilibrated with a solvent having an ionic strength of 0.2 mol per kg and a pH of 7.0-7.5. Apply to the column about 2.5 mg of each polysaccharide in a volume of about 1.5 ml and elute at about 20 ml per hour. Collect fractions of about 2.5 ml and determine the content of polysaccharide by a suitable method.

Use cross-linked agarose for chromatography and apply a suitable immunochemical method (2.2.14) to establish the elution pattern of both polysaccharide(s). The vaccine complies with the test if:

— 65.0 per cent of group A polysaccharide is eluted before K_0 of 0.50,

— 75.0 per cent of group C polysaccharide is eluted before K_0 of 0.50.

Assay

Carry out an assay as stated under each polysaccharide present in the vaccine.

Use measurement of phosphorus (2.7.1) to determine the content of polysaccharide A and measurement of sialic acid (2.7.1) to determine the content of polysaccharide C. To

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determine sialic acid, use as reference solution a 150 mg/l solution of *N-acetylneuraminic acid.*

The vaccine contains not less than 70.0 per cent and not more than 130.0 per cent of the quantity of each polysaccharide stated on the label.

Labelling. The label states (1) the groups of polysaccharides (A, C) present in the vaccine; (2) the number of µg of polysaccharide per human dose; (3) antimicrobial preservative (if added).

Storage temperature. Time within which the vaccine must be used after reconstitution.

Meningococcal Group A, C, W135 and Y Conjugate Vaccine

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Meningococcal group A, C, W135 and Y Conjugate Vaccine is a freeze-dried preparation, or a preparation consisting of freeze-dried and liquid components or a full liquid preparation, of purified capsular polysaccharides covalently linked to a carrier protein. The polysaccharides are obtained from suitable strains of Neisseria meningitidis group A, group C, group W135 and group Y that are capable ofconsistently producing polysaccharides.

N. meningitidis group A polysaccharide consists of partly *O*-acetylated repeating units of *N*- acetylmannosamine, linked with α-(1→6) phosphodiester bonds.

N. meningitidis group C polysaccharide consists of partly *O*-acetylated repeating units of sialic acid, linked with α-(2→9) glycosidic bonds.

N. meningitidis group W135 polysaccharide consists of partly *O*-acetylated alternating units of sialic acid and D-galactose, linked with α-(2→6) and α-(1→4) glycosidic bonds.

N. meningitidis group Y polysaccharide consists of partly *O*-acetylated alternating units of sialic acid and D-glucose, linked with α-(2→6) and α- (1→4) glycosidic bonds.

The carrier protein used may vary for the various polysaccharide conjugates contained in a multivalent vaccine.

Production

General provisions

The production method shall have been shown to yield consistently meningococcal group A, C, W135 and Y conjugate vaccines of adequate safety and immunogenicity in man. The production of the meningococcal polysaccharides and of the carrier protein(s) is based on a seed-lot system.

The production method is validated to demonstrate that the product, if tested, would comply with tests for safety and efficacy.

During development studies, it shall be demonstrated that the vaccine consistently induces a T-cell-dependent B-cell immune response to each polysaccharide. If the manufacturing process is modified, it shall be demonstrated by appropriate *in vitro* methods that the characteristic properties of the vaccine are not affected.

The stability of the conjugated bulks and/or final lot and meningococcal polysaccharides is evaluated using suitable indicator tests. Such tests may include determination of molecular size, quantification of polysaccharide content and free polysaccharide content in the conjugate.

Taking into account the results of stability testing, release requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

SEED LOTS

The strains of *N. meningitidis* used for the master seed lots shall be identified by historical records that include information on their origin and by their biochemical and serological characteristics.

Master Seed

A quantity of live bacterial suspension of *N. meningitidis* derived from the original strain has been processed as a single lot and it is of uniform composition. It is used for the preparation of working seed lots. Master seed lots shall be maintained in liquid form, frozen at or below - 45° or can be freeze-dried and stored at or below 4°.

Working Seed

The working seed lots are cultured on solid or liquid media that do not contain blood-group substances or ingredients of mammalian origin. The inoculum may undergo 1 or more subcultures in liquid medium before being used for inoculating the final medium. The liquid media used and the final medium are semi-synthetic and free from substances precipitated by cetrimonium bromide (hexadecyltrimethylammoniumbromide) and do not contain blood-group substances or high-molecular-mass polysaccharides.

Cultures from each working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot. The strains have the following characteristics:

— colonies obtained from a culture are rounded, uniform in shape and smooth with a mucous, opalescent, greyish appearance;

— Gram staining reveals characteristic Gram- negative diplococci in 'coffee-bean' arrangement;

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— the oxidase test is positive;

— the culture utilises glucose and maltose;

— suspensions of the culture agglutinate with suitable specific antisera.

The bacterial purity of the culture is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

PROPAGATION AND HARVEST

The working seed lots are cultured on media that do not contain blood-group substances or ingredients of mammalian origin. The inoculum may undergo 1 or more subcultures in liquid medium before being used for inoculating thefinal medium. The liquid media used and the final medium are semi-synthetic and free from substances precipitated by cetrimoniumbromide(hexadecyltrimethylammonium bromide) and do not contain blood-group substances or high- molecular-mass polysaccharides.

The bacterial purity of the culture is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

The cultures are centrifuged, and the polysaccharides precipitated from the supernatantby addition of cetrimonium bromide. The obtained precipitate is harvested and may be stored at -15 ° or below awaiting further purification.

PURIFIED POLYSACCHARIDES

The polysaccharides are purified, after dissociation of the complex of polysaccharide and cetrimonium bromide, using suitable procedures to remove successively nucleic acids, proteins and lipopolysaccharides.

The final purification step consists of suitable procedure precipitation of the polysaccharides, which are then dried in vacuo and stored at -15° or below. The loss on drying is determined by thermogravimetry (2.4.31) or another suitable method and the value is used to calculate the results of the other chemical tests with reference to the dried substance.

Only purified polysaccharides that comply with the following requirements may be used in the preparation of the conjugate.

Protein (2.7.1 or other suitable methods). Mmaximum 10 mg per gram of purified polysaccharide, calculated with reference to the dried substance.

Nucleic acids (2.7.1 or other suitable methods). Maximum 10 mg per gram of purified polysaccharide, calculated with reference to the dried substance.

***O*-Acetyl groups** (2.7.1). Minimum 2 mmol per gram of purified polysaccharide for group A, minimum 1.5 mmol per gram of purified polysaccharide for group C, minimum 0.3 mmol per gram of purified polysaccharide for groups W135 and Y, all calculated with reference to the dried substance.

Phosphorus (2.7.1). Minimum 80 mg per gram of purified polysaccharide for group A, calculated with reference to the dried substance.

Sialic acid (2.7.1). Minimum 800 mg per gram of purified polysaccharide for group C and minimum 560 mg per gram of purified polysaccharide for groups W135 and Y, all calculated with reference to the dried substance, using *N-acetylneuraminic acid R* to prepare the reference solution.

Calcium. If a calcium salt is used during purification, a determination of calcium is carried out on the purified polysaccharide; the content is within the limits approved for the particular product. Where validation studies have demonstrated removal of calcium, the test on purified polysaccharides may be omitted.

Residual reagents. Where applicable, suitable tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagentis established for the particular product and each batch of polysaccharide must be shown to comply with this limit. Where validation studies have demonstrated removal of residual reagents, the test on purified polysaccharides may be omitted.

Molecular-size or molecular-mass distribution. The molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (2.4.16) combined with an appropriate detection system. An acceptable value is established for each purified polysaccharide. Each batch must be shown to comply with this limit.

Identification and serological specificity. The identity and serological specificity are determined by a suitable immunochemical method (2.2.14) or another suitable method, for example 1H nuclear magnetic resonance spectrometry (2.4.34). Identity and purity of each polysaccharide shall be confirmed; it shall be shown that there is not more than 1 per cent m/m of group-heterologous *N. meningitidis* polysaccharide.

Bacterial endotoxins (2.2.3). The content is within the limits approved by the competent authority for the particular product.

Water (2.3.43). Where applicable, the values are within the limits approved for each group, determined by a suitable method. If loss on drying (by TGA or other suitable methods) is performed on the purified polysaccharide, residual water determination is not necessary.

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MODIFIED MENINGOCCOCAL POLYSACCHARIDES

Before conjugation, the polysaccharides can be depolymerised by chemical or mechanical means followed by a concentration step to obtain polysaccharides of a desired molecular size range. Polysaccharides ordepolymerisedpolysaccharides are modified by an activation process.

MONOVALENT BULK CONJUGATE

The conjugate is obtained by the covalent binding of activated polysaccharides to the appropriate carrier protein.

Only a bulk conjugate that complies with the following requirements may be used in the preparation of the final bulk vaccine. For each test, limits of acceptance are established, and each batch of conjugate must be shown to comply with these limits.

Saccharide. The saccharide content is determined by a suitable physical or chemical method or byan immunochemical method. The value complies with the requirement approved for each conjugate.

Protein. The protein content is determined by a suitable physical or chemical method (for example, 2.7.1 or other suitable methods). The value complies with the requirement approved for each conjugate.

Saccharide-to-protein ratio. Determine the ratio by calculation. The value complies with the requirement approved for each conjugate.

Free saccharide. The free polysaccharide content is determined after separation from the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration, or other validated methods. The value complies with the requirement approved for each conjugate.

Free carrier protein. The free carrier protein content is determined by a suitable method, either directly or by calculation from the results of other tests. The value complies with the requirement approved for each conjugate.

Residual reagents. Where applicable, suitable tests are carried out to determine residues of reagents used during conjugation. An acceptable value for each reagent is established for the particular product and each batch of monovalent bulk conjugate must be shown to comply with this limit. Where validation studies have demonstrated removal of residual reagents, the test on monovalent bulk conjugate may be omitted.

Molecular size or molecular-mass distribution. The molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (2.4.16) combined with an appropriate detection system. An acceptable value is established for each conjugate. Each batch must be shown to comply with this limit.

Identification. Each polysaccharide conjugate is identified by an immunochemical method (2.2.14) or another suitable method.

CARRIER PROTEINS

The carrier protein is chosen in a way so that when the polysaccharide is conjugated it is able to induce a T-cell dependent immune response. The carrier proteins are produced by culture of suitable microorganism; the bacterial purity of the culture is verified. The culture may be inactivated. The carrier protein is purified by a suitable method.

Identification. The carrier protein is identified by a suitable immunochemical method (2.2.14)

Diphtheria CRM_{197} protein. Suitable tests are carried-out, for validation or routinely, todemonstrate that the product is non-toxic. The protein obtained contains not less than 90% of diphtheria CRM_{197} protein, when tested by liquid chromatography (2.4.14) or any suitable method. The carrier protein shall be characterized by a suitable chemical or physicochemical method like SDS-PAGE, HPLC, isoelectric focusing, aminoacid sequencing, circular dichroism, fluorescence spectroscopy, peptide mapping or mass spectrometry, as appropriate.

Diphtheria toxoid. Diphtheria toxoid isproduced as stated under Diphtheria vaccine (Adsorbed) and complies with the requirements prescribed there for bulk purified toxoid.

Tetanus toxoid. Tetanus toxoid is produced as stated under Tetanus vaccine (Adsorbed) and complies with the requirements prescribed there for bulk purified toxoid except that the antigenic purity is not less than 1500 Lf per mg of protein nitrogen.

Pyrogens (2.2.8) or **Bacterial endotoxins (BET)** (2.2.3). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated in vitro test such as the bacterial endotoxin test.

FINAL BULK VACCINE

Where a final bulk vaccine is formulated as a release intermediate, it complies with the following requirement and is within the limits approved for the particular product.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Sterility (2.2.11). It complies with the test for sterility, carried out using 10 mL or the equivalent of 100 doses for each medium, whichever is less.In agreement with the competent authority, replacement of the sterility test by a bioburden test with a low bioburden limit based on batch data and process

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validation may be acceptable provided that a sterilising filtration is performed later in the production process.

FINAL LOT

Only a final lot that is within the limits approved for the particular product and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Identification

Conjugate from each group present in the vaccine is identified by a suitable immunochemical method (2.2.14).

Tests

Free saccharide. The free polysaccharide content for each group is determined by a suitable physico-chemical (for example liquid chromatography (2.4.14)) or immunochemical (for example enzyme-linked immunosorbent assay (ELISA) (2.2.14)) method. The free polysaccharide content is within the limits approved by the competent authority for the particular product. Where justified and authorised, the test may be carried out on the monovalent bulk conjugate only.Other suitable alternative methods measuring active substance content (conjugated saccharide content) can also be used in place of free saccharide test.

Molecular-size or molecular-mass distribution. The molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (2.4.16) combined with an appropriate detection system. An acceptable value is established for each conjugate. Where justified and authorised, the test may be carried out on the monovalent bulk conjugate only.

Sterility (2.2.11). It complies with the test for sterility.

Water (2.3.43). Where applicable, not more than 3,0 per cent of moisture content by thermogravimetry, Karl Fischer or any other suitable method.

Bacterial endotoxins (2.2.3). The content is within the limits approved by the competent authority for the particular product.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by chemical method or any other suitable method. The content is not less than the minimum amount shown to be effective and isnotgreater than 115 per cent of that stated on the label.

Aluminium(2.3.9). Not more than 1,25 mg per single human dose. When aluminium compounds are used as an adjuvant.

Assay

Saccharide. The total polysaccharide content for each group is determined by a suitable physico- chemical (for example liquid chromatography (2.4.14)) or immunochemical (for example enzyme-linked immunosorbent assay (ELISA) (2.2.14)) method.

The content of each group is within the limits approved by the competent authority for the particular product.

Labelling. The label states (1) the nominal amount of polysaccharide for each group (A, C, W135 and Y) per single human dose; (2) the type and amount of carrier protein per single human dose.

Mumps Vaccine (Live)

Mumps Vaccine (Live) is a freeze-dried preparation of a suitable attenuated strain of mumps virus. The vaccine is reconstituted immediately before use to give a clear liquid that may be coloured owing to the presence of a pH indicator.

Production

General provisions

The production of vaccine is based on a virus seed-lot system and, if the virus is propagated in human diploid cells, a cell-bank system. The production method shall have been shown to yield consistently live mumps vaccines of adequate immunogenicity and safety in man.

Unless otherwise justified and authorised, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy even with authorized exceptions, the number of passages beyond the level used for clinical studies shall not exceed five.

The production method is validated to demonstrate that the product, if tested, would comply with the tests for safety and efficacy.

Substrate for virus propagation

The virus is propagated in human diploid cells or in primary cultures of chick embryo cells derived from a chicken flock free from specified pathogens.

SEED LOT

The strain of mumps virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. To avoid unnecessary use of monkeys in the test for neurovirulence, Virus seed lots are prepared in large quantities and stored at temperatures below -20° if freeze-dried, or below -60° if not freeze-dried.

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Only a seed lot that complies with the following tests may be used for virus propagation.

Identification

The master and working seed lots are identified as mumps virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The virus concentration of the master and working seed lots is determined to ensure consistency of production.

Extraneous agents (2.7.3). The working seed lot complies with the tests for seed lots.

Neurovirulence (2.7.5). The master/working seed lot complies with the test for neurovirulence of live virus vaccines. *Macaca* and *Cercopithecus* monkeys are suitable for the test.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled. Suitable animal (but not human) serum may be used in the growth media. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. Not less than 500 ml of the production cell culture is set aside as uninfected cell culture (control cells). The viral suspensions are harvested at a time appropriate to the strain of virus being used.

Only a single harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification

The single harvest contains virus that is identified as mumps virus by serum neutralization in cell culture, using specific antibodies.

Virus concentration. The virus concentration in the single harvest is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

Bacterial and fungal contamination. Single harvest complies with sterility test (2.2.11) should be processed further.

Control cells. The control cells comply with a test for extraneous agents (2.7.3).

Mycoplasma (2.7.4).Complies with the test for absence of mycoplasma.

FINAL BULK VACCINE

Single harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. The final bulk vaccine complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

FINAL LOT

A minimum virus concentration for release of the product is established such as to ensure, in the light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the tests for minimum virus concentration for release, with the following requirement for thermal stability and with each of the requirements given below under Identification and Tests and Assay may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Thermal stability. Maintain samples of the final lot of freeze-dried vaccine in the dry state at 37 ± 1° for 7 days. Determine the virus concentration as described under Assay in parallel for the vaccine held at 37 ± 1° for 7 days and for vaccine stored at 2° to 8°. The virus concentration of the vaccine exposed to 37 ± 1° for 7 days is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

Identification

When the vaccine is reconstituted as stated on the label is mixed with specific mumps antibodies, it is no longer able to infect susceptible cell cultures.

Tests

Water (2.3.43). Not more than 3.0 per cent, determined by Karl Fischer, semi-micro determination of water or by any suitable validated method.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.2.14).

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Ovalbumin. If the mumps component is produced in chick embryos, the vaccine contains not more than 1 µg of ovalbumin per single human dose, determined by a suitable immunochemical method (2.2.14).

Assay

Titrate the vaccine for infective virus at least in triplicate, using at least five cell cultures for each 0.5 $\log_{10}$ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated virus concentration is not less than that stated on the label; the minimum virus concentration stated on the label is not less than 5×10^3 $CCID_{50}$ per human dose. The assay is not valid if the confidence limits (P = 0.95) of the logarithm of the virus concentration is greater than ± 0.3.

Mumps vaccine (Live) RS is suitable for use as a reference preparation.

Labelling. The label states (1) the strain of virus used for the preparation of the vaccine; (2) the type and origin of the cells used for the preparation of the vaccine; (3) the minimum virus concentration and; (4) the time within which the vaccine must be used after reconstitution.

Pertussis Vaccine (Whole Cell)

Pertussis Vaccine is a sterile saline suspension of inactivated whole cells of one or more strains of *Bordetella pertussis*.

Production

General provisions

Inactivated *B. pertussis* suspension

Production is based on a seed-lot system. One or more strains of *B. pertussis* with known origin and history are used. Strains, culture medium and cultivation method are chosen in such a way that agglutinogens 1, 2 and 3 are present in the final vaccine. Each strain is grown for 24 to 72 hours in a liquid medium or on a solid medium; the liquid medium used in the final cultivation stage does not contain blood or blood products. Human blood or blood products are not used in any culture media. The bacteria are harvested, washed to remove substances derived from the medium and suspended in a 0.9 per cent w/v solution of *sodium chloride* or other *suitable isotonic solution*. The opacity of the suspension is determined not later than 2 weeks after harvest by comparison with the *reference preparation* of Opacity and used as the basis of calculation for subsequent stages in vaccine preparation. Single harvests are not used for the final bulk vaccine unless they have been shown to contain *B. pertussis* cells with the same characteristics with regard to growth and agglutinogens, as the parent strain and to be free from contaminating bacteria and fungi. The bacteria are killed and detoxified in controlled conditions by means of a suitable chemical agent or by heating or by a combination of these methods. Freedom from live *B. pertussis* is tested using a suitable culture medium. The suspension is maintained at 5 ± 3° for a suitable period to diminish its toxicity.

FINAL BULK VACCINE

Suitable quantities of the inactivated single harvests are pooled to prepare the final bulk vaccine. Suitable antimicrobial preservatives may be added. The bacterial concentration of the final bulk vaccine does not exceed that corresponding to an opacity of 20 IU per single human dose. If 2 or more strains of *B. pertussis* are used, the composition of consecutive lots of the final bulk vaccine shall be consistent with respect to the proportion of each strain as measured in opacity units.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for specific toxicity, free formaldehyde and antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Identification

Identify pertussis vaccine by agglutination of the bacteria in the vaccine by antisera specific to *B. pertussis*.

Tests

Specific toxicity

Use not less than 10 healthy mice each weighing between 14 to 16 g for the vaccine group and for the saline control. Use mice of the same sex or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 hours before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 ml, containing a quantity of the vaccine equivalent to not

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less than half the single human dose. Inject each mouse of the control group with 0.5 ml of a 0.9 per cent w/v sterile solution of *sodium chloride*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection, 72 hours and 7 days after the injection. The vaccine complies with the test if (a) at the end of 72 h the total mass of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60.0 per cent of that per control mouse; and (c) not more than 5.0 per cent of the vaccinated mice die during the test. The test may be repeated and the results of the tests combined.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not more than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Assay

Carry out the assay of Pertussis Vaccine as described below :

Biological assay of pertussis vaccine

The potency of Pertussis Vaccine is determined by comparison of the dose necessary to protect mice against the effects of a lethal dose of *B. pertussis* challenge culture, administered intracerebrally, with the dose of a reference preparation, calibrated in International Units, required to give the same level of protection. For this comparison, the Standard preparation of Pertussis vaccine & a suitable strain of *B. pertussis* (e.g.18323, to be used as challenge strain), are required.

Reference preparation

The reference preparation is an International standard of Pertussis vaccine, consisting of a freeze dried vaccine or another suitable preparation, calibrated in comparison to International standard, from time to time. The International Unit is the activity contained in a stated amount of the International standard, which consists of a quantity of dried pertussis vaccine. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Use healthy mice of a suitable strain, weighing between 13 and 16 g from the same stock. Distribute the mice randomly, in six to eight groups of not less than 16 and not more than 24 and four groups of 10. The mice should all be of the same sex or the males and females should be distributed equally between the groups. Half of the groups of 16 to 24 should receive the reference preparation and the other half should receive the vaccine under examination. The four groups of 10 each should be used for the LD_{50} titration of challenge suspension.

Use at least, three dilutions of the reference vaccine and similar dilutions of the vaccine under examination. In each case the dilutions are so selected that the dilution protecting 50 per cent of the mice (ED_{50}) is as near as possible to middle of the dilution range. For example, suggested dilutions are (1/8, 1/40 and 1/200 of the human dose of the vaccine under examination) and (0.5 IU, 0.1 IU and 0.02 IU or any other suitable standardized dilutions, of the reference preparation), each dose being contained in a volume, not exceeding 0.5 ml. For each dilution use 16 to 24 mice and inject intraperitoneally, into each mouse one dose of the dilution.

Select a suitable strain of *B. pertussis* (e.g. 18323), capable of causing the death of mice within 14 days of intracerebral injection. Make two subcultures after reviving the strain on a suitable medium (e.g. B.G medium) and suspend the harvested growth in a solution containing 1 per cent w/v of *casein hydrolysate* (e.g. casamino acid) and 0.85 per cent w/v of *sodium chloride* and having a pH of 7.0 to 7.2 or in another suitable solution. Determine the opacity of the suspension by using 5th International reference preparation for opacity (10 OU) and/or spectrophotometrically. Alternatively, aliquots of challenge suspension frozen in liquid nitrogen with a suitable preservative like 10 per cent DMSO may be used, to avoid heterogenicity. After 14 to 17 days of immunization, inject intracerebrally, a dose of 0.02 to 0.03 ml of the challenge dilution randomly, into each immunized mouse. The challenge should contain, approximately 1,00,000 organisms and 100 to 1000 LD_{50} per dose, in a volume of not more than 0.03 ml. In the same way, inject 4 groups of 10 control mice each, for LD_{50} titration of challenge preparation, prepared by a series of dilutions, from the dilution selected for challenge. The challenge should be completed within 2 to 2.5 hours of preparation. Exclude any mouse from consideration, that dies within 3 days of challenge. Count the number of mice surviving in each of the groups, after 14 days. On the basis of the numbers of animals surviving in each of the groups of 16 to 24 mice, calculate the potency of vaccine under examination, against the potency of reference preparation. Seed a suitable highest dilution of the challenge suspension, into each of two B.G medium plates, before and after challenge. Incubate the plates at 37° for 48 to 72 hours and calculate the number of colony forming units (CFUs).

Calculate the potency of the vaccine by Probit analysis and LD_{50} of the challenge suspension by Reed and Munch Method.

The test is not valid unless: a) for both the vaccine under examination and the reference preparation, the ED_{50} (protective

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dose), lies between the largest and the smallest doses given to the mice; b) the number of animals, which die in the four groups of 10 injected with the challenge suspension and its dilutions indicate that the challenge dose contains 100 to 1000 LD_{50} and 1 LD_{50} contains not more than 300 colony forming units; c) and the statistical analysis shows no deviation from linearity or parallelism, in terms of significance of the scope of dose response curve; d) the vaccine passes the requirements for potency, if the test results of a statistically valid assay show that the estimated potency of the vaccine is not less than 4.0 IU per single human dose and the lower fiducial limit ($P = 0.95$) of estimated potency is not less than 2.0 IU.

The test may be repeated once, but when more than one test is performed the results of all valid tests must be combined, in the estimate of potency.

Labelling. The label states (1) the minimum number of International Units per single human dose; (2) that the vaccine must be shaken before use; (3) that the vaccine is not to be frozen.

Plague Vaccine

Formolised Plague Vaccine

Plague Vaccine is a sterile suspension of killed plague bacilli, *Yersinia pestis*, of the 195/P strain grown in a suitable enriched medium such as acid hydrolysate of casein, harvested and killed by the addition of formaldehyde. It may contain a suitable preservative.

Plague Vaccine contains in each ml of human dose not less than 250 mouse median effective immunising doses (250 ED_{50}).

Description. A turbid, golden yellow or brownish liquid with or without flakes or clumps.

Tests

pH (2.4.24). 6.8 to 7.4.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Potency. Carry out the biological assay of plague vaccine described below.

Biological Assay of Plague Vaccine

The potency of plague vaccine is estimated by determining the dose necessary to protect mice against a lethal dose of a virulent strain of *Yersinia pestis*.

Test animals. Use white mice, 6 to 7 weeks old, each weighing between 20 and 28 g and of a strain susceptible to plague infection. The selected strain of mice should be such that an infective dose of 6 to 12 organisms of a virulent strain of *Y. pestis* per mouse given subcutaneously produces a mortality of not less than 80 per cent of the animals used. The animals should be healthy and free from intercurrent infection with organisms such as *Salmonella*.

Suggested Method

Selection of suitable virulent strain. A freeze-dried virulent culture of *Y. pestis*, established to be suitable for challenge, is revived by subculturing 0.5 ml in 9.5 ml of *nutrient broth* contained in another test-tube and incubating at 28° for exactly 48 hours. Such a culture should contain 300 to 600 million organisms per ml. Make 10-fold dilutions in *nutrient broth* and test for virulence. Use a 10^{-7} dilution containing 6 to 12 organisms in 0.2 ml as the test infective dose per animal. Those strains which produce a mortality of 80 per cent among the animals tested with this dose are considered to be virulent.

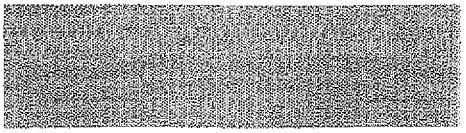
Standard challenge dose. Freshly reconstitute the freeze-dried culture and dilute with *nutrient broth* to a strength such that 0.2 ml contains 60 to 120 organisms.

Measurement of protective power. Prepare a series of five graded doses of the preparation under examination arranged in such a manner that the 50 per cent protective dose (ED_{50}) lies about the middle of the selected series. For each dose a batch of 16 mice is used. Inject subcutaneously the selected dose in two equal parts with an interval of 7 days between them. Seven days after the second half of the dose, inject subcutaneously into each group of mice the standard challenge dose. At the same time inject into 10 control mice, 8 to 9 weeks old and weighing between 28 and 30 g, the standard challenge dose.

Observe the animals for 15 days and record the number of deaths in each group. Carry out a post-mortem on the dead animals and look for signs of plague in them. If plague organisms are not seen, such deaths are excluded from the calculation. The test is not valid unless the number of such deaths is not more than 1. At the end of the period of observation kill all the surviving animals and examine for signs of plague. Calculate the median effective immunising dose, ED_{50}, by standard statistical methods. The vaccine passes the test if it has an ED_{50} of 0.004 ml or less per mouse.

Storage. Store at a temperature between 2° and 8°. The vaccine should not be allowed to freeze. When stored under the prescribed conditions the vaccine is expected to retain its potency for not less than 3 years from the date of initiation of the potency test.

Labelling. The label states (1) ED_{50} unitage per dose; (2) the name and proportion of any preservative added; (3) that the contents should be shaken well before use; (4) that the vaccine should not be allowed to freeze.



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Pneumococcal Polysaccharide Conjugate Vaccine (Adsorbed)

Pneumococcal polysaccharide conjugate vaccine (adsorbed) is a sterile suspension of purified capsular polysaccharides obtained from *Streptococcus pneumoniae* serotypes individually conjugated to a carrier protein. The carrier protein used may vary for the various polysaccharide conjugates contained in a multivalent vaccine. The vaccine may be adsorbed on a suitable adjuvant or adsorbant.

Each serotype, produced from suitable pathogenic strains of *S. pneumoniae*, is grown in an appropriate medium.

The individual polysaccharides are purified through suitable purification methods (for example centrifugation, precipitation, ultra filtration and column chromatography). The methods applied for purification should be approved by the National Regulatory Authority. Each polysaccharide has a defined composition and a defined molecular size range.

The choice of polysaccharide depends on the frequency of the serotypes responsible for acute pathologies and their geographical distribution. The vaccines contain immunochemically different capsular polysaccharides.

Production

General provisions

The production method shall have been shown to yield consistently *S. pneumoniae* conjugate vaccines of adequate safety and immunogenicity in human. The production of polysaccharides and of the carrier is based on a seed lot system.

The strains of *S. pneumoniae* used for preparing the polysaccharide should be agreed with the National Regulatory Authority. Each strain should have been shown to be capable of producing polysaccharide of the appropriate serotype through suitable tests during seed characterization (including serotyping) and product characterization (Nuclear Magnetic Resonance).

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.2.1).

During development studies and whenever revalidation of the manufacturing process is necessary, it shall be demonstrated by tests in animals that the vaccine consistently induces a T-cell-dependent B-cell immune response.

The stability of the conjugated bulk and/or final lot and pneumococcal saccharide is evaluated using suitable indicator tests. Such tests may include determination of molecular size, quantification of saccharide content and free polysaccharide content in the conjugate.

SEED LOT

The bacterial strains used for master seed lot should be identified by a record of its history, including the source from which it was obtained and the tests made to determine the characteristics of the strain. Cultures obtained from the working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot.

Purity of the bacterial cultures should be verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of gram stained smears and culture agglutination with suitable specific antisera.

If materials of animal origin are used in the medium for seed production, preservation of strain viability for freeze-drying or for frozen storage, then they should comply with the World Health Orgaziazation Guidelines on Transmissible Spongiform and should be approved by the National Regulatory Authority.

Pneumococcal polysaccharides

Each strain of *S. pneumoniae* serotypes is individually grown in a liquid medium that does not contain high-molecular-mass polysaccharides; if any ingredient of the medium contains blood-group substances, the process is validated to demonstrate that after the purification step they are no longer detectable. The components of animal origin, if used in media, should comply with the WHO Guidelines on Transmissible Spongiform Encephalopathies and should be approved by the National Regulatory Authority. The consistency of growth of *S. pneumoniae* should be demonstrated by monitoring growth rate, pH and the final yield of polysaccharide.

The bacterial purity of the culture is verified by suitable methods. The culture is inactivated. Each polysaccharide is separated from the liquid culture and purified by suitable methods. If any contamination is found, the culture or any product derived from it should be discarded. The killing process should also be adequately validated. Volatile matter, including water, in the purified polysaccharide is determined by a suitable method such as thermogravimetry (2.4.31); semi-micro determination of water (2.3.43) or, where applicable, determination of solvent and/or alcohol content by spectrometry. The values are used to calculate the results of other chemical tests with reference to the dried substance, as prescribed below.

The purified polysaccharide should be stored at appropriate temperatures to ensure stability.

Only polysaccharides that comply with the following requirements may be used in the preparation of the conjugate. Percentage contents of components, determined by the methods prescribed below, are as shown in Table 1 of Pneumococcal Polysaccharide Vaccine (Liquid/Adsorbed) monograph.

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Identification

Each polysaccharide is identified by an immunochemical method (2.2.14) such as countercurrent immunoelectrophoresis, rate nephelometry or any other suitable method such as ELISA or Gel diffusion or other suitable methods, for example Nuclear magnetic resonance spectrometry (2.4.34) (either H^1 or C^{13}).

Protein (2.7.1). Depending upon the serotype used, not more than the limit approved for the product, calculated with reference to the dried substance.

Nucleic acids (2.7.1). Depending upon the serotype used, not more than the limit approved for the product, calculated with reference to the dried substance.

Molecular size or molecular mass distribution. Molecular size or molecular mass distribution is determined by size exclusion chromatography combined with an appropriate detection system. An acceptable value is established for each pneumococcal polysacharide. Each batch should comply with this limit.

Bacterial endotoxin (2.2.3). Not more than 0.5 IU of endotoxin per microgram of polysaccharide.

Residual reagents. Where applicable, suitable tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established for the particular product and each batch of polysaccharide must be shown to comply with this limit. Wherever validation studied have demonstrated removal of residual reagents, the test on polysaccharides may be omitted.

Composition tests. Depending upon the chemical composition of a pneumococcal polysaccharide serotype, following tests may be applicable. The values are within the limits approved. Suitable limits for some pneumococcal polysaccharide serotype are given in pneumococcal polysaccharide vaccine. These limits are based on colorimetric estimations and are determined on dried weight basis. These composition tests may be determined using other suitable methods such as NMR. Suitable limits for such tests may be approved by National Regulatory Authority.

Pyrogen (2.2.8). Complies with the test for Pyrogen, the limits shall be fixed based on the clinical development batches. This test can be omitted if bacterial endotoxin test is validated.

MODIFIED PNEUMOCOCCAL POLYSACCHARIDE

Before conjugation the polysaccharide can be depolymerised by chemical or mechanical means followed by a concentration step to obtain polysaccharides of a desired molecular size range. The chemical method chosen should be approved by the National Regulatory Authority. Polysaccharides or depolymerized polysaccharides are modified by an activation process. Only modified polysaccharides that comply with the following requirements may be used in the preparation of the conjugate.

Molecular size or molecular mass distribution. In the case of a size-reduced modified pneumococcal polysaccharide, the molecular size or molecular mass distribution is determined by size exclusion chromatography combined with an appropriate detection system. An acceptable value is established for each modified pneumococcal polysacharide. Each batch should comply with this limit.

Degree of oxidation. Where applicable the degree of oxidation is represented by the ratio of moles of saccharide repeat unit per mole of aldehyde and determined by a suitable method. The values are within the limits approved for each serotype.

Carrier Protein

The carrier protein is produced by culture of suitable (including inducible recombinant) micro-organisms with verified purity. The culture is inactivated. The strain used for the production of carrier protein is revealed for its identity, origin and short history. The strain used is approved by the National Regulatory Authority. Components of animal origin, if used, should comply with WHO guideline on Transmissible Spongiform Encephalopathies (TSE). The certificate for TSE-free should be issued.

The protein carrier should also be characterized before using for conjugation as a carrier protein (one time activity during consistency batches). Test methods used to characterize such proteins to ensure that they are non toxic and to determine their purity and concentration should be approved by National Regulatory Authority.

The carrier protein is purified by a suitable method. Suitable tests are carried out, for validation or routinely, to demonstrate that, where applicable, the product is free from specific toxins.

Where Diphtheria toxoid is used, it is produced as described in the monograph *Diphtheria vaccine adsorbed* and complies with the requirements prescribed therein for bulk purified toxoid, except that the test for sterility is not required.

Where CRM 197 is used as the carrier protein, it is not less than 90 per cent pure, determined by a suitable method.

Where tetanus toxoid is used as the carrier protein, it is produced as described in the monograph *Tetanus vaccine adsorbed* and complies with the requirements prescribed therein for purified bulk toxoid, except that the antigenic purity is not less than 1500 Lf per milligram of protein nitrogen and that the test for sterility is not required.

Where protein D is used, a specific strain of *E.coli* is modified by a plasmid carrying the protein D coding sequence in order to express this outer surface protein of *Haemophilus influenzae*. The modified strain is grown in a suitable liquid medium to express protein D. At the end of cultivation, protein

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D is purified by suitable methods. The product contains not less than 95 per cent of protein D.

Only a carrier protein that complies with the following requirements may be used in the preparation of the conjugate.

Identification

The carrier protein is identified by a suitable immunochemical method (2.2.14).

Bacterial Endotoxins (2.2.3). Not more than 1 IU per microgram of protein.

Carrier protein. Not less than 90 per cent of the total protein content, determined by a suitable method.

Residual Reagents. Appropriate method to be used to ascertain the absence or to establish the limit of reagent/ chemical used during purification. Wherever validation studies have demonstrated removal of residual reagents, the test on carrier protein may be omitted.

MONOVALENT BULK CONJUGATE

The conjugate is obtained by the covalent binding of activated polysaccharides to the carrier protein.

The conjugate purification procedures are designed to remove residual reagents used for conjugation. The removal of residual reagents is confirmed by suitable tests or by validation of the purification process.

Only a bulk conjugate that complies with the following requirements may be used in the preparation of the final bulk vaccine. For each test, limits of acceptance are established and each batch of conjugate must be shown to comply with these limits.

Saccharide content. The polysaccharide content is determined by a suitable physical or chemical method or by an Immunochemical method (2.2.14). The value complies with the requirement approved for each serotype.

Protein. The protein content is determined by a suitable physical or chemical method. The value complies with the requirement approved for each serotype.

Saccharide-to-protein ratio. Determine the saccharide-to-protein ratio by calculation. The value complies with the requirement approved for each serotype.

Free saccharide. Unbound polysaccharide is determined after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods.

A value consistent with adequate immunogenicity as shown in clinical trials is established for each serotype and each lot must be shown to comply with this limit.

Free carrier protein. Determine the content by a suitable method, either directly or by deriving the content by calculation from the results of other tests. The value complies with the requirement approved for each serotype.

Molecular size or molecular mass distribution. Molecular size or molecular mass distribution is determined by size exclusion chromatography combined with an appropriate detection system. An acceptable value is established for the bulk conjugate of each polysaccharide. Each batch should comply with this limit.

Residual reagents. Where applicable, suitable tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established for the particular product and each batch of conjugate polysaccharide must be shown to comply with this limit. Where validation studies have demonstrated removal of residual reagent, the test on conjugate polysaccharide may be omitted.

Sterility (2.2.11). *It complies with the test for sterility, carried* out using 10 ml for each medium or the equivalent of 100 doses for each medium, whichever is less.

Specific toxicity of the carrier protein. It complies with the test for specific toxicity carried out using a validated method where appropriate (e.g. when tetanus or diphtheria toxoids have been used). Absence of specific toxicity of the carrier protein may also be accessed through validation of the production process.

Bacterial endotoxins (2.2.3). Not more than 0.75 IU of endotoxin per microgram of polysaccharide.

ADSORBED MONOVALENT BULK CONJUGATE

An aluminium containing adjuvant may be added to each of the monovalent bulk conjugates prior to formulation of the final bulk. Once the conjugates are adsorbed on a sterile adjuvant, sterility is assured by aseptic processing.

Only an adsorbed monovalent bulk conjugate that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification

Each adsorbed polysaccharide conjugate is identified by an immunochemical method (2.2.14) or other suitable methods.

Sterility (2.2.11). It complies with the test for sterility, carried out using 10 ml for each medium or the equivalent of 100 doses for each medium, whichever is less.

Saccharide. The polysaccharide content is determined by a suitable physical or chemical method or by an immunochemical method (2.2.14). The value complies with the requirement approved for each serotype.

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Free saccharide. Centrifuge the adsorbed monovalent bulk conjugate. In the supernatant the unbound polysaccharide is determined after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic liquid chromatography, ultrafiltration or other validated methods. An acceptable value consistent with adequate immunogenicity as shown in clinical trials is established for each serotype and each lot must be shown to comply with this limit.

Degree of Adsorption. If any adjuvant is used the degree of adsorption of each polysaccharide conjugate is assessed.

FINAL BULK VACCINE

A final bulk vaccine may be formulated from the individually adsorbed monovalent bulk conjugate, or from the mixture of the monovalent bulk conjugates that is adsorbed on an aluminium-containing adjuvant.

Where a final bulk vaccine is formulated as a release intermediate, it complies with the following requirements and is within the limits approved for the particular product.

Only a final bulk vaccine that complies with the following requirements and is within the limits approved for the particular product may be used in the preparation of the final lot.

Sterility (2.2.11). It complies with the test for sterility, carried out using 10 ml for each medium or the equivalent of 100 doses for each medium, whichever is less.

FINAL LOT

Only a final lot that is within the limits approved for the particular product and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Identification

Carry out an identification test to determine each polysaccharide content by a suitable immunochemical method (2.4.14) or by any other suitable validated method.

Carry out an identification test to determine carrier protein(s) present in the vaccine by a suitable validated method.

Tests

pH (2.4.24). If the vaccine is a liquid preparation, the pH of each final lot should be within the range of values found for vaccine lots shown to be safe and effective in the clinical trials and in stability studies. For a lyophilized preparation, the pH should be measured after reconstitution with the appropriate diluent.

Total protein content. The protein content of the final vaccine should be determined by means of an appropriate validated assay and comply with limits for the particular product.

Total saccharide content. The total saccharide content of the final vaccine should be determined by means of an appropriate validated assay and comply with limits for the particular product.

Extractable volume. Not less than the nominal volume of the intended dose.

Aluminium (2.3.9). Not more than 1.25 mg per single human dose.

Sterility (2.2.11). It complies with the test for sterility.

Bacterial endotoxins (2.2.3). Not more than 12.5 IU per single human dose, unless otherwise justified and authorized.

Preservative content. If the preservative is added in the vaccine, its amount should be determined using a validated method approved by National Regulatory Authority.

General safety test (Innocuity). The final vaccine should comply with the test for abnormal toxicity for immunosera and vaccines for human use.

Assay

Saccharide content. The polysaccharide content for each serotype is determined by a suitable immunochemical method (for example, nephelometry assay or ELISA). The vaccine contains not less than 70 per cent and not more than 130 per cent of the quantity stated on the label for each polysaccharide. The confidence limits (P = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated content.

Labeling. The label states (1) the pneumococcal serotype and carrier protein present in each single human dose; (2) the number of micrograms of each polysaccharide per single human dose; (3) the type and nominal amount of carrier protein per single human dose; (4) if applicable, the name and amount of adsorbent; (5) if applicable, that the vaccine must be shaken before use; (6) if applicable, that the vaccine must not be frozen; (7) temperature recommended during storage and transport; (8) route of administration.

Pneumococcal Polysaccharide Vaccine (Liquid/Adsorbed)

Pneumococcal Polysaccharide Vaccine consists of a mixture of equal parts of purified capsular polysaccharide of various serotype antigens prepared from suitable pathogenic strains of *Streptococcus pneumoniae* in different desired combinations whose capsules have been shown to be made up of polysaccharides that are capable of inducing satisfactory levels of specific antibodies in man. It contains upto 23 immunochemically different capsular polysaccharides listed in the Table 1.

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Table 1- Specifications on monovalent bulk polysaccharides (per cent contents):

Molecular Type*	Proteins	Nucleic acids	Total	Phosphorus Nitrogen	Molecular size K_0 CL-4B**	CL-2B***	Uronic acids	Hexo-samines	Methyl-pentoses	O-acetyl Groups
1	≤2	≤2	3.5-6	0-1.5	≤0.15		≥45			≥1.8
2	≤2	≤2	0-1	0-1.0	≤0.15		≥15		≥38	
3	≤5	≤2	0-1	0-1.0	≤0.15		≥40			
4	≤3	≤2	4-6	0-1.5	≤0.15			≥40		
5	≤7.5	≤2	2.5-6.0	≤2		≤0.60	≥12	≥20		
6B	≤2	≤2	0-2	2.5-5.0		≤0.50			≥15	
7F	≤5	≤2	1.5-4.0	0-1.0	≤0.20				≥13	
8	≤2	≤2	0-1	0-1.0	≤0.15		≥25			
9N	≤2	≤1	2.2-4	0-1.0	≤0.20		[3]20	≥28		
9V	≤2	≤2	0.5-3	0-1.0		≤0.45	[3]15	≥13		
10A	≤7	≤2	0.5-3.5	1.5-3.5		≤0.65		≥12		
11A	≤3	≤2	0-2.5	2.0-5.0		≤0.40				≥9
12F	≤3	≤2	3-5	0-1.0	≤0.25			≥25		
14	≤5	≤2	1.5-4	0-1.0	≤0.30			≥20		
15B	≤3	≤2	1-3	2.0-4.5		≤0.55		≥15		
17A or 17F	≤2	≤2	0-1.5	0-3.5		≤0.45			≥20	
18C	≤3	≤2	0-1	2.4-4.9	≤0.15				≥14	
19A	≤2	≤2	0.6-3.5	3.0-7.0	≤0.45			≥12	≥20	
19F	≤3	≤2	1.4-3.5	3.0-5.5	≤0.20			≥12.5	≥20	
20	≤2	≤2	0.5-2.5	1.5-4.0		≤0.60		≥12		
22F	≤2	≤2	0-2	0-1.0		≤0.55	≥15		≥25	
23F	≤2	≤2	0-1	3.0-4.5	≤0.15				≥37	
33F	≤2.5	≤2	0-2	0-1.0		≤0.50				

* The different types are indicated using the Danish nomenclature

** Cross linked agarose for chromatography R

*** Cross linked agarose for chromatography R1

Production

General provisions

Production of the vaccine is based on a well defined seed-lot system for each type. The production method shall have been shown to yield consistently pneumococcal polysaccharide vaccines of acceptable immunogenicity and safety in man.

The production method is validated to demonstrate that the product, if tested, would comply with the tests for abnormal toxicity of vaccines for human use, modified as follows for the test on guinea-pig; inject 10 human dose into each guinea-pig and observe for 12 days.

Monovalent bulk polysaccharides

The bacteria are grown in a suitable liquid medium that does not contain blood-group substances or high-molecular-mass polysaccharides. The bacterial purity of the culture is verified and the culture is inactivated with *phenol*. Impurities are removed by such techniques as fractional precipitation, enzymatic digestion and ultrafiltration. The polysaccharide is obtained by fractional precipitation, washed, and dried in a vacuum to a residual moisture content shown to be favourable to the stability of the polysaccharide. The residual moisture content is determined by drying under reduced pressure over *diphosphorus pentoxide* or by thermogravimetric analysis and

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the value obtained is used to calculate the results of the tests shown below with reference to the dried substance. The monovalent bulk polysaccharide is stored at a suitable temperature in conditions that avoid the uptake of moisture.

Only a monovalent bulk polysaccharide that complies with the following requirements may be used in the preparation of the final bulk vaccine. Percentage contents of components, determined by the methods prescribed below, are shown in the Table 1.

The purified polysaccharides comply with the following tests as applicable:

Protein (2.7.1). Comply with the test for protein.

Nucleic acids (2.7.1). Comply with the test for nucleic acids.

Total nitrogen (2.3.30). Comply with the test for total nitrogen.

Phosphorus (2.7.1). Comply with the test for phosphorus.

Uronic acids (2.7.1). Comply with the test for uronic acids.

Hexosamine (2.7.1). Comply with the test for hexosamine.

Methylpentoses (2.7.1). Comply with the test for methylpentoses.

O-Acetyl groups (2.7.1). Comply with the test for O-acetyl groups.

Sterility (2.2.11). Comply with the test for sterility.

Molecular size. Molecular size is determined by gel filtration or high performance size-exclusion chromatography (HPSEC) (2.4.16) using cross linked Agarose for chromatography R or chromatography Agarose for chromatograph R1, either alone or Multiple angle light laser scattering (MALLS) or any other suitable method.

Identification

Confirm the identity of the monovalent bulk polysaccharide by immunochemical method (2.2.14)(except for polysaccharides 7F, 14 and 33F).

Specificity

For establishing the specificity, no reaction should occur, when the antigens are tested against all the antisera specific for the other polysaccharides of the vaccine, including factor sera for distinguishing types within groups. The polysaccharides are tested at a concentration of 50 µg/ml using a method capable of detecting 0.5 µg/ml.

FINAL BULK VACCINE

The final bulk vaccine is obtained by aseptically mixing the different polysaccharide powders. The uniform mixture is aseptically dissolved in a suitable isotonic solution so that one human dose of 0.5 ml contains 25 µg of each polysaccharide. An antimicrobial preservative may be added. The solution is sterilized by filtration through a bacteria-retentive filter.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk vaccine is distributed and filled aseptically into sterile containers (vials or ampoules). Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for phenol and for antimicrobial preservative have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot. When consistency of production has been established on a suitable number of consecutive batches, the assay may be replaced by a qualitative test that identifies each polysaccharide, provided that an assay has been performed on each monovalent bulk polysaccharide used in the preparation of the final lot.

Identification

The assay also serves to identify the vaccine.

Tests

Sterility (2.2.11). Complies with the test for sterility.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject each of the rabbit with 1 ml of a dilution of the vaccine containing 2.5 µg per ml of each polysaccharide or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be within the limits approved by the National Regulatory authority.

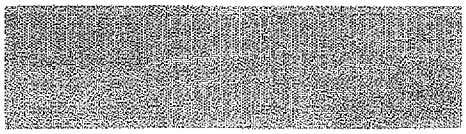
Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Phenol (2.3.36). Not more than 2.5 g/l.

pH (2.4.24). 4.5 to 7.4.

Assay

Determine the content of each polysaccharide by a suitable biochemical, physicochemical or immunochemical method (2.2.14), using antisera specific for each polysaccharide



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contained in the vaccine, including factor sera for types within groups, and purified polysaccharides of each type as standards.

The vaccine contains not less than 70.0 per cent and not more than 130.0 per cent of the quantity stated on the label for each polysaccharide. The confidence interval (P = 0.95) of the assay is not less than 80.0 and not more than 120.0 per cent of the estimated content.

Labelling. The label states (1) the number of µg of each polysaccharide per human dose; (2) the total amount of polysaccharide in the container.

Poliomyelitis Vaccine (Inactivated)

Poliomyelitis Vaccine (Inactivated) is a liquid preparation of suitable strains of human polioviruses 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method.

Production

General provisions

The production method should consistently yield vaccines of acceptable safety and immunogenicity in man.

Production of the vaccine is based on a virus seed-lot system. Cell lines are used according to a cell-bank system. If primary, secondary or tertiary monkey kidney cells are used, production complies with the requirements indicated below.

Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more passages from the master seed lot than was used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy.

Substrate for virus propagation

The virus is propagated in a human diploid cell line (2.7.2), in a continuous cell line (2.7.2) or in primary, secondary or tertiary monkey kidney cells.

Primary, secondary or tertiary monkey kidney cells. The following special requirements for the substrate for virus propagation apply to primary, secondary or tertiary monkey kidney cells.

Monkeys used in the preparation of kidney cell cultures for production and control of the vaccine. The animals used are of a species approved by the competent authority, in good health and, unless otherwise justified and authorised, have not been previously employed for experimental purposes. Kidney cells used for vaccine production and control are derived from monitored, closed colonies of monkeys bred in captivity, not from animals caught in the wild; a previously approved seed lot prepared using virus passaged in cells from wild monkeys may, subject to approval by the competent authority, be used for vaccine production if historical data on safety justify this.

Monitored, closed colonies of monkeys. The monkeys are kept in groups in cages. Freedom from extraneous agents is achieved by the use of animals maintained in closed colonies that are subject to continuous and systematic veterinary and laboratory monitoring for the presence of infectious agents. The supplier of animals is certified by the competent authority. Each monkey is tested serologically at regular intervals during a quarantine period of not less than 6 weeks imposed before entering the colony and then during its stay in the colony. The monkeys used are shown to be tuberculin-negative and free from antibodies to simian virus 40 (SV40) and simian immunodeficiency virus. If *Macaca* spp. monkeys are used for production, the monkeys are also shown to be free from antibodies to herpesvirus B (*Cercopithecine herpesvirus 1*) infection. Human herpesvirus 1 has been used as an indicator for freedom from herpesvirus B antibodies on account of the danger of handling herpesvirus B (*Cercopithecine herpesvirus 1*).

Monkeys from which kidneys are to be removed are thoroughly examined, particularly for evidence of tuberculosis and herpesvirus B (*Cercopithecine herpesvirus 1*) infection. If a monkey shows any pathological lesion relevant to the use of its kidneys in the preparation of a seed lot or vaccine, it is not to be used nor are any of the remaining monkeys of the group concerned unless it is evident that their use will not impair the safety of the product.

All the operations described in this section are conducted outside the area where the vaccine is produced.

Monkey cell cultures for vaccine production. Kidneys that show no pathological signs are used for preparing cell cultures. Each group of cell cultures derived from a single monkey forms a separate production cell culture giving rise to a separate single harvest.

The primary monkey kidney cell suspension complies with the test for mycobacteria ; disrupt the cells before carrying out the test.

If secondary or tertiary cells are used, it shall be demonstrated by suitable validation tests that cell cultures beyond the passage level used for production are free from tumorigenicity.

SEED LOT

Each of the three strains of poliovirus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

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Only a working seed lot that complies with the following requirements may be used for virus propagation.

Identification

Each working seed lot is identified as human poliovirus 1, 2 or 3 by virus neutralisation in cell culture using specific antibodies.

Virus concentration. The virus concentration of each working seed lot is determined to define the quantity of virus to be used for inoculation of production cell cultures.

Extraneous agents (2.7.3). The working seed lot complies with the requirements for seed lots for virus vaccines. In addition, if primary, secondary or tertiary monkey kidney cells have been used for isolation of the strain, measures are taken to ensure that the strain is not contaminated with simian viruses such as simian immunodeficiency virus, simian virus 40, filoviruses and herpesvirus B (*Cercopithecine herpesvirus 1*). A working seed lot produced in primary, secondary or tertiary monkey kidney cells complies with the requirements given below under Virus Propagation and Harvest for single harvests produced in such cells.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells or viruses are being handled. Approved animal serum (but not human serum) may be used in the cell culture media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. Not less than 500 ml of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells); where continuous cell lines in a fermenter are used for production, 200×10^6 cells are set aside to prepare control cells; where primary, secondary or tertiary monkey kidney cells are used for production, a cell sample equivalent to at least 500 ml of the cell suspension, at the concentration employed for vaccine production, is taken to prepare control cell cultures.

Only a single harvest that complies with the following requirements may be used in the preparation of the vaccine. The tests for Identification and Sterility may be carried out instead on the purified, pooled monovalent harvest. After demonstration of consistency of production at the stage of the single harvest, the test for virus concentration may be carried out instead on the purified, pooled monovalent harvest.

Control cells. The control cells of the production cell culture comply with a test for Identification (if a cell-bank system is used for production) and with the requirements for extraneous agents, where primary, secondary or tertiary monkey kidney cells are used, the tests in cell cultures are carried out as shown below under Test in Rabbit Kidney Cell Cultures and Test in Cercopithecus Kidney Cell Cultures).

Test in rabbit kidney cell cultures. Test a sample of at least 10 ml of the pooled supernatant fluid from the control cultures for the absence of herpesvirus B (*Cercopithecine herpesvirus 1*) and other viruses in rabbit kidney cell cultures. The dilution of supernatant in the nutrient medium is not greater than 1:4 and the area of the cell layer is at least 3 cm^2 per ml of inoculum. Set aside one or more containers of each batch of cells with the same medium as non-inoculated control cells. Incubate the cultures at 37° and observe for at least 2 weeks. The test is not valid if more than 20 per cent of the control cells are discarded for non-specific, accidental reasons.

Test in Cercopithecus kidney cell cultures. Test a sample of at least 10 ml of the pooled supernatant fluid from the control cultures for the absence of SV40 virus and other extraneous agents by inoculation onto cell cultures prepared from the kidneys of cercopithecus monkeys, or other cells shown to be at least as sensitive for SV40, by the method described under *Test in Rabbit Kidney Cell Cultures*. The test is not valid if more than 20 per cent of the control cell cultures are discarded for non-specific, accidental reasons.

Identification

The single harvest is identified as containing human poliovirus 1, 2 or 3 by virus neutralisation in cell cultures using specific antibodies.

Virus concentration. The virus concentration of each single harvest is determined by titration of infectious virus in cell cultures.

Bacterial and fungal contamination. The single harvest complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

Mycoplasmas (2.7.4). The single harvest complies with the test for mycoplasmas, carried out using 10 ml or polymerase chain reaction or any other validated method shall be used.

Test in rabbit kidney cell cultures. Where primary, secondary or tertiary monkey kidney cells are used for production, test a sample of at least 10 ml of the single harvest for the absence of herpesvirus B (*Cercopithecine herpesvirus 1*) and other viruses in rabbit kidney cell cultures as described for the control cells.

Test in Cercopithecus kidney cell cultures. Where primary, secondary or tertiary monkey kidney cells are used for production, test a sample of at least 10 ml of the single harvest for the absence of SV40 virus and other extraneous agents. Neutralise the sample by a high-titre antiserum against the specific type of poliovirus. Test the sample in primary

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cercopithecus kidney cell cultures or cells that have been demonstrated to be at least as susceptible for SV40. Incubate the cultures at 37° and observe for 14 days. At the end of this period, make at least one subculture of fluid in the same cell culture system and observe both primary cultures and subcultures for an additional 14 days.

PURIFICATION AND PURIFIED MONOVALENT HARVEST

Several single harvest of the same type may be pooled and may be concentrated. The monovalent harvest or pooled monovalent harvest is purified by validated methods. If continuous cell lines are used for production, the purification process shall have been shown to reduce consistently the content of substrate-cell DNA to less than 10 ng per single human dose. This substrate cell DNA content test may be omitted with the agreement of the national control authority, if the manufacturing process is validated to achieve this specification.

Only a purified monovalent harvest that complies with the following requirements may be used for the preparation of the inactivated monovalent harvest.

Identification

The virus is identified by virus neutralisation in cell cultures using specific antibodies or by determination of D-antigen.

Virus concentration. The virus concentration is determined by titration of infectious virus.

Specific activity. The ratio of the virus concentration or the D-antigen content, determined by a suitable immunochemical method (2.2.14) to the total protein content (specific activity) of the purified monovalent harvest is within the limits approved for the particular product.

INACTIVATION AND INACTIVATED MONOVALENT HARVEST

Several purified monovalent harvests of the same type may be mixed before inactivation. To avoid failures in inactivation caused by the presence of virus aggregates, filtration is carried out before and during inactivation; inactivation is started within a suitable period, preferably not more than 24 h and in any case not more than 72 h, of the prior filtration. The virus suspension is inactivated by a validated method that has been shown to inactivate poliovirus without destruction of immunogenicity; during validation studies, an inactivation curve with at least four points (for example, time 0, 24, 48, and 96 h) is established showing the decrease in concentration of live virus with time. If formaldehyde is used for inactivation, the presence of an excess of formaldehyde at the end of the inactivation period is verified.

Only an inactivated monovalent harvest that complies with the following requirements may be used in the preparation of a trivalent pool of inactivated monovalent harvests or a final bulk vaccine.

Test for effective inactivation. After neutralisation of the formaldehyde with sodium bisulphite (where applicable), verify the absence of residual live poliovirus by inoculation on suitable cell cultures of two samples of each inactivated monovalent harvest, corresponding to at least 1500 human doses. Take one sample not later than three-quarters of the way through the inactivation period and the other at the end. Inoculate the samples in cell cultures such that the dilution of vaccine in the nutrient medium is not greater then ¼ and the area of the cell layer is at least 3 cm^2 per ml of inoculum. Set aside one or more containers with the same medium as non-inoculated control cells. Observe the cell cultures for at least 3 weeks. Make not fewer than two passages from each container, one at the end of the observation period and the other 1 week before; for the passages, use cell culture supernatant and inoculate as for the initial sample. Observe the subcultures for at least 2 weeks. No sign of poliovirus multiplication is present in the cell cultures. At the end of the observation period, test the susceptibility of the cell culture used by inoculation of live poliovirus of the same type as that present in the inactivated monovalent harvest.

Bacterial and fungal contamination. The inactivated monovalent harvest complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

D-antigen content. The content of D-antigen determined by a suitable immunochemical method (2.2.14) is within the limits approved for the particular preparation.

FINAL BULK VACCINE

The final bulk vaccine is prepared directly from the inactivated monovalent harvests of human polioviruses 1, 2 and 3 or from a trivalent pool of inactivated monovalent harvests. If a trivalent pool of inactivated monovalent harvests is used, a test for effective inactivation is carried out on this pool instead of on the final bulk vaccine. A stabiliser and an antimicrobial preservative may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

Inactivation. Before addition of any antimicrobial preservative, a sample of at least 1500 ml or, for a purified and concentrated

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vaccine, the equivalent of 1500 doses is tested for residual live poliovirus in cell cultures, as described for the inactivated monovalent harvest. If the final bulk vaccine is prepared from a trivalent pool of inactivated monovalent harvests, the test for inactivation is carried out on that pool rather than on the final bulk vaccine.

Provided that the test for inactivation has been performed with satisfactory results on the monovalent harvests, it may be omitted on the trivalent pool of inactivated monovalent harvests or on the final bulk vaccine.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde and antimicrobial preservative and the *in vivo* assay have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot. Provided that the test for bovine serum albumin has been performed with satisfactory results on the trivalent pool of inactivated monovalent harvests or on the final bulk vaccine, it may be omitted on the final lot.

Identification

The vaccine is shown to contain human polioviruses 1, 2 and 3 by a suitable immunochemical method such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

Tests

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Protein content (2.3.49). Not more than 10 µg of protein per human dose.

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.2.14).

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Bacterial endotoxins (2.2.3). Not more than 5 IU per human dose.

Assay

D-antigen content. As a measure of consistency of production, determine the D-antigen content for human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.2.14) using an appropriate reference preparation calibrated in D-antigen units. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product.

Guideline on waiving off the *in vivo* assay of poliomyelitis vaccine (inactivated) and its combinations

This guideline is applicable to vaccines derived from wild strains of poliovirus. The validation should be carried out for each product before waving off the in vivo assay, and should be repeated wherever a substantial change takes place in the manufacturing process that may affect the in vitro or in vivo assays.

This guideline is intended to promote waving off the *in vivo* assay wherever it can be demonstrated for a given product that the *in vitro* assay (D-antigen determination) provides sufficient assurance of satisfactory potency of vaccine for routine batch control.

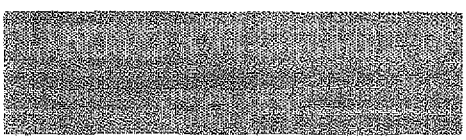
In vivo assay in rats is considered the preferred method. For vaccines that are assayed using chicks or guinea-pigs and that have an established record of production history, the *in vivo* assay may be waived off if the rat assay is also performed on the batches included in the validation study described below. For vaccines not yet approved, the results of the rat assay on all final bulks should be included in all data generated for demonstration of consistency of production before waving off the *in vivo* assay.

Once the *in vivo* assay has been waived, batches of vaccine will be released on the basis of the *in vitro* assay, and the *in vivo* assay should not be used as an alternative for the release of a batch that fails the *in vitro* assay. Repetition of the *in vitro* assay may be performed according to an authorized procedure.

PROCEDURE

The following conditions should be satisfied before performance of the validation study:

- appropriate experience with the rat assay;
- full validation of the D-antigen assay that is linearity, repeatability, intermediate precision and accuracy;
- establishment of acceptance criteria for the D-antigen assay based on a suitable number of consecutive final lots;
- establishment of production consistency on recent final bulks using the currently approved *in vivo* assay; the final bulks should correspond to the final lots used to establish the acceptance criteria for the D-antigen assay and should represent different inactivated harvests of each of the 3 types of poliovirus.



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The validation study should be performed on:

— a final bulk/lot that is representative of the current production method;

— 2 sub-potent batches prepared, for example, by heating normal vaccine or mixing it with heat-treated vaccine; the sub-potent batches should have expected titres of about half that of the representative final bulk/lot.

These batches are assayed using as reference standard a homologous production batch:

— by the currently approved *in vivo* assay for the vaccine;

— by the rat assay where this is not the currently approved in *vivo assay*;

— by the D-antigen assay.

Waiving off the *in vivo* assay is acceptable if the representative final bulk/lot complies with the *in vivo* and *in vitro* assays and the sub-potent batches fail to comply. If a sub-potent batch fails to comply with the D-antigen assay but complies with the *in vivo* assay, the latter may be repeated.

***In vivo* test.** The capacity of the vaccine to induce the formation of neutralizing antibodies is determined *in-vivo* by one of the following methods:

Test in chicks or guinea-pigs. Prepare a suitable series of at least three dilutions of the vaccine under examination using a suitable *buffered saline solution*. Inject 0.5 ml of the dilutions intramuscularly into groups of ten 3 week-old chickens or groups of ten guinea-pigs, each weighing between 250 and 350 g, using a separate group for each dilution of vaccine. Bleed the animals on the fifth or sixth day after the injection and separate the sera. Examine the sera for the presence of neutralising antibody, at a dilution of 1 in 4, to each of the human polioviruses 1, 2 and 3. Mix 100 $CCID_{50}$ of virus with the dilution of serum and incubate at 37° for 4 h 30 min to 6 h. Keep at 5 ± 3° for 12 to 18 h. Inoculate the mixtures into cell cultures for the detection of unneutralised virus and read the results up to 7 days after inoculation. For each group of animals, note the number of sera which have neutralising antibody and calculate the dilution of the vaccine giving an antibody response in 50.0 per cent of the animals. Carry out in parallel a control test using a suitable *reference preparation*.

The vaccine complies with the test if a dilution of 1 in 100 or more produces an antibody response for each of the three types of virus in 50.0 per cent of the animals.

Test in rats. A suitable *in vivo* assay method consists of intramuscular injection into the hind limb(s) of not fewer than 3 dilutions of the vaccine under examination and a reference vaccine, using for each dilution a group of 10 specific pathogen-free rats of the suitable strain. Use of 4 dilutions is often necessary to obtain valid results for all 3 serotypes. The number of animals per group must be sufficient to obtain results that meet the validity criteria; groups of 10 rats are usually sufficient although valid results may be obtained with fewer animals per group. The weight of individual animal must not vary by more than 10.0 per cent from the group mean. An inoculum of 0.5 ml per rat is used. The dose range is chosen such that a dose response to all 3 poliovirus types is obtained. Bleed the animals after 20 to 22 days. Neutralising titres against all 3 polivirus types are measured separately using 100 CCID50 of the Sabin strains as challenge viruses. Vero or Hep2 as indicator cells, and neutralization conditions of 3 hours at 35° to 37° followed by 18 hours at 2° to 8°. Results are read microscopically and/or after fixation and staining after 7 days of incubation at 35°. For a valid antibody assay, the titre of each challenge virus must be shown to be within the range of 10 to 1000 $CCID_{50}$ and the neutralizing antibody titre of a control serum must be within 2 two fold dilutions of the geometric mean titre of the serum. The potency is calculated by comparison of the preparation of responders for the vaccine under examination and the reference vaccine by the probit method or, after validation, using a parallel-line model. For the probit method it is necessary to establish a cut-off neutralising antibody titre for each poliovirus type to define a responder. Due to interlaboratory variation, it is not possible to define cut-off values that could be applied by all laboratories. Rather, the cut-off values are determined for each laboratory based on a minimum series of 3 tests with the reference vaccine. The mid-point on a log 2 scale of the minimum and maximum geometric mean titres of the series of 3 or more tests is used as the cut-off value. For each of the 3 poliovirus types, the potency of the vaccine is not significantly less than that of the *reference preparation*. The test is not valid unless (1) for both the test and reference vaccines the ED_{50} lies between the smallest and the largest doses given to the animals; (2) the statistical analysis shows no significant deviation from linearity or parallelism; (3) the fiducial limits of the estimated relative potency fall between 25.0 per cent and 400.0 per cent of the estimated potency.

Labelling. The label states (1) the types of poliovirus contained in the vaccine; (2) the nominal amount of virus of each type (1, 2 and 3), expressed in units of D-antigen per single human dose; (3) the cell substrate used to prepare the vaccine.

Poliomyelitis Vaccine, Live (Oral)

Oral Poliomyelitis Vaccine is a preparation of approved strains of live attenuated poliovirus type 1, 2 or 3 grown in *in vitro* cultures of approved cells, containing any one type or any combination of the three types of Sabin strains, prepared in a form suitable for oral administration. The vaccine is a clear liquid that may be coloured owing to the presence of a pH indicator.

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Production

General provisions

The vaccine strains and the production method should consistently yield vaccines that are both immunogenic and safe in man.

The production of vaccine is based on a virus seed-lot system. Cell lines are used according to a cell-bank system. If primary monkey kidney cells are used, production complies with the requirements indicated below. Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more than two passages from the master seed lot.

Substrate for virus propagation

The virus is propagated in human diploid cells (2.7.2) or in continuous cell lines (2.7.2) or in primary monkey kidney cells (including serially passaged cells from primary monkey kidney cells). Continuous cell lines are approved by the competent authority.

Primary monkey kidney cells. *The following special requirements for the substrate for virus propagation apply to primary monkey kidney cells.*

Monkeys used for preparation of kidney cell cultures and for testing of virus. If the vaccine is prepared in monkey kidney cell cultures, animals of a species approved by the competent authority, in good health, and not previously employed for experimental purposes shall be used.

The monkeys shall be kept in well-constructed and adequately ventilated animal rooms in cages spaced as far apart as possible. Adequate precautions shall be taken to prevent cross-infection between cages. Not more than two monkeys shall be housed per cage and cage-mates shall not be interchanged. The monkeys shall be kept in the country of manufacture of the vaccine in quarantine groups for a period of not less than 6 weeks before use. A quarantine group is a colony of selected, healthy monkeys kept in one room, with separate feeding and cleaning facilities, and having no contact with other monkeys during the quarantine period. If at any time during the quarantine period the overall death rate of a shipment consisting of one or more groups reaches 5 per cent (excluding deaths from accidents or where the cause was specifically determined not to be an infectious disease), monkeys from that entire shipment shall continue in quarantine from that time for a minimum of 6 weeks. The groups shall be kept continuously in isolation, as in quarantine, even after completion of the quarantine period, until the monkeys are used. After the last monkey of a group has been taken, the room that housed the group shall be thoroughly cleaned and decontaminated before being used for a fresh group. If kidneys from near-term monkeys are used, the mother is quarantined for the term of pregnancy.

Monkeys from which kidneys are to be removed shall be anaesthetised and thoroughly examined, particularly for evidence of tuberculosis and *Cercopithecid herpesvirus 1* (B virus) infection.

If a monkey shows any pathological lesion relevant to the use of its kidneys in the preparation of a seed lot or vaccine, it shall not be used, nor shall any of the remaining monkeys of the quarantine group concerned be used unless it is evident that their use will not impair the safety of the product.

All the operations described in this section shall be conducted outside the areas where the vaccine is produced.

The monkeys used shall be shown to be free from antibodies to simian virus 40 (SV40) and simian immunodeficiency virus. If *Macaca* spp. are used for production, the monkeys shall also be shown to be free from antibodies to cercopithecid herpesvirus 1 (B virus). Human herpesvirus has been used as an indicator for freedom from B virus antibodies on account of the danger of handling cercopithecid herpesvirus 1 (B virus).

Monkey kidney cell cultures for vaccine production. Kidneys that show no pathological signs are used for preparing cell cultures. If the monkeys are from a colony maintained for vaccine production, serially passaged monkey kidney cell cultures from primary monkey kidney cells may be used for virus propagation, otherwise the monkey kidney cells are not propagated in series. Virus for the preparation of vaccine is grown by aseptic methods in such cultures. If animal serum is used in the propagation of the cells, the maintenance medium after virus inoculation shall contain no added serum.

Each group of cell cultures derived from a single monkey or from fetuses from no more than ten near-term monkeys is prepared and tested as an individual group.

SEED LOT

The strains of poliovirus used shall be identified by historical records that include information on the origin and subsequent manipulation of the strains.

Working seed lots are prepared by a single passage from a master seed lot and at an approved passage level from the original Sabin virus. Virus seed lots are prepared in large quantities and stored at a temperature below -60°.

Only a virus seed lot that complies with the following requirements may be used for virus propagation.

Identification

Each working seed lot is identified as poliovirus of the given type, using specific antibodies.

Virus concentration. Determined by the method described below, the virus concentration is the basis for the quantity of virus used in the neurovirulence test.

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Extraneous agents (2.7.3). If the working seed lot is produced in human diploid cells (2.7.2) or in continuous cell lines (2.7.2) it complies with the requirements for seed lots for virus vaccines. If the working seed lot is produced in primary monkey cells, it complies with the requirements given below under Propagation and Harvest and Monovalent Pooled Harvest and with the tests in adult mice, suckling mice and guinea-pigs given under Tests for extraneous agents in viral vaccines for human use.

Working seed lot shall be free from detectable DNA sequences from simian virus 40 (SV40).

Neurovirulence (2.7.6). Each master and working seed lot complies with the test for neurovirulence of poliomyelitis vaccine (oral) in monkeys. Furthermore, the seed lot shall cease to be used in vaccine production if the frequency of failure of the monovalent pooled harvests produced from it is greater than predicted statistically. This statistical prediction is calculated after each test on the basis of all the monovalent pooled harvests tested; it is equal to the probability of false rejection on the occasion of a first test (i.e. 1 per cent), the probability of false rejection on retest being negligible. If the test is carried out only by the manufacturer, the test slides are provided to the control authority for assessment.

Genetic markers. Each working seed lot is tested for its *replicating properties at temperatures ranging from 36° to 40°* as described under Monovalent Pooled Harvest.

PROPAGATION AND HARVEST

All processing of the cell-banks and subsequent cell-cultures is done under aseptic conditions in an area where no other cells are handled. Approved animal (but not human) serum may be used in the media, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from live extraneous agents. The cell-culture medium may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. Not less than 5 per cent and not more than 1000 ml of the cell cultures employed for vaccine production are set aside as uninfected cell cultures (control cells); special requirements, given below, apply to control cells when the vaccine is produced in primary monkey cells The virus suspension is harvested not later than 4 days after virus inoculation. After inoculation of the production cell culture with the virus working seed lot, inoculated cells are maintained at a fixed temperature, shown to be suitable, within the range 33° to 35°; the temperature is maintained constant to ±0.5°; control cell cultures are maintained at 33° to 35° for the relevant incubation periods.

Only a single virus harvest that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

Virus concentration. The virus concentration of virus harvests is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

Extraneous agents (2.7.3). Complies with tests for extraneous agents.

Control cells. The control cells of the production cell culture from which the virus harvest is derived comply with a test for identity and with the requirements for extraneous agents or, where primary monkey cells are used, as shown below.

Primary monkey kidney cells. *The following special requirements apply to virus propagation and harvest in primary monkey kidney cells.*

Cell cultures. On the day of inoculation with virus seed, each cell culture is examined for degeneration caused by an infective agent. If, in this examination, evidence is found of the presence in a cell culture of any extraneous agent, the entire group of cultures concerned shall be rejected.

On the day of inoculation with the virus working seed lot, a sample of at least 30 ml of the pooled fluid removed from the cell cultures of the kidneys of each single monkey or from fetuses from not more than ten near-term monkeys is divided into two equal portions. One portion of the pooled fluid is tested in monkey kidney cell cultures prepared from the same species, but not the same animal, as that used for vaccine production. The other portion of the pooled fluid is, where necessary, tested in monkey kidney cell cultures from another species so that tests on the pooled fluids are done in cell cultures from at least one species known to be sensitive to SV40. The pooled fluid is inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet is at least 3 cm^2 per ml of pooled fluid. At least one bottle of each kind of cell culture remains uninoculated to serve as a control. If the monkey species used for vaccine production is known to be sensitive to SV40, a test in a second species is not required. Animal serum may be used in the propagation of the cells, provided that it does not contain SV40 antibody, but the maintenance medium after inoculation of test material contains no added serum except as described below.

The cultures are incubated at a temperature of 35° to 37° and are observed for a total period of at least 4 weeks. During this observation period and after not less than 2 weeks incubation, at least one subculture of fluid is made from each of these cultures in the same cell culture system. The subcultures are also observed for at least 2 weeks.

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Serum may be added to the original culture at the time of subculturing, provided that the serum does not contain SV40 antibody.

Fluorescent-antibody techniques may be useful for detecting SV40 virus and other viruses in the cells.

A further sample of at least 10 ml of the pooled fluid is tested for cercopithecid herpesvirus 1 (B virus) and other viruses in rabbit kidney cell cultures. Serum used in the nutrient medium of these cultures shall have been shown to be free from inhibitors of B virus. Human herpesvirus has been used as an indicator for freedom from B virus inhibitors on account of the danger of handling cercopithecid herpesvirus 1 (B virus). The sample is inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet is at least 3 cm^2 per ml of pooled fluid. At least one bottle of the cell cultures remains uninoculated to serve as a control.

The cultures are incubated at a temperature of 35° to 37° and observed for at least 2 weeks.

A further sample of 10 ml of the pooled fluid removed from the cell cultures on the day of inoculation with the seed lot virus is tested for the presence of extraneous agents by inoculation into human cell cultures sensitive to measles virus.

The tests are not valid if more than 20 per cent of the culture vessels have been discarded for non-specific accidental reasons by the end of the respective test periods.

If, in these tests, evidence is found of the presence of an extraneous agent, the single harvest from the whole group of cell cultures concerned is rejected.

If the presence of *Cercopithecid herpesvirus 1* (B virus) is demonstrated, the manufacture of oral poliomyelitis vaccine shall be discontinued and the competent authority shall be informed. Manufacturing shall not be resumed until a thorough investigation has been completed and precautions have been taken against any reappearance of the infection, and then only with the approval of the competent authority.

If these tests are not done immediately, the samples of pooled cell-culture fluid shall be kept at a temperature of -60° or below, with the exception of the sample for the test for B virus, which may be held at 4°, provided that the test is done not more than 7 days after it has been taken.

Control cell cultures. On the day of inoculation with the virus working seed lot 25 per cent (but not more than 2500 ml) of the cell suspension obtained from the kidneys of each single monkey or from not more than ten near-term monkeys is taken to prepare uninoculated control cell cultures. These control cell cultures are incubated in the same conditions as the inoculated cultures for at least 2 weeks and are examined during this period for evidence of cytopathic changes. The tests are not valid if more than 20 per cent of the control cell cultures have been discarded for non-specific, accidental reasons. At the end of the observation period, the control cell cultures are examined for degeneration caused by an infectious agent. If this examination or any of the tests required in this section shows evidence of the presence in a control culture of any extraneous agent, the poliovirus grown in the corresponding inoculated cultures from the same group shall be rejected.

Tests for haemadsorbing viruses. At the time of harvest or within 4 days of inoculation of the production cultures with the virus working seed lot, a sample of 4 per cent of the control cell cultures is taken and tested for haemadsorbing viruses. At the end of the observation period, the remaining control cell cultures are similarly tested. The tests are made as described in *(2.7.3), Tests for extraneous agents in viral vaccines for human use.*

Tests for other extraneous agents. At the time of harvest, or within 7 days of the day of inoculation of the production cultures with the working seed lot, a sample of at least 20 ml of the pooled fluid from each group of control cultures is taken and tested in two kinds of monkey kidney cell culture, as described above.

At the end of the observation period for the original control cell cultures, similar samples of the pooled fluid are taken and the tests referred to in this section in the two kinds of monkey kidney cell culture and in the rabbit cell cultures are repeated, as described above under Cell cultures.

If the presence of *Cercopithecid herpesvirus 1* (B virus) is demonstrated, the production cell cultures shall not be used and the measures concerning vaccine production described above must be undertaken.

The fluids collected from the control cell cultures at the time of virus harvest and at the end of the observation period may be pooled before testing for extraneous agents. A sample of 2 per cent of the pooled fluid is tested in each of the cell culture systems specified.

Single harvests

Tests for neutralised single harvests in monkey kidney cell cultures. A sample of at least 10 ml of each single harvest is neutralised by a type-specific poliomyelitis antiserum prepared in animals other than monkeys. In preparing antisera for this purpose, the immunising antigens used shall be prepared in non-simian cells.

Half of the neutralised suspension (corresponding to at least 5 ml of single harvest) is tested in monkey kidney cell cultures prepared from the same species, but not the same animal, as that used for vaccine production. The other half of the neutralised suspension is tested, if necessary, in monkey kidney cell cultures from another species so that the tests on

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the neutralised suspension are done in cell cultures from at least one species known to be sensitive to SV40.

The neutralised suspensions are inoculated into bottles of these cell cultures in such a way that the dilution of the suspension in the nutrient medium does not exceed 1 in 4. The area of the cell sheet is at least 3 cm^2 per ml of neutralised suspension. At least one bottle of each type of cell culture remains uninoculated to serve as a control and is maintained by nutrient medium containing the same concentration of the specific antiserum used for neutralisation.

Animal serum may be used in the propagation of the cells, provided that it does not contain SV40 antibody, but the maintenance medium, after the inoculation of the test material, contains no added serum other than the poliovirus neutralising antiserum, except as described below.

The cultures are incubated at a temperature of 35° to 37° and observed for a total period of at least 4 weeks. During this observation period and after not less than 2 weeks incubation, at least one subculture of fluid is made from each of these cultures in the same cell-culture system. The subcultures are also observed for at least 2 weeks.

Serum may be added to the original cultures at the time of subculturing, provided that the serum does not contain SV40 antibody.

Additional tests are made for extraneous agents on a further sample of the neutralised single harvests by inoculation of 10 ml into human cell cultures sensitive to measles virus.

Fluorescent-antibody techniques may be useful for detecting SV40 virus and other viruses in the cells.

The tests are not valid if more than 20 per cent of the culture vessels have been discarded for non-specific accidental reasons by the end of the respective test periods.

If any cytopathic changes occur in any of the cultures, the causes of these change are investigated. If the cytopathic changes are shown to be due to unneutralised poliovirus, the test is repeated. If there is evidence of the presence of SV40 or other extraneous agents attributable to the single harvest, that single harvest is rejected.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

MONOVALENT POOLED HARVEST

Monovalent pooled harvests are prepared by pooling a number of satisfactory single harvests of the same virus type. Monovalent pooled harvests from continuous cell lines may be purified. Each monovalent pooled harvest is filtered through a bacteria-retentive filter.

Only a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification

Each monovalent pooled harvest is identified as poliovirus of the given type, using specific antiserum.

Virus concentration

The virus concentration is determined by the method described below and serves as the basis for calculating the dilutions for preparation of the final bulk, for the quantity of virus used in the neurovirulence test and to establish and monitor production consistency.

Genetic markers. A ratio of the replication capacities of the virus in the monovalent pooled harvest is obtained over a temperature range between 36° and 40° in comparison with the seed lot or a reference preparation for the marker tests and with appropriate rct/40- and rct/40+ strains of poliovirus of the same type. The incubation temperatures used in this test are controlled to within ±0.1°. The monovalent pooled harvest passes the test if, for both the virus in the harvest and the appropriate reference material, the titre determined at 36° is at least 5.0 log greater than that determined at 40°. If growth at 40° is so low that a valid comparison cannot be established, a temperature in the region of 39.0° to 39.5° is used, at which temperature the reduction in titre of the reference material must be in the range 3.0 to 5.0 log of its value at 36°; the acceptable minimum reduction is determined for each virus strain at a given temperature. If the titres obtained for one or more of the reference viruses are not concordant with the expected values, the test must be repeated.

Neurovirulence (2.7.6). Each monovalent pooled harvest complies with the test for neurovirulence of poliomyelitis vaccine (oral). If the test is carried out only by the manufacturer, the test slides are provided to the competent authority for assessment. The TgPVR21 transgenic mouse model provides a suitable alternative to the monkey neurovirulence test for neurovirulence testing of types 1, 2 or 3 vaccines once a laboratory qualifies as being competent to perform the test and the experience gained is to the satisfaction of the competent authority. The test is carried out using a standard operating procedure approved by the competent authority. A suitable procedure *(Neurovirulence test of type 1, 2 or 3 live poliomyelitis vaccines (oral) in transgenic mice susceptible to poliovirus)* is available from WHO, Quality and Safety of Biologicals, Geneva.

Primary monkey kidney cells. *The following special requirements apply to monovalent pooled harvests derived from primary monkey kidney cells.*

Retroviruses. The monovalent pooled harvest is examined using a reverse transcriptase assay. No indication of the presence of retrovirus is found.

Test on rabbits. A sample of the monovalent pooled harvest is tested for cercopithecid herpesvirus 1 (B virus) and other

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viruses by injection of not less than 100 ml into not fewer than 10 healthy rabbits each weighing between 1.5 and 2.5 kg. Each rabbit receives not less than 10 ml and not more than 20 ml, of which 1 ml is given intradermally at multiple sites, and the remainder subcutaneously. The rabbits are observed for at least 3 weeks for death or signs of illness.

All rabbits that die after the first 24 h of the test and those showing signs of illness are examined by autopsy, and the brain and organs removed for detailed examination to establish the cause of death.

The test is not valid if more than 20.0 per cent of the inoculated rabbits show signs of intercurrent infection during the observation period. The monovalent pooled harvest passes the test if none of the rabbits shows evidence of infection with B virus or with other extraneous agents or lesions of any kind attributable to the bulk suspension.

If the presence of B virus is demonstrated, the measures concerning vaccine production described above under Cell cultures are taken.

Test on guinea pigs. Administer to not less than five guinea-pigs, each weighing between 350 and 450 g, 0.1 ml of the monovalent pooled harvest by intracerebral injection and 0.5 ml by intraperitoneal injection. Measure the rectal temperature of each animal on each working day for 6 weeks. At the end of the observation period carry out autopsy on each animal.

In addition, administer to not fewer than five guinea-pigs 0.5 ml by intraperitoneal injection and observe as described above for 2 to 3 weeks. At the end of the observation period, carry out a passage from these animals to not fewer than five guinea pigs using blood and a suspension of liver or spleen tissue. Measure the rectal temperature of the latter guinea pigs for 2 to 3 weeks. Examine by autopsy all animals that, after the first day of the test, die or are killed because they show disease or show for three consecutive days a body temperature higher than 39°; carry out histological examination to detect infection with Marburg virus; in addition, inject a suspension of liver or spleen tissue or of blood intraperitoneally into not fewer than three guinea pigs. If any signs of infection with Marburg virus are noted, confirmatory serological tests are carried out on the blood of the affected animals. The monovalent pooled harvest complies with the test if not fewer than 80.0 per cent of the guinea pigs survive to the end of the observation period and remain in good health and no animal shows signs of infection with filoviruses virus.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more satisfactory monovalent pooled harvests and may contain more than one virus type. Suitable flavouring substances and stabilisers may be added.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

FINAL LOT

Only a final lot that complies with the following requirement for thermal stability and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Thermal stability. Expose samples of the final lot at 37° for 48 hours. Determine the total virus concentration as described under Assay in parallel for the heated vaccine and for unheated vaccine. The estimated difference between the total virus concentration of the unheated and heated vaccines is not greater than 0.5 $\log_{10}$ infectious virus units ($CCID_{50}$) per single human dose.

Identification

The vaccine is shown to contain poliovirus of each type stated on the label, using specific antibodies.

Tests

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Assay

Titrate for infectious virus at least in triplicate using the method described below. Use an appropriate virus reference preparation to validate each assay. If the vaccine contains more than one poliovirus type, titrate each type separately, using appropriate type-specific antiserum (or preferably a monoclonal antibody) to neutralise each of the other types present.

For a trivalent vaccine, the estimated mean virus titres must be: not less than $1 \times 10^{6.0}$ infectious virus units ($CCID_{50}$) per single human dose for type 1; not less than $1 \times 10^{5.0}$ infectious virus units ($CCID_{50}$) for type 2; and not less than $1 \times 10^{5.8}$ infectious virus units ($CCID_{50}$) for type 3.

For monovalent or divalent vaccine, the minimum virus titres are decided by the competent authority.

Method. Groups of eight to twelve flat-bottomed wells in a microtitre plate are inoculated with 0.05 ml of each of the selected dilutions of virus followed by a suitable cell suspension of the Hep-2 (Cincinnati) line. The plates are incubated at a suitable temperature. Examine the cultures on days 7 to 9.

The assay is not valid if (a) the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is greater than

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±0.3; (b) the virus concentration of the reference preparation differs by more than 0.5 log $CCID_{50}$ from the assigned value.

Labelling. The label states (1) the types of poliovirus contained in the vaccine; (2) the minimum amount of virus of each type contained in one single human dose; (3) the cell substrate used for the preparation of the vaccine; (4) that the vaccine is not to be injected.

Rabies Antiserum

Antirabies Serum; Antirabic Serum

Rabies Antiserum is a preparation containing the specific globulin or its derivatives obtained by purification of hyperimmune serum or plasma of healthy horses or other suitable animals having the specific activity of neutralising the rabies virus. It may contain a suitable preservative.

Rabies Antiserum contains not less than 300 Units per ml.

Description. A clear, colourless or pale yellow liquid free from suspended particles or cream-coloured powder or pellet to be reconstituted with diluent supplied by the manufacturer.

Identification

Specifically neutralizes the standard strain of rabies virus rendering it harmless to susceptible animals or by any other suitable *in vitro* method.

Tests

Potency. Carry out the biological assay of rabies antiserum described below.

Biological Assay of Rabies Antiserum

The potency of rabies antiserum is determined by comparing the dose necessary to protect mice against a lethal intracerebral dose of rabies virus with the dose of the Standard Preparation of rabies antiserum necessary to give the same protection.

Standard Preparation

The standard preparation is a dried serum or other suitable preparation the potency of which has been determined in relation to the International Standard.

Suggested Method

Test animals. Use healthy mice of either sex from the same stock weighing between 10 and 14 g, but animals of the same sex should be used in any given test.

Test virus. Any suitable strain of rabies virus of known potency such as the CVS strain may be used.

Test dose of virus. The test dose of virus is such that each mouse receives by intracerebral injection between 20 and 1000 LD_{50} preferably about 100 LD_{50}. To determine the number of LD_{50} of virus used, mix the virus dilution used in the test with an equal quantity of a 2 per cent v/v solution of *heat-inactivated normal horse serum* in *water* and maintain the mixture at 37° for 1 hour. Prepare 10-fold dilutions in a 2 per cent v/v solution of *heat-inactivated normal horse serum* and inject them into mice. Carry out this test at the same time as the test for determining the potency of the rabies antiserum. The test is not valid unless the quantity of virus used lies between 20 and 1000 LD_{50}.

Determination of potency of the rabies antiserum. Prepare a series of six 2-fold dilutions of the Standard Preparation and of the preparation under examination with *water* containing 2 per cent v/v of *heat inactivated normal horse serum*. To each dilution add a quantity of a suspension of the test virus containing the test dose and keep the mixtures at 37° for 1 hour. Inject intracerebrally 0.03 ml of each mixture into 10 mice.

Observe the mice for 14 days after the injection. Mice dying before the fifth day after inoculation with the virus are eliminated from the test; all the mice dying between the fifth and fourteenth days after showing signs of rabies are considered to have died of rabies; mice living upto the fourteenth day but showing signs of rabies are also counted as having died from rabies. Calculate the result of the test by standard statistical methods. The preparation under examination passes the test if, in a single comparative assay, it is found to have 80 or more Units per ml. If it is found to have less than 80 Units per ml, two more similar assays may be carried out; the preparation passes the test if, in both these additional assays, it is found to have 80 or more Units per ml.

Other tests. Complies with the tests stated under Antisera.

Storage. As stated under Antisera.

Labelling. The label states (1) the number of Units per ml in case of liquid preparation; (2) the total volume in the container; (3) the name and volume of the diluent to be used for reconstituting the freeze-dried preparation; (4) the recommended dose; (5) the name and concentration of any added preservative; (6) the animal species in which the antiserum has been raised; (7) that the liquid preparation should not be allowed to freeze.

Rabies Vaccine, Human

Rabies Vaccine for Human use is a freeze-dried or liquid (adsorbed) preparation of a suitable approved, strain of fixed rabies virus grown in an approved cell culture/ embryos of duck/chicken and inactivated by a validated method.

The freeze-dried vaccine is reconstituted immediately before use as stated on the label to give a clear or slightly opalescent

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solution/suspension. It may be coloured owing to the presence of a pH indicator.

The vaccine complies with the General Requirements of Vaccines for Human Use.

Production

General provisions

The vaccine is produced on the basis of virus seed lot system and if a cell line is used for virus propagation, a cell-bank system shall be followed. The production method shall have been shown to yield consistently vaccines that comply with the requirements for immunogenicity, safety and stability. Unless otherwise justified and authorized, the virus in the final vaccine shall not have undergone more passages from the master seed lot than was used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy.

Substrate for virus propagation

The virus is propagated in any suitable approved cell substrate like a human diploid cell line (2.7.2), a continuous cell line, or in duck embryos or in cultures of chicken embryos derived from a flock certified as free from specified pathogens(2.7.7).

SEED LOT

The strain of rabies virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

Working seed lots are prepared by not more than five passages from the master seed lot.

Only a working seed lot that complies with the following requirements may be used for virus propagation.

Identification

Each working seed lot is identified as rabies virus using specific antibodies by an approved method.

Virus concentration. The virus concentration of each working seed lot is determined by a cell culture method using immunofluoresence or any other approved method.

Extraneous agents (2.7.3). The working seed lot complies with the requirements for the virus seed lots.

Mycoplasma (2.7.4).Complies with the test for absence of mycoplasma.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures are done under aseptic conditions in an area where no other cells are handled. Approved animal (but not human) serum may be used in the media, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum; the media may contain human albumin. Serum proteins, if present are reduced to an acceptable level by a suitable method of purification. Serum and trypsin used in the preparation of cell suspension and media are shown to be free from infectious extraneous agents. The cell culture media may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. Not less than 5 per cent or 500 ml which ever is more of the cell cultures employed for vaccine production are set aside as uninfected cell cultures (control cells). The virus suspension is harvested on one or more occasions during incubation. Multiple harvests from the same production cell culture may be pooled and considered as a single harvest.

When vaccine is prepared in embryonated eggs, the egg proteins are minimized by an appropriate method of purification. The eggs are inoculated with virus seed by the yolk sac route. The infected sterile living embryos are harvested, minced and emulsified in suitable diluent, and stabilizer with aseptic precautions. Emulsions are centrifuged, supernatants are collected and stored as raw virus harvest at a suitable temperature.

Viral harvests that comply with the following requirements are pooled in the preparation of the inactivated viral harvest.

Identification

The single harvest contains virus that is identified as rabies virus using specific antibodies by an approved method.

Virus concentration. Titrate for infective virus in cell cultures or by any other approved method. The titre is used to monitor consistency of production.

Control cells. The control cells of the production cell culture from which the single harvest is derived should comply with a test for identification and with the requirements for extraneous agents (2.7.3).

Control eggs. Control eggs shall be tested for freedom from haemagglutinating agents, and other extraneous agents.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

PURIFICATION AND INACTIVATION

The virus harvests may be concentrated and/or purified by suitable methods; the virus harvest is inactivated by a validated method at a fixed, well defined stage of the process which may be before, during or after any concentration or purification. The method shall have been shown to be capable of inactivating rabies virus without destruction of the immunogenic activity. If betapropiolactone is used, the concentration shall at no time exceed 1:3500.

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Cell culture vaccines

Only an inactivated viral suspension that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Inactivation. Inactivation is confirmed by carrying out an amplification test for residual infectious rabies virus, not more than 4 days after inactivation or on the sample frozen after inactivation and stored at -70°. Inoculate a quantity of inactivated viral suspension equivalent to not less than 25 vaccine doses into cell cultures of the same type as those used for production of the vaccine. Make a passage after 7 days. Maintain the cultures for a further period of 14 days and then examine the cell cultures for rabies virus using an immune fluorescence test. No rabies virus is detected. Alternatively, 5 ml of each culture fluid is pooled on days 14 and 21 and 0.03 ml is inoculated intracerebrally into each of the 10 mice weighing 12 to 15 g. The mice are observed for 14 days for symptoms caused by rabies virus, and mice showing symptoms of rabies are sacrificed and virus presence is confirmed by immunofluorescence test or tested for live virus in cell culture by immunofluorescence test or method of equal sensitivity. No rabies virus should be detected.

Residual host-cell DNA (2.2.15). If a continuous cell line is used for virus propagation, the content of residual host-cell DNA, determined using a suitable method, should not be greater than 10 ng per single human dose.

Embryonated egg vaccine

Only concentrated viral suspensions that comply with the test for sterility, antigen content and endotoxin requirements may be used for preparation of bulk for inactivation.

Inactivation is confirmed by carrying out Mice Inoculation Test for residual infectious rabies virus, not more than four days after inactivation or on the sample frozen 4 days after inactivation are stored at −70°. Inoculate intracerebrally 0.03 ml into each of 20 mice weighing between 12 and 15 g. The mice are observed for 14 days for symptoms caused by rabies virus and mice showing symptoms of rabies are sacrificed and virus presence is confirmed by an immunofluorescence test or tested for live virus in cell culture by immunofluorescence test or method of equal sensitivity. No rabies virus should be detected.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more inactivated viral suspensions. An approved stabilizer may be added to maintain the activity of the product during and after freeze-drying. Thiomersal can be used as preservative.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antigen content. Determine the antigen content by a suitable approved *in vitro* or *in vivo* method. The content should be within the limits approved for the particular product.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile containers and can be freeze-dried in case of lyophilized products. The containers are then sealed so as to prevent contamination and the introduction of moisture.

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for inactivation has been carried out with satisfactory results on the inactivated virus suspension and the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, these tests may be omitted on the final lot.

Identification

The vaccine is shown to contain rabies virus antigen by a suitable immunochemical method using specific antibodies, alternatively, the Assay also serves to identify the vaccine.

Tests

Inactivation. Inoculate a quantity equivalent to not less than 25 human doses of vaccine into cell cultures of the same type as those used for production of the vaccine. Make a passage after 7 days. Maintain the culture for a further 14 days and then examine the cell culture for rabies virus using an immunofluorescence test. No rabies virus is detected. Alternatively, inject 0.03 ml of the vaccine intracerebrally into each of the 10 mice weighing between 12 and 15g. Neither symptoms of disease in the central nervous system nor death occurs in any of the animal within 14 days. If the inactivation test is already performed on inactivated virus used for final lot, it may be omitted from the test on final lot.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Bacterial endotoxins (2.2.3). Less than 25 IU per single human dose. Or

Pyrogens (2.2.8). Complies with the test for pyrogens. Unless otherwise justified and authorized, inject into each rabbit a single human dose of the vaccine diluted to ten times its volume.

Water (2.3.43). Not more than 3.0 per cent determined by an approved method.

Accelerated degradation. The potency determined by method described under "Assay" of a sample of the preparation under

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examination after storage at 37° for 4 weeks is not less than 2.5 units per single human dose. This may not be mandatory for lot release, once the consistency of the product is approved by National Regulatory Authority.

Bovine serum albumin (for cell culture vaccine). Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.2.14).

Aluminium content (for gel absorbed vaccine) (2.3.9). Not more than 1.25 mg per single human dose.

Ovalbumin (for egg based vaccines). Not more than 1 µg of ovalbumin per human dose, determined by a suitable technique using a suitable reference preparation of ovalbumin.

Residual host-cell DNA (for continuous cell line vaccines) (2.2.15). Should not be greater than 10 ng per single human dose.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Glycoprotein estimation. Determine the Glycoprotein content by using any validated immunochemical method as approved by national regulatory Authority.

Assay

The Standard preparation is the international standard or another suitable preparation, the potency of which has been determined in relation to the International standard. The potency of rabies vaccine is determined by comparing the dose necessary to protect mice against a lethal intracerebral dose of rabies vaccine necessary to provide the same protection. For this comparison, a reference preparation of rabies vaccine, calibrated in international units, and a suitable preparation of rabies virus for use as the challenge preparation are necessary.

The international unit is the activity contained in a stated quantity of the international standard. The equivalence in international units of the international standard is stated by the World Health Organization.

The test described below uses a parallel-line model with at least three points for the vaccine under examination and the reference preparation.

Test animals. Use mice of a suitable strain, drawn from a uniform stock three to four weeks old, weighing between 11 and 15 g. Distribute the mice into six groups of at least 16 mice each and four groups of 10 mice each and must be of the same sex or the sexes must be equally distributed among the groups.

Throughout the test all mice that die before the fifth day after challenge are excluded from the test and all mice that die with signs of rabies between the fifth and fourteenth day after challenge are counted as failing to resist the challenge.

The strain of mice suitable for the test is such that when 0.03 ml containing 5 to 50 LD_{50} of the challenge virus suspension is injected intracerebrally per mouse there is 100 per cent mortality.

Standard challenge virus suspension. A working pool of the challenge virus strain is prepared by injecting intracerebrally 0.03ml of a 10 fold dilution of the CVS strain of rabies virus in 2 per cent v/v sterile inactivated normal horse serum in water for injection or another suitable diluent approved by the competent authority into a suitable number of test animals. The animals when moribund after showing characteristic signs of rabies are sacrificed and their brains harvested aseptically. They are then washed in chilled saline solution to remove blood clots. A 10 per cent suspension of the brains is prepared in a suitable diluent approved by the competent authority and thoroughly homogenised. After centrifuging lightly, the supernatant liquid is distributed into sterile vials and freeze dried. The sealed and freeze-dried supernatant liquid containing vials are stored at -20°. When stored under prescribed conditions the virus titre of the freeze-dried preparation may be expected to be maintained for not less than 3 years. Alternatively, the washed brains are homogenised in a suitable diluent approved by the competent authority to give 10 per cent suspension. It is then centrifuged lightly, distributed into sterile ampoules or sterile plastic vials and sealed. The sealed ampoules or plastic vials can be stored at –60° or below. When stored under prescribed conditions, the virus titre may be expected to be maintained for not less than one year. Storage time needs to be validated by the manufacturer.

Virus titre of the challenge virus. Prepare ten fold serial dilutions of the standard challenge virus suspension. Using the four groups of 10 mice each, inject 0.03 ml of the virus suspension intracerebrally into each mouse, using a different group for each suspension. Observe the mice for 14 days. Calculate the virus titre of the standard challenge virus suspension in LD_{50} per dose of 0.03 ml by standard statistical methods.

Determination of potency of the vaccine. Reconstitute the standard preparation with a suitable diluent. Prepare at least three 5-fold serial dilutions of the solution of the standard preparation and three 5-fold serial dilutions of the vaccine under examination. For both, the standard preparation and the preparation under examination, the serial dilutions should be prepared in such a way that the lowest dilution protects more than 50 per cent of the injected mice. Allocate one dilution to each of the six groups of 16 mice each. Inject intraperitoneally each mouse in each group with dilutions of the vaccine and reference preparation and repeat the injections. After 7 days, prepare identical dilutions of the vaccine and reference preparation and repeat the injections.

After a further 7 days, inject each vaccinated mouse intracerebrally with 0.03 ml of the standard challenge virus

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suspension such that on the basis of preliminary titration, 0.03 ml contains between 5 to 50 LD_{50}. Observe the mice for 14 days and record the number of mice surviving the challenge in each group. Calculate the potency of the preparation under examination by standard statistical methods.

The vaccine complies with the test if the estimated potency is not less than 2.5 IU per single human dose.

The test is not valid unless (a) for both the preparation under examination and the standard preparation, the 50 per cent protective dose lies between the largest and smallest doses given to the mice; (b) there is not deviation from linearity or parallelism of the dose response lines, the confidence limit (P = 0.95) are not less than 25.0 per cent and not more than 400 per cent of the estimated potency; (c) the titre of the challenge virus suspension lies between 5 to 50 LD_{50}.

Labelling. The label states the biological origin of the cells used for the preparation of the vaccine.

Rotavirus Vaccine (Live Attenuated, Oral)

Rotavirus vaccine (live attenuated, oral) is a sterile preparation of one or more virus serotypes, grown in an approved suitable cell substrate and presented in a form suitable for oral administration.

The vaccine is presented as clear liquid or as freeze-dried preparation to be reconstituted immediately before use, as stated on the label, to give clear or slightly turbid liquid. The vaccine ready for administration may be coloured owing to the presence of a pH indicator.

Production

General provision

The vaccine strains and the production method should show consistency in yield comparable with the vaccine of acceptable clinical safety and efficacy in infants. The vaccine formulation should have acid neutralization capacity to avoid inactivation of live viruses by gastric juice, artificial. In case of freeze-dried vaccine, acid neutralizing capacity of the diluent and its stability should be established.

Production of the vaccine is based on a virus seed-lot system. Cell lines are used according to a cell-bank system; unless otherwise justified and authorised, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

If purification steps are present, the reduction of selected process related impurities and residuals such as residual host cell proteins, endotoxins, bovine serum, trypsin and antibiotics is monitored to establish consistency of purification process. If the consistency of production has been validated, the monitoring programme could be conducted based on a reduced frequency of testing, but not on a batch-to-batch basis with the agreement of the national regulatory authority. For residual cellular host cell DNA, quantity of DNA and size distribution of DNA should be evaluated during assessment of manufacturing consistency and process validation.

REFERENCE PREPARATION

A suitable reference preparation that is representative of batches of vaccine shown to be effective in clinical trials is established for use in tests to determine virus concentration. The differences in the composition and characteristics of rotavirus vaccines mean that there will be a specific reference preparation for each vaccine.

Substrate for virus propagation

The virus is propagated in a suitable continuous or diploid cell line or primary cell culture approved by National Regulatory Authority.

VIRUS SEED LOTS

Each strain of rotavirus used should be identified by historical records that include information on the origin of each strain and its subsequent manipulation including the method of attenuation, whether the strain has been biologically cloned prior to generation of the master seed lot, genetic sequence information, the phenotypic and genotypic stability of the master and working seed lots when passaged up to the single harvest level, and the passage level at which attenuation for humans was demonstrated by clinical trials. Virus sees lots are stored at temperature below -20° if freeze-dried, or below -60° if not freeze-dried.

Only a working seed lot that complies with the following requirements may be used for virus propagation.

Identification. Each working seed lot is identified to be of the required rotavirus type by an immunological assay using serotype specific antibodies or by a molecular identity test such as polyacrylamide gel electrophoresis of RNA, RNA/RNA hybridisation, or restriction-enzyme mapping of genetic sequences of polymerase chain reaction (PCR)-amplified VP7 gene segments.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

Virus concentration. The virus concentration of each working seed lot is determined to define the quantity of virus to be used for inoculation of production cell cultures. Direct cell-culture based methods or immune focus assay or nucleic acid

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amplification techniques (NAT) such as PCR quantification of virus replication in cell culture may be used.

Extraneous agents (2.7.3). Each working seed lot complies with the requirements for virus seed lots for viral vaccine.

VIRUS PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells or viruses are being handled. Suitable animal (but not human) serum may be used in the culture media, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production.

For infection with the virus strain, the range of multiplicity of infection, temperature, pH, time period of incubation may vary from strain to strain, a defined range should be established by the manufacturer.

Stored virus intermediate culture

Where a stored virus intermediate culture, prepared from the working seed lot, is used for inoculation, on the day of inoculation not less than 5 per cent or 500 ml of the cell cultures employed, whichever is greater, are set aside as uninfected cell cultures (control cells). Stored virus intermediate cultures are harvested at a time appropriate to the strain of virus and stored at temperatures below -60°.

Only a stored virus intermediate culture that complies with the following requirements may be used for virus propagation.

Identification. Each stored virus intermediate culture is identified by rotavirus type by an immunological assay using serotype specific antibodies or by a molecular identity test such as NAT.

Bacterial and fungal contamination. Each stored virus intermediate culture complies with the test for sterility (2.2.11), using 10 ml for each medium.

Mycoplasma (2.7.4).Complies with the test for absence of mycoplasma.

Virus concentration. The virus concentration of each stored virus intermediate culture is determined by titration of infectious virus in cell cultures as prescribed under Assay to monitor consistency of production. Direct cell-culture based methods or immune focus assay or NAT such as PCR quantification of virus replication in cell culture may be used.

Extraneous agents (2.7.3). Each stored virus intermediate culture complies with the tests for extraneous agents.

Control cells (2.7.3). The control cells of the production cell culture from which each stored virus intermediate culture is derived, comply with a test for identity and with the requirements for extraneous agents and sterility.

MONOVALENT VIRUS POOL

On the day of inoculation with the virus working seed lot or stored virus intermediate culture, the cell suspension or cell monolayer used to prepare cell culture for growing of rotavirus, an amount of processed cell suspension equivalent to at least 5 per cent or 500 ml of cell suspension, whichever is greater, are set aside as uninfected cell cultures (control cells). If bioreactor technology is used, the size and handling of the cell sample to be examined is approved by the regulatory authority.

A monovalent virus pool may be the result of single harvest or pool of more than one single harvest from multiple numbers of roller bottles, tissue culture flasks, cell factories or bioreactors. Samples of monovalent virus pools should be taken for testing and stored at –20° or below.

Monovalent virus pool that complies with the following requirements may be used for further processing.

Control cells (2.7.3). The control cells of the production cell culture from which each single harvest is derived comply with a test for identity and with the requirements for extraneous agents.

Identification. Each single harvest or monovalent pooled harvest is identified by rotavirus type by an immunological assay using serotype specific antibodies or by a molecular identity test such as Nucleic acid amplification test.

Bacterial and fungal contamination. Monovalent virus pool complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

Mycoplasma contamination (2.7.4). The stored virus intermediate culture complies with the test for mycoplasmas, carried out using 10 ml.

Virus concentration.The virus concentration of monovalent virus pool is determined by titration of infectious virus in cell cultures as prescribed under Assay to monitor consistency of production. Direct cell-culture based methods or immune focus assay or NAT such as PCR quantification of virus replication in cell culture may be used.

Extraneous agents (2.7.3). Monovalent virus pool complies with the tests for extraneous agents.

CLARIFIED MONOVALENT VIRUS POOL (MONOVALENT BULK)

The monovalent virus pool may be clarified or filtered to remove cell debris and may be further purified. Clarified

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monovalent virus pool may be stabilized using appropriate stabilizer or it may be un-stabilized and may be stored at suitable conditions.

Only a clarified monovalent virus pool, stabilized or un-stabilized, that complies with the following requirements may be used in the next step of vaccine preparation.

Bacterial and fungal contamination. The clarified monovalent virus pool complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

Virus concentration. The virus concentration of the clarified monovalent virus pool is determined by titration of infectious virus in cell cultures as prescribed under Assay to monitor consistency of production. Direct cell-culture based methods or immune focus assay or NAT such as PCR quantification of virus replication in cell culture may be used.

Residual cellular DNA. Not more than 100 µg of cellular DNA per human dose for viruses grown in continuous cells lines.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more satisfactory stabilized or unstabilized bulk based on virus concentration of bulk and vaccine potency. Final bulk vaccine may contain one or more than one virus serotype. Suitable stabilizers or diluents may be added.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Bacterial and fungal contamination. The final bulk vaccine complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile containers and may be freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to avoid contamination and the introduction of moisture.

An approved minimum virus concentration for release of the product is established for each virus type to ensure, in light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity. The manufacturers should provide sufficient data to the national regulatory authority on the stability of the product under appropriate conditions of storage and shipping.

For freeze-dried vaccine an expiry date should be established for the diluents based upon stability data. If an antacid is to be used, the stability of the rotavirus in the presence of the antacid should be confirmed.

Only a final lot that complies with the following requirement is satisfactory. For freeze-dried vaccine, both final lot and its diluent, as applicable, should be tested and should fulfill the following requirement.

Visual inspection of final containers. Each container of final lot should be inspected visually and containers showing abnormalities should be rejected.

Appearance. The physical appearance of the freeze-dried or liquid vaccine conforms with respect to its form and colour as defined by the manufacturer and approved by the national regulatory authority. For freeze-dried vaccines, a visual inspection should be performed with the freeze-dried vaccine, its diluent and the reconstituted vaccine.

Identity. The vaccine is shown to contain rotavirus of each type stated on the label by an immunological assay using specific antibodies or by a molecular identity test. If PCR is used for the assay, this may serve as the identity test.

Bacterial and fungal contamination. The liquid or reconstituted vaccine complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

pH (2.4.24). The pH of the final lot tested in a pool of final containers meets appropriate limit set by the manufacturer to guarantee virus stability. In case of freeze-dried vaccines, pH should be measured after reconstitution of the vaccine with the diluent.

Water. Not more than 3.0 per cent for each final lot of freeze-dried vaccine.

Virus concentration. As described under Assay

Assay

Rotavirus vaccine potency should be assayed for infectivity in a sensitive assay in a cell culture system. An immunofocus or plaque forming assay may be used in MA-104, Vero or other sensitive cells to determine virus concentration. The assay is based on the visualization of infected areas (plaques or focus of infection) of a cell monolayer directly or by probing with rotavirus-specific antibodies. Results should be recorded as focus-forming units (FFU/ml) or plaque-forming units (PFU/ml).

The assay of rotavirus vaccine is carried out by inoculation of suitable cell cultures with dilutions of the vaccine and evaluation of the rotavirus concentration, either by visualisation of infected areas of a cell monolayer or by comparison of the capacity of the vaccine to produce viral RNA following infection of cells with the corresponding capacity of an approved reference preparation.

For the assay based on visualisation of infected areas of a cell monolayer. Titrate the vaccine for infective virus using at least 3 separate containers. Titrate the contents of 1 container of an appropriate virus reference preparation in triplicate to validate each assay. If the vaccine contains more than 1

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rotavirus type, titrate each type separately using a method of suitable specificity. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory.

Calculate the individual virus concentration for each container of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods.

The assay is not valid if:

— the confidence interval (P=0.95) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ±0.3 log $CCID_{50}$ (or an equivalent value expressed with a unit suitable for the method used for the assay);

— the virus concentration of the reference preparation differs by more than 0.5 log $CCID_{50}$ (or an equivalent value expressed with a unit suitable for the method used for the assay) from the established value.

The assay is repeated if the confidence interval (P=0.95) of the combined virus concentration of the vaccine is greater than ±0.3 log $CCID_{50}$ (or an equivalent value expressed with a unit suitable for the method used for the assay); data generated from valid assays only are combined by the usual statistical method to calculate the virus concentration of the sample. The confidence interval (P = 0.95) of the combined virus concentration is not greater than ± 0.3 log $CCID_{50}$ (or an equivalent value expressed with a unit suitable for the method used for the assay).

Where justified and authorized by the local national regulatory authority, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

For the assay based on comparison of the capacity of the vaccine to produce viral RNA. Following infection of cells with the corresponding capacity of an approved reference preparation, a suitable number of cell cultures in a microtitre plate are infected in parallel with serial dilutions of the vaccine and the reference preparation. After incubation to allow virus replication, viral RNA in the individual wells is released from the cells and quantified by NAT such as real-time quantitative reverse-transcriptase polymerase chain reaction (qPCR) technology.

Not less than 3 separate containers of the vaccine are assayed against a container of the reference preparation titrated in triplicate.

Calculate the individual virus concentration for each container of vaccine against the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods.

The combined estimate of the virus concentration for the 3 containers of vaccine is not less than that stated on the label.

The assay is not valid unless:

— the negative external NAT control is unambiguously negative;

— the positive external NAT control is unambiguously positive;

— the negative matrix control (uninfected cells) is unambiguously negative;

— the positive matrix control (cells spiked with viral RNA) is unambiguously positive;

— the statistical analysis shows a significant slope and no significant deviations from linearity or parallelism of the dose-response curves.

The assay is repeated if the confidence interval (P=0.95) of the combined virus concentration of the vaccine is greater than ±0.3 log infectious units; data generated from valid assays only are combined by the usual statistical methods to calculate the virus concentration of the sample. The confidence interval (P = 0.95) of the combined virus concentration is not greater than ± 0.3 log infectious units.

Thermal Stability. Maintain not less than 3 containers of the final lot at an elevated temperature for a defined time period, using conditions found suitable for the particular product as approved by the local national regulatory authority. Determine the virus concentration as described under assay in parallel for the heated vaccine and for vaccine maintained at the temperature recommended for storage. The virus concentration of the containers that have been heated does not decrease by more than an approved amount during the period of exposure. For a multivalent vaccine, if there is no significant difference in the virus loss between serotypes, the loss may be determined from total virus concentration.

Labelling. The label states (1) the type or types of rotavirus contained in the vaccine; (2) the nominal amount of each type of virus contained in 1 single human dose; (3) the cell substrate used for the preparation of the vaccine; (4) the time within which the vaccine must be used after reconstitution (for freeze-dried); (6) expiry date; (7) the vaccine is for oral administration, only.

Rubella Vaccine (Live)

Rubella Vaccine (Live) is a freeze-dried preparation of a suitable attenuated strain of rubella virus. The vaccine is reconstituted immediately before use to give a clear liquid or may be coloured owing to the presence of a pH indicator.

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Production

General provisions

The production of vaccine is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to yield consistently live rubella vaccines of adequate immunogenicity and safety in man. Unless otherwise justified and authorised, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy.

Substrate for virus propagation

The virus is propagated in human diploid cells (2.7.2).

SEED LOT

The strain of rubella virus used in the production of rubella vaccine shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

To avoid unnecessary use of monkeys in the test for neurovirulence virus seed lots are prepared in large quantities and stored at temperatures below -20° if freeze-dried, or below -60° if not freeze-dried.

Only a seed lot that complies with the following tests may be used for virus propagation.

Identification

The master and working seed lots are identified as rubella virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The virus concentration of the master and working seed lots is determined to ensure consistency of production.

Extraneous agents (2.7.3). The working seed lot complies with the tests for seed lots.

Neurovirulence (2.7.5). The master/working seed lot complies with the test for neurovirulence of live virus vaccines. *Macaca* and *Cercopithecus* monkeys are suitable for the test.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled. Suitable animal (but not human) serum may be used in the growth medium, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. Not less than 500 ml of the production cell culture is set aside as uninfected cell culture (control cells). The temperature of incubation is controlled during the growth of the virus. The virus suspension is harvested, on one or more occasions, within 28 days of inoculation. Multiple harvests from the same production cell culture may be pooled and considered as a single harvest.

Only a single harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification

The single harvest contains virus that is identified as rubella virus by serum neutralization in cell culture, using specific antibodies.

Virus concentration. The virus concentration in the single harvest is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

Extraneous agents (2.7.3). The single harvest or virus pool complies with the test for extraneous agents.

Control cells. The control cells comply with a test for identification and with the tests for extraneous agents (2.7.3).

Mycoplasma (2.7.4).Complies with the test for absence of mycoplasma.

FINAL BULK VACCINE

Single harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabilizer may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

A minimum virus concentration for release of the product is established such as to ensure, in the light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the tests for minimum virus concentration for release, with the following requirement for

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thermal stability and with each of the requirements given below under Identification and Tests may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Thermal stability. Maintain samples of the final lot of freeze-dried vaccine in the dry state at 37 ± 1° for 7 days. Determine the virus concentration as described under Assay in parallel for the vaccine held at 37 ± 1° for 7 days and for vaccine stored at 2° to 8°. The virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

Identification

When the vaccine reconstituted as stated on the label is mixed with specific rubella antibodies, it is no longer able to infect susceptible cell cultures.

Tests

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.2.11).

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

Water (2.3.43). Not more than 3.0 per cent, determined by Karl Fischer, semi-micro determination of water or by any suitable validated method.

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.2.14).

Assay

Titrate the vaccine for infective virus at least in triplicate, using at least five cell cultures for each 0.5 $\log_{10}$ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated rubella virus concentration is not less than that stated on the label; the minimum rubella virus concentration stated on the label are not less than 1×10^3 $CCID_{50}$ per single human dose or 3.0 $LOG_{10}CCID_{50}$ per single human dose. The assay is not valid if the confidence limits (P = 0.95) of the logarithm of the virus concentration are greater than ± 0.3.

Rubella vaccine (Live) RS is suitable for use as a reference preparation.

Labelling. The label states (1) the strain of virus used for the preparation of the vaccine; (2) the type and origin of the cells used for the preparation of the vaccine; (3) the minimum virus concentration; (4) the time within which the vaccine must be used after reconstitution; (5) that the vaccine must not be given to a pregnant woman and that a woman must not become pregnant within two months.

Scorpion Venom Antiserum

Anti Scorpion Venom Serum; Scorpion Antivenin

Scorpion Venom Antiserum is a sterile preparation containing antitoxic globulins or their derivatives that have the power of specifically neutralizing the venom of one or more species of scorpion. It is obtained from the serum or plasma of healthy equines or other suitable animals immunized against venoms of specified species of scorpion. It is a liquid preparation or a freeze dried product. It may contain a suitable preservative.

Description. A clear to slightly opalescent colorless or pale yellow liquid free from suspended particles or cream colored powder or pellet which when reconstituted with the diluent supplied by the manufacturer with freeze-dried product yields a clear, colorless or pale yellow liquid.

Identification

Specifically renders the corresponding venom or venoms harmless to susceptible animals. It may also be identified or by any other alternate suitable *in vitro* method.

Tests

Other tests. Complies with the tests stated under Antisera.

Assay

Potency. Each milliliter of the preparation under examination contains sufficient antivenom globulin to neutralize not less than 1 mg of *Hottentotta tumulus* (Red scorpion) venom.

Preparation of 1 per cent venom. Allow the vials containing lyophilized venoms to reach room temperature in order to avoid condensation of water which may cause errors in weighing. Prepare 10 mg per ml of the venom solutions separately in *saline solution, sterile*. Shake the solutions very gently to avoid foaming and centrifuge at 20,000 g in cold conditions for 10 minutes. Decant the supernatant slowly and sterilize using 0.22 µm cellulose nitrate membrane. Store the filtered venom solutions in pre-sterilized glass vials.

LD50 of venom. The quantity of venom, when injected intravenously, causes death of 100 per cent of animal population in a group after 24 hours.

Take graded quantities of the 1 per cent venom solution insuch a manner that the middle dilution contains the doseexpected to be the LD50 in sufficient quantity of salinesolution, sterile to make volume up to 5 ml. Inject a dose of 0.5ml intravenously, of each dilution into at least 5 mice, eachweighing 18-20 g, per dilution. Observe the mice for 24 hours and calculate LD50 using suitable statistical method.

Suitable venom. Due to limitations to prepare a reference standard for scorpion venoms, considering their individual seasonal and geographical variations observed, suitable

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venom should be prepared by adjusting the venom to a specific LD50 value before using the same for the potency testing of antiserum. Scorpion venom should be adjusted to 50 µg as one LD50. Adjust the LD50 of Scorpion venon from reconstituted (1.0 per cent) venon to desired LD50.

Determination of potency of the anti scorpion venom serum. The potency of the scorpion venom antiserum is determined by estimating the ability of the antiserum to protect mice or other suitable animals against the lethal effect of a fixed dose of suitable venom of the relevant species of the venom.

Prepare challenge dose of venom, in such a way that 1.0 ml contains the specified amount of venom to be neutralized, from 0.2 per cent stock solution of venom. Prepare appropriately diluted serum such that 1.0 ml neutralizes the specified amount of the venom. Mix equal quantities of venom and antiserum dilution and incubate at 37° for 30 minutes. Inject a dose of 0.5 ml intravenously, of each dilution into at least 2 mice, each weighing 18-20 g. Observe for survival of mice after 48 hours. Inject one MLD and negative control.

Storage. Store the freeze-dried preparation in a cool, dark place. Store the liquid preparation at a temperature of 2° to 8° and do not expose to light. Do not allow to freeze.

Labeling. The label states (1) the volume of contents and in case of a freeze-dried preparation, the directions for reconstitutions; (2) the species of scorpion against whose venom the antiserum is effective; (3) the name of the animal species from which the antiserum has been obtained; (4) the name and proportion of any preservative added; (5) that in case of a liquid preparation, it should not be allowed to freeze.

Snake Venom Antiserum

Snake Antivenin; Snake Antivenom Serum; Snake Venom Antitoxin

Each milliliter of snake venom antiserum contains sufficient antitoxic globulins to neutralize not less than 0.6 mg of Indian cobra (*Naja naja*) and Russell's viper (*vipera russelli*) suitable venoms and not less than 0.45 mg of common krait (*Bangarus caeruleus*) and Saw scaled viper (*Echis carinatus*) suitable vnoms. It may be obtained from the serum or plasma of healthy equines or other suitable animals immunised against venoms of specific species usually elapine and viperine. It may be a purified liquid preparation or a freeze-dried product. It may contain a suitable preservative.

Description. A clear to slightly opalescent, colourless or pale yellow liquid, free from suspended particles or cream-coloured powder or pellet which when reconstituted with the diluent supplied by the manufacturer with the freeze-dried product yields a clear, colourless or pale yellow liquid.

Identification

Specifically renders the corresponding venom or venoms harmless to susceptible animals. It may also be identified by any other alternate suitable *in vitro* method.

Tests

Potency. In view of the numerous toxic fractions in venoms and varying requirements of different localities, no standard for potency is prescribed.

The potency of the snake venom antiserum is determined by estimating the ability of the antiserum to protect mice or other suitable animals against the lethal effect of a fixed dose of a reference preparation of snake venom of the relevant species or by comparing its ability to do so with that of a reference preparation of antiserum of established potency at two or more dose levels of the venom.

Other tests. Complies with the tests stated under Antisera.

Storage. Store the freeze-dried preparation in a cool, dark place and avoid exposure to excessive heat. Store the liquid preparation at a temperature between 2° and 8°. It should not be allowed to freeze.

Labelling. The label states (1) the volume of the contents and in case of freeze-dried preparation, the directions for reconstitution; (2) the species of snake against whose venom the antiserum is effective; (3) the animal species from which the antiserum has been obtained; (4) the name and proportion of any preservative added; (5) that in case of a liquid preparation, it should not be allowed to freeze.

Tetanus Antitoxin

Tetanus Antitoxin is a preparation containing the specific antitoxic globulins or their derivatives obtained by purification of hyperimmune serum of horses or other suitable animals and have the specific activity of neutralising the toxin formed by *Clostridium tetani*. The liquid preparation may contain a suitable antimicrobial preservative.

Tetanus Antitoxin has a potency of not less than 1000 Units per ml when intended for prophylactic use and not less than 3000 Units per ml when intended for therapeutic use.

Description. A clear, colourless or pale yellow liquid or a freeze-dried, cream-coloured powder or pellet.

Identification

Specifically neutralises and renders the toxin formed by *C. tetani* harmless to susceptible animals or by any other suitable *in vitro* test.

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Tests

Other tests. Complies with the tests stated under Antisera.

Potency. Carry out the biological assay of tetanus antitoxin described below.

Biological Assay of Tetanus Antitoxin

The potency of tetanus antitoxin is determined by comparing the dose necessary to protect mice against the paralytic effects of a fixed dose of tetanus toxin with the dose of the Standard Preparation of tetanus antitoxin necessary to give the same protection. For this purpose the Standard Preparation of tetanus antitoxin and a suitable preparation of toxin, for use as a test toxin, are required. The test dose of the toxin is determined in relation to the Standard Preparation and the potency of the preparation under examination is then determined in relation to the Standard Preparation using the test toxin.

Standard Preparation

The Standard Preparation is the 2nd International Standard for Tetanus antitoxin, equine, established in 1969, consisting of freeze-dried hyperimmune horse serum (supplied in ampoules containing 1400 Units), or another suitable preparation the potency of which has been determined in relation to the International Standard.

Suggested Method

NOTE — The severity of the tetanic paralysis to be regarded as the end-point is such that the paralysis is readily recognised but not sufficiently extensive to cause significant suffering. For humane reasons the animals should be examined at least twice a day and should be killed as soon as the end-point is reached.

Test animals. Use healthy mice, weighing between 17 to 22 g, from the same stock.

Preparation of test toxin. Prepare tetanus toxin from a sterile filtrate of an 8 to 10 day culture of *C. tetani*. Test toxin may be prepared by adding this filtrate to *glycerin* in the proportion of 1 volume of the filtrate to 1 or 2 volumes of *glycerin*. This solution of test toxin is stored below 0°. Test toxin may also be prepared in stable form by saturating the filtrate with *ammonium sulphate*, collecting the resulting precipitate, drying it over *phosphorus pentoxide* at a pressure of 1.5 to 2.5 kPa and reducing it to a fine powder. The powder so obtained is preserved in the dry condition at a low temperature either in sealed ampoules or *over phosphorus pentoxide* at a pressure of 1.5 to 2.5 kPa.

Determination of test dose of toxin *(Lp/10 dose)*. First determine the Limes paralyticum/10 (Lp/10) dose of the test toxin. This is the smallest quantity of the toxin which when mixed with 0.1 Unit of the Standard Preparation and injected subcutaneously into mice causes tetanic paralysis within 4 days. Prepare a solution of the Standard Preparation in a suitable liquid such that 1 ml contains 0.5 Unit. Accurately measure or weigh a quantity of the test toxin and dilute it with, or dissolve it in, a suitable liquid. Prepare mixtures such that each contains 2.0 ml of the solution of the Standard Preparation (1 Unit), one of a series of graded volumes of the solution of the toxin, and sufficient of a suitable liquid to give a final volume of 5.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 60 minutes and then inject 0.5 ml of each mixture subcutaneously into mice, six mice being used for each mixture, and observe the mice for 4 days.

The test (Lp/10) dose of the toxin is the amount present in 0.5 ml of that mixture that contains the smallest amount of toxin sufficient to cause tetanic paralysis in all six mice injected with it within 4 days.

Determination of potency of the antitoxin. Prepare a solution of the Standard Preparation in a suitable liquid such that it contains 0.5 Unit per ml. Accurately measure or weigh a quantity of the test toxin and dilute it with, or dissolve it in, a suitable liquid so that 1.0 ml contains five times the Lp/10 dose as previously determined. Prepare mixtures such that each contains 2.0 ml of the solution of the test toxin and one of a series of graded volumes of the preparation under examination, and sufficient of a suitable liquid to give a final volume of 5.0 ml. Prepare similar mixtures containing 2.0 ml of the test toxin and one of a series of graded volumes of the solution of the Standard Preparation centred on that volume (2.0 ml) that contains 1 Unit. Allow the mixtures to stand at room temperature, protected from light, for 60 minutes and then inoculate 0.5 ml of each mixture subcutaneously into each mouse, six mice being used for each mixture, and observe the mice for 4 days. The mixture that contains the largest volume of the preparation under examination that fails to protect the mice from paralysis contains 1 Unit. The test is not valid unless all the mice injected with mixtures containing 2.0 ml or less of the solution of the Standard Preparation show paralysis and all those injected with more do not. Calculate the potency of the preparation under examination in Units per ml.

Storage. As stated under Antisera.

Labelling. The label states (1) the number of Units per ml; (2) species of animal from which the preparation has been made; (3) whether the preparation is for prophylactic or for therapeutic use; (4) the recommended dose; (5) the name and proportion of any added preservative; (6) that the preparation, if liquid, should not be allowed to freeze; (7) that the preparation, if dried, should be used immediately after reconstitution in the stated quantity of the diluent supplied by the manufacturer.

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Tetanus Vaccine (Adsorbed)

Tetanus Vaccine (Adsorbed) is a preparation of tetanus formol toxoid adsorbed on mineral carrier. The formol toxoid is prepared from the toxin produced by the growth of *Clostridium tetani*.

Production

General provisions

The maximum number of Lf per single human dose of tetanus vaccine is 25. The production method is validated to demonstrate that the product, if tested, would comply with the following specific toxicity test.

Specific toxicity. Use five normal, healthy guinea pigs weighing between 250 g and 350 g which have been maintained for at least one week on a uniform, unrestricted diet, have not lost weight during this period and have not been previously treated with any material that will interfere with the test. Weigh the animals separately and record their weights. Inject subcutaneously into each animal five times the single human dose stated on the label. Weigh all the animals at weekly intervals for 3 weeks. The vaccine passes the test if none of the animals shows any symptoms of tetanus toxaemia or dies from tetanus within 21 days or loses weight at the end of the test. If more than one animal dies from non-specific causes or loses weight repeat the test. If an animal dies or loses weight in the second test, the vaccine fails the test.

BULK PURIFIED TOXOID

For the production of tetanus toxin, from which toxoid is prepared, seed cultures are managed in a defined seed-lot system in which toxinogenicity is conserved and, where necessary, restored by deliberate reselection. A highly toxinogenic strain of *C. tetani* with known origin and history is grown in a suitable liquid medium. At the end of cultivation, the purity of each culture is tested and contaminated cultures are discarded. Toxin-containing culture medium is collected aseptically. The toxin content (Lf per ml) is checked to monitor consistency of production. Single harvests may be pooled to prepare the bulk purified toxoid. The toxin is purified to remove components likely to cause adverse reactions in humans. The purified toxin is detoxified with formaldehyde by a method that avoids destruction of the immunogenicity of the toxoid and reversion of toxoid to toxin, particularly on exposure to heat. Alternatively, purification may be carried out after detoxification.

Only bulk purified toxoid that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

Absence of tetanus toxin. Inject subcutaneously at least 500 Lf of purified toxoid in a volume of 1 ml into each of five healthy guinea pigs, each weighing 250 to 350 g, that have not previously been treated with any material that will interfere with the test. The bulk purified toxoid passes the test if none of the animals shows any symptoms of tetanus toxaemia or dies from tetanus within 21 days or loses weight at the end of the test. If more than one animal dies from non-specific causes or loses weight repeat the test. If an animal dies or loses weight in the second test the toxoid fails the test.

Irreversibility of toxoid. Using the buffer for the final vaccine (without adjuvant), prepare a dilution of the bulk purified toxoid containing the same toxoid concentration as the final vaccine. Divide the dilution into two equal parts. Keep one of them at 2° to 8° and the other at 34° to 37° for 6 weeks. Test both dilutions by injecting 5 ml of incubated samples subcutaneously to 5 guinea pigs weighing 250 g to 350 g that have not previously treated with any material that will interfere with the test and observe the animals for 21 days. The toxoid complies with the test if neither sample produces any sign of a toxic reaction attributable to tetanus toxin.

Antigenic purity. Not less than 1,000 Lf per mg of protein nitrogen.

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption of a suitable quantity of bulk purified toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Specific toxicity. Use five normal, healthy guinea-pigs weighing 250 to 350 g which have been maintained for at least one week on a uniform, unrestricted diet, have not lost weight during this period and have not been previously treated with any material that will interfere with the test. Weigh the animals separately and record their weights. Inject subcutaneously into each animal five times the single human dose stated on the label. Weigh all the animals at weekly intervals for 3 weeks. The vaccine passes the test if none of the animals shows any symptoms of tetanus toxaemia or dies from tetanus within 21 days or loses weight at the end of the test. If more than one animal dies from non-specific causes or loses weight repeat the test. If an animal dies or loses weight in the second test, the vaccine fails the test.

The specific toxicity test on the final bulk may be omitted for routine lot release once consistency of production has been

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established to the satisfaction of the National Regulatory Authority.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

pH (2.4.24). 6.0 to 7.0.

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for antimicrobial preservative, free formaldehyde and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Identification

Tetanus toxoid is identified by a suitable immunochemical method (2.2.14). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine under examination sufficient *sodium citrate* to give a 10 per cent solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable tetanus antitoxin, giving a precipitate.

Tests

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if *aluminium hydroxide* or *hydrated aluminium phosphate* is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the quantity stated on the label.

Sterility (2.2.11). Complies with the test for sterility.

Potency of tetanus component

Assay. Determine by either of the following methods.

(1) Inject subcutaneously on each of two occasions separated by an interval of not more than 4 weeks, one-tenth of the stated human dose diluted to 1 ml with *saline solution* into each of 9 normal, healthy guinea pigs weighing between 250 and 350 g. Not more than 2 weeks after the second injection, collect the serum from each animal and carry out the biological test for tetanus antitoxin, described under Tetanus antitoxin or any other method approved by National Regulatory Authority.

Sera of at least 6 guinea pigs out of 9 should contain not less than 0.5 Unit of tetanus antitoxin per ml.

(2) Carry out the biological assay of adsorbed Tetanus Vaccine (Adsorbed) described below.

After establishing consistency/stability of product by any of the following multiple dilution assay, a single dilution assay based on serological methods or other principles may be used. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 40 IU (i.e. of the minimum potency of a single human dose), that elicits a minimal protective effect, and comparing its effect with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine ($P < 0.05$), the potency of the test vaccine is satisfactory. Single dilution assay is a qualitative approach and provides an assurance that the lower limit of the estimated potency is in excess of the minimum requirement. It does not give information on the dose response curves and their linearity, parallelism and significant slope. Where a single dilution is used, production and test consistency over time are monitored via suitable indicators and by carrying out a full multiple-dilution assay periodically as approved by National Regulatory Authority.

The lower 95 per cent confidence limit of the estimated potency should be not less than 40 IU per single human dose. Because the minimum potency requirement now applies to lower confidence limit of 95 per cent interval, there is no requirement to achieve a 95 per cent confidence interval narrower than 50-200 per cent.

Biological Assay of Adsorbed Tetanus Vaccine

The potency of adsorbed tetanus vaccine is determined by comparing the dose of the vaccine required to protect guinea-pigs or mice from the paralytic effects or death of a subcutaneous injection of tetanus toxin with the dose of the Standard preparation needed to give the same protection. For this comparison, the Standard preparation of adsorbed tetanus toxoid and a suitable preparation of tetanus toxin, for use as a challenge toxin, are necessary.

The lower limit of the 95.0 per cent confidence interval of estimated potency is not less than 40 IU per single human dose. Confidence intervals greater than 50 to 200 per cent is acceptable provided that the lower limit of the 95.0 per cent confidence interval of the estimated potency is still above the minimum potency requirement for a single human dose.

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Standard preparation

The Standard preparation is International standard for Tetanus toxoid, adsorbed or another suitable preparation, the potency of which has been determined in relation to the International standard.

Suggested method

The paralysis method is not obligatory and the lethal method may be used. For this method the number of animals and the procedure are identical with those described for the paralysis method but the end-point is the death of the animal rather than the onset of paralysis and the LD_{50} dose is used instead of the Lp_{50} dose. Either of the methods once standardized can be used for determination of potency of tetanus component.

(A) ***Test on guinea pigs***

Test animals. Use healthy guinea-pigs from the same stock and weighing 250 to 350 g. Distribute them into six groups of not fewer than sixteen each. The guinea-pigs should all be of the same sex or the males and females should be distributed equally between the groups. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardised, include four further groups of five guinea pigs to serve as unvaccinated controls.

Challenge toxin. Select a preparation of tetanus toxin containing not less than 50 times the 50 per cent paralytic dose per ml or between 50 to 200 LD_{50} of toxin dose in case of lethal challenge assay. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the paralytic dose or lethal dose for every assay.

Preparation of the challenge toxin solution. Immediately prior to use, prepare from the challenge toxin by dilution with *phosphate buffered saline pH 7.4* or *1 per cent peptone water* a challenge toxin solution containing fifty times the 50 per cent paralytic dose per ml or between 50 to 200 LD_{50} of toxin dose. If necessary, dilute portions of this challenge toxin solution 16, 50 and 160 fold with the same buffer solution.

Determination of potency. Prepare in *saline solution* three dilutions of the vaccine under examination and three dilutions of a solution of the Standard preparation such that, for each, the dilutions form a series differing by not more than 2.5 fold steps and in which the dilutions of intermediate concentration, when injected subcutaneously in 1 ml volumes into guinea-pigs, protect approximately 50 per cent of the animals from the paralytic effects of the subcutaneous injection of the quantity of tetanus toxin prescribed for this test. Allocate the six dilutions one to each of the six groups of sixteen guinea pigs and inject subcutaneously 1.0 ml of each dilution into each guinea pig in the group to which that dilution is allocated. After 28 days inject each animal subcutaneously with 1.0 ml of the *challenge toxin solution* containing fifty times the 50 per cent paralytic dose or between 50 to 200 LD_{50} of toxin dose in case of lethal challenge assay. If necessary, allocate the challenge toxin solution and the three dilutions made from it one to each of the four groups of five guinea pigs and inject subcutaneously 1.0 ml of each toxin solution into each guinea-pig in the group to which that toxin solution is allocated. Examine the guinea pigs twice daily, remove and euthanized all animals showing definite signs of tetanus paralysis. Count the number of guinea pigs without paralysis 5 days after injection of the challenge toxin and calculate the potency of the vaccine under examination relative to the potency of the Standard preparation on the basis of the number of animals without paralysis in each of the six groups of sixteen, using standard statistical methods.

Reading and interpretation of results. In order to minimise suffering in the test animals, it is recommended to note the degree of paralysis on a scale such as that shown below. The scale gives typical signs when injection of the challenge toxin is made in the dorsal region, close to one of the hind legs. Grade T3 is taken as the end-point, but with experience grade T2 can be used instead. Tetanus toxin produces in the toxin-injected hind leg paresis followed by paralysis that can be recognized at an early stage. The tetanus grades in mice are characterized by the following signs:

- T1: slight stiffness of 1 forelimb, but difficult to observe;
- T2: Paresis of 1 forelimb, which still can function;
- T3: Paralysis of 1 forelimb. The animal moves reluctantly, the body is often slightly banana shaped owing to scoliosis;
- T4: the forelimb is completely stiff and the toes are immovable. The muscular contraction of the forelimb is very pronounced and usually scoliosis is observed.
- T5: tetanus seizures, continuous tonic spasm of muscles;
- D: death.

The test is not valid unless (a) for both the vaccine under examination and the Standard preparation, the 50 per cent protective doses lie between the largest and smallest doses of the preparations given to the guinea pigs; (b) if applicable, the number of paralysed animals among the four groups of five injected with the challenge toxin solution and its dilutions indicate that the challenge was approximately 50 times the 50 per cent paralytic dose or between 50 to 200 LD_{50} of toxin dose in case of lethal challenge assay; (c) the fiducial limits of assay lie between 50.0 per cent and 200.0 per cent of the estimated potency; (d) the statistical analysis shows no deviations from linearity or parallelism. The test may be repeated any number of times but when more than one test is performed the results of all valid tests must be combined in the estimate of potency.

(B) ***Test on mice***

Test animals. Use healthy mice from the same stock, weighing between 14 and 20 g. Distribute them into six groups of sixteen

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each. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardised, include four further groups of six mice to serve as unvaccinated controls. The mice should all be of the same sex or the males and females should be distributed equally among the groups.

Challenge toxin. Select a preparation of tetanus toxin containing not less than 100 times the 50 per cent paralytic dose per ml or between 50 to 200 LD_{50} of toxin dose in case of lethal challenge assay.

Preparation of the challenge toxin solutions. Immediately prior to use prepare from the challenge toxin by dilution with *phosphate buffered saline pH 7.4* or *1 per cent peptone water* a challenge toxin solution containing fifty times the 50 per cent paralytic dose in each 0.5 ml or between 50 to 200 LD_{50} of toxin dose in case of lethal challenge assay. If necessary, dilute portions of this challenge toxin solution 16, 50 and 160 fold with the same buffer solution.

Determination of potency. Prepare in *saline solution* three dilutions of the vaccine under examination and three dilutions of a solution of the Standard preparation such that, for each, the dilutions form a series differing by not more than 2.5 fold steps and in which the dilutions of intermediate concentration, when injected subcutaneously in 0.5 ml volumes into mice, protect approximately 50 per cent of the animals from the paralytic effects of the subcutaneous injection of the quantity of tetanus toxin prescribed for this test. Allocate the six dilutions one to each of the six groups of sixteen mice and inject subcutaneously 0.5 ml of each dilution into each mouse in the group to which the dilution is allocated. After 28 days inject each animal subcutaneously with 0.5 ml of the *challenge toxin solution* containing fifty times the 50 per cent paralytic dose or between 50 to 200 LD_{50} of toxin dose in case of lethal challenge assay. If necessary, allocate the challenge toxin solution and the three dilutions made from it one to each of the four groups of six mice and inject subcutaneously 0.5 ml of each *toxin solution* into each mouse in the group to which that toxin solution is allocated. Count the number of mice without paralysis 4 days after injection of the challenge toxin and calculate the potency of the vaccine under examination relative to the potency of the Standard preparation on the basis of the numbers of animals without paralysis in each of the six groups of sixteen, using standard statistical methods.

Reading and interpretation of results. In order to minimise suffering in the test animals, it is recommended to note the degree of paralysis on a scale such as that shown below. The scale gives typical signs when injection of the challenge toxin is made in the dorsal region, close to one of the hind legs. Grade T3 is taken as the end-point, but with experience grade T2 can be used instead. Tetanus toxin produces in the toxin-injected hind leg paresis followed by paralysis that can be recognized at an early stage. The tetanus grades in mice are characterized by the following signs:

- T1: slight stiffness of toxin-injected hind leg, only observed when the mouse is lifted by the tail;
- T2: Paresis of the toxin-injected hind leg, which still can function for walking;
- T3: Paralysis of the toxin-injected hind leg, which does not function for walking;
- T4: the toxin-injected hind leg is completely stiff with immovable toes;
- T5: tetanus seizures, continuous tonic spasm of muscles;
- D: death.

The test is not valid unless (a) for both the vaccine under examination and the Standard preparation, the 50 per cent protective doses lie between the largest and smallest doses of the preparations given to the mice; (b) if applicable, the number of paralysed animals among the four groups of six injected with the challenge toxin solution and its dilutions indicate that the challenge was approximately 50 times the 50 per cent paralytic dose or between 50 to 200 LD_{50} of toxin dose in case of lethal challenge assay; (c) the fiducial limits of the assay lie between 50.0 per cent and 200.0 per cent of the estimated potency; (d) the statistical analysis shows no deviation from linearity or parallelism. The test may be repeated any number of times but when more than one test is performed the results of all valid tests must be combined in the estimate of potency.

(C) *Determination of antibodies in guinea pigs*

Test animals. Use in the test healthy guinea –pigs from the same stock, each weighing 250-350 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs in not fewer than 6 equal groups, with not less than 6 animals in each group; use groups containing a number of animals sufficient to obtain results that fulfill the requirements for a valid assay. Use a further group of non-vaccinated guinea-pigs of the same origin to provide a negative serum control. If test consistency has been demonstrated, a reference negative serum control may be used.

Immunization. Prepare in saline solution dilutions of the vaccine under examination and dilution of the Standard preparation such that, for each, the dilutions from a series differing by 2.5 to 5 fold steps. Allocate the six dilutions one to each group and inject subcutaneously 1.0 ml of each dilution into each guinea pig of the group to which each dilution is allocated. After 35-42 days bleed each vaccinated and control guinea pig using a suitable method.

Preparation of serum samples. For preparation of serum samples, the following technique has been found suitable. Invert the tubes containing blood samples 6 times and allow

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to stand at 37° for 2 h, then at 4° for 2 hours, centrifuge at room temperature at 800 g for 20 min. transfer the serum to sterile tubes and store at a temperature below -20°. At least 40.0 per cent yield of serum is obtained by this procedure.

Determination of antibody titre. The ELISA and ToBI tests shown below are given as examples of immunochemical methods that have been found suitable for the determination of antibody tire.

Determination of antibody titre in guinea pig serum by enzyme-linked immunosorbent assay (ELISA). Dilutions of test and reference sera are made on ELISA plates coated with tetanus toxoid. A positive guinea-pig serum control and a negative guinea-pig serum control are included on each plate to monitor the assay performance. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig-IgG is added followed by a peroxidase substrate. Optical density is measured and the relative antibody titre is calculated using the usual statistical methods.

Requirements of a valid assay. The test is not valid assay:

- the confidence limits (P=0.95) are not less than 50.0 per cent and not more than 200.0 per cent of the estimated potency;
- the statistical analysis shows a significant slope and no deviation from linearity and parallelism of the dose-response curves.

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimation of potency.

Reagents and equipment

ELISA plates: 96 wells, columns 1-12, rows A-H.

Clostridium tetani guinea-pig antiserum (for vaccines-human use) reference preparation (positive control serum).

Peroxidase conjugate. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig IgG.

Tetanus toxoid

Carbonate coating buffer pH 9.6. Dissolve 1.59 g of *anhydrous sodium carbonate* and 2.93 g of *sodium hydrogen carbonate* in 1000 ml of *water*. Distribute into 150 ml bottles and sterilise by autoclaving at 121° for 15 min.

Phosphate buffered saline pH 7.4 (PBS). Dissolve with stirring 80.0 g of *sodium chloride*, 2.0 g of *potassium dihydrogen phosphate*, 14.3 g of *disodium hydrogen phosphate dihydrate* and 2.0 g of *potassium chloride* in 1000 ml of *water*. Store at room temperature to prevent crystallisation. Dilute to 10 times its volume with *water* before use.

Citric acid solution. Dissolve 10.51 g of *citric acid* in 1000 ml of *water* and adjust the solution to pH 4.0 with a 400 g/l solution of *sodium hydroxide*.

Washing buffer. PBS *containing 0.05 per cent w/v of polysorbate 20*.

Diluent block buffer. PBS containing 0.05 per cent w/v of *polysorbate 20* and 25 g/l of dried skimmed milk.

Peroxidase substrate. Shortly before use, dissolve 10 mg of *diammonium 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate)* (ABTS) in 20 ml of *citric acid solution*. Immediately before use add 5 µl of *strong hydrogen peroxide solution*.

Method

The description below is given as an example of a suitable plate lay-out but others may be used. Wells 1A-H are for negative control serum and wells 2A-H and 3A-H are for positive control serum for assay monitoring. Wells 4-12A-H are for test samples.

Coat each well of the ELISA plates with 100 µl of *tetanus toxoid solution* (0.5 Lf/ml in carbonate coating buffer).

Allow to stand overnight at 4° in a humid atmosphere. To avoid interference from temperature gradient, do not stack more than 4 plates high. On the following day, wash the plates thoroughly with *washing buffer*. Block the plates by addition of 100 µl of *diluent block buffer* to each well. Incubate in a humid atmosphere at 37° for 1 h. Wash the plates thoroughly with *washing buffer*. Place 100 µl of *diluent block buffer* in each well of the plates, except those of row A. Prepare suitable dilutions of *negative control serum, positive control serum* (from about 0.01 IU/ml) and test sera. Allocate the *negative control serum* to column 1, *positive control serum* to columns 2 and 3 and test sera to columns 4-12 and add 100 µl of each serum to the first 2 wells of the column to which it is allocated. Using a multi channel micropipette, make twofold serial dilutions from row B down the plate to row H by transferring 100 µl to the following well. Discard 100 µl from the last row so that all wells contain 100 µl. Incubate at 37° for 2 h. Wash thoroughly with *washing buffer*. Prepare a suitable dilution (a 1 in 2000 dilution has been found suitable) of *peroxidase conjugate* in *diluent block buffer* and add 100 µl to each well. Incubate at 37° in a humid atmosphere for 1 h. Wash the plates thoroughly with *washing buffer*. Add 100 µl of peroxidase substrate to each well. Allow to stand at room temperature, protected from light, for 30 min. Read the plates at 405 nm in the same order as addition of substrate was made.

Determination of antibody titre in guinea-pig serum by toxin- or toxoid-binding inhibition (ToBI). Tetanus toxin or toxoid is added to serial dilutions of test and reference sera; the serum/antigen mixtures are incubated overnight. To determine unbound toxin or toxoid, the mixtures are transferred to an ELISA plate coated with tetanus antitoxin. *Peroxidase-conjugated equine anti-tetanus IgG* is added followed by a *peroxidase substrate*. Optical density is measured and the

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antibody titre is calculated using the usual statistical methods. A *positive control serum* and a *negative control serum* are included on each plate to monitor assay performance.

Reagents and equipment

Round-bottomed, rigid polystyrene microtitre plates.

Flat-bottomed ELISA plates.

Tetanus toxin or tetanus toxoid.

Clostridium tetani guinea-pig antiserum (for vaccines-human use) reference preparation.

Equine anti-tetanus IgG.

Peroxidase-conjugated equine anti-tetanus IgG.

Carbonate buffer pH 9.6. Dissolve 1.5 g of *anhydrous sodium carbonate,* 2.39 g of *sodium hydrogen carbonate* and 0.2 g of *sodium azide* in 1000 ml of *water,* adjust to pH 9.6 and autoclave at 121° for 20 min.

Sodium acetate buffer pH 5.5. Dissolve 90.2 g of *anhydrous sodium acetate* in 900 ml of *water,* adjust to pH 5.5 using a saturated solution of *citric acid monohydrate* and dilute to 1000 ml with *water.*

Phosphate buffered saline pH 7.2 (PBS). Dissolve 135.0 g of *sodium chloride,* 20.55 g of *disodium hydrogen phosphate dihydrate* and 4.80 g of *sodium dihydrogen phosphate monohydrate* in *water* and dilute to 15 litres with the same solvent. Autoclave at 100° for 60 min.

Diluent buffer. PBS containing 0.5 per cent w/v of *bovine albumin* and 0.05 per cent w/v of *polysorbate 80.*

Block buffer. PBS containing 0.5 per cent w/v of *bovine albumin.*

Tetramethylbenzidine solution. 0.6 per cent w/v solution of *tetramethyl-benzidine* in *alcohol.* The substance dissolves within 30-40 min at room temperature.

Peroxidase substrate. Mix 90 ml of *water,* 10 ml of *sodium acetate buffer* pH 5.5, 1.67 ml of *tetramethylbenzidine solution* and 20 µl of *strong hydrogen peroxide solution.*

Washing solution. Tap water containing 0.5 g/l of *polysorbate 80.*

Method

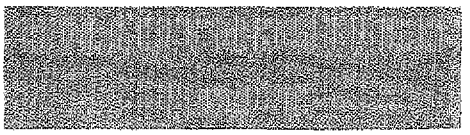
Block the round-bottomed polystyrene microtitre plates by placing in each well 150 µl of *block buffer.* Cover the plates with a lid or sealer. Incubate in a humid atmosphere at 37° for 1 h. Wash the plates thoroughly with *washing solution.* Place 100 µl of *PBS* in each well. Place 100 µl of *reference guinea pig tetanus antitoxin* in the first well of a row. Place 100 µl of *undiluted test sera* in the first well of the required number of rows. Using a multichannel micropipette, make it two fold serial dilutions across the plate (up to column 10), by transfer of 100 µl to the following well. Discard 100 µl from the last column so that all wells contain 100 µl. Prepare 0.1 Lf/ml solution of *tetanus toxin* or toxoid using *PBS* a diluent. Add 40 µl of this solution to all wells except those column 12. The wells of row 11 are a positive control. Add 40 µl of *PBS* to the wells of column 12 (negative control). Shake the plates gently and cover them with lids. Coat the ELISA plates: immediately before use make a suitable dilution of *equine anti-tetanus IgG* in *carbonate buffer pH 9.6* and add 100 µl to all wells. Incubate the 2 series of plates overnight in a humid atmosphere at 37°. To avoid temperature gradient effects, do not stack more than 4 plate high. Cover the plates with lids. On the following day, wash the ELISA plates thoroughly with *washing solution.* Block the plates by placing in each well 125 µl of *block buffer.* Incubate at 37° in a humid atmosphere *for* 1 h. Wash the plates thoroughly with *washing solution.* Transfer 100 µl of the pre-incubation mixture from the polystyrene plates to the corresponding wells of the ELISA plates, starting with column 12 and then from 1 to 11. Cover the plates with a lid. Incubate at 37° in a humid atmosphere for 2 h. Wash the ELISA plates thoroughly with *washing solution.* Make a suitable dilution (a 1 in 4000 dilution has been found suitable) of the *peroxidase-conjugated equine anti-tetanus IgG* in *diluent buffer.* Add 100 µl of the dilution to each well and cover the plates with a lid. Incubate at 37° in a humid atmosphere *for* 1.5 h. Wash the ELISA plates thoroughly with *washing solution.* Add 100 µl of *peroxidase substrate* to each well. A blue colour develops. Incubate the plates at room temperature. Stop the reaction at a given time (within 10 min) by the addition of 100 µl of 2 M *sulphuric acid* to each well in the same order as the addition of substrate. The colour changes from blue to yellow. Measure the absorbance (2.4.7) at 450 nm immediately after addition of the *sulphuric acid* or maintain the plates in the dark until reading.

(D) Any other validated serological assay in guinea-pigs/mice approved by National Regulatory Authority.

Labelling. The label states (1) the human dose (ml); (2) the minimum units per single human dose or the minimum International Units per single human dose if potency test done by challenge method; (3) the name and the amount of the adsorbent and preservative; (4) that the vaccine must be shaken before use; (5) that the vaccine is not to be frozen.

Tick-borne Encephalitis Vaccine (Inactivated)

Tick-borne Encephalitis Vaccine (Inactivated) is a liquid preparation of a suitable strain of tick-borne encephalitis virus grown in cultures of chick-embryo cells or other suitable cell cultures and inactivated by a suitable, validated method.



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Production

General provisions

The vaccine complies with the General Requirements of Vaccines for Human Use.

Production of the vaccine is based on a virus seed lot system. The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man. The virus in the final vaccine shall not have undergone more passages from the master seed lot than the virus in the vaccine used in clinical trials.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity and immunogenicity.

Substrate for virus propagation

The virus is propagated in chick embryo cells prepared from eggs derived from a chicken flock free from specified pathogens (2.7.7) or in other suitable cell cultures.

SEED LOT

The strain of virus used is identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are stored at or below -60°.

Only a seed lot that complies with the following requirements may be used for virus propagation.

Identification

Each seed lot is identified as containing the vaccine strain of tick-borne encephalitis virus by a suitable immunochemical method, preferably using monoclonal antibodies.

Virus concentration. The virus concentration of each seed lot is determined by titration in suitable cell cultures to monitor consistency of production.

Extraneous agents (2.7.3). Each seed lot complies with the requirements for extraneous agents in viral vaccines for human use; the tests in cell cultures are carried out in human and simian cells only.

Mycoplasma (2.7.4).Complies with the test for absence of mycoplasma.

PROPAGATION AND HARVEST

All processing of the cell cultures if performed under aseptic conditions in an area where no other cells are being handled. Serum and trypsin used in the preparation of cell suspensions and media used must be shown to be free from extraneous [illegible] culture media may contain a pH indicator [illegible] at the lowest

employed for vaccine production is set aside as uninfected cell cultures (control cells).

Only a single harvest that complies with the following requirements may be used in the preparation of the inactivated harvest.

Identification

The single harvest is shown to contain tick-borne encephalitis virus by a suitable immunochemical method, preferably using monoclonal antibodies, or by virus neutralization in cell cultures.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

Mycoplasma (2.7.4). Complies with the test for mycoplasmas carried out using 1 ml for each medium.

Control cells. The control cells comply with the tests for extraneous agents (2.7.3). If the vaccine is produced using a cell-bank system, the control cells comply with a test for identification.

Virus concentration. Determine the virus concentration by titration in suitable cell culture to monitor consistency of production.

Inactivation

To avoid interference, viral aggregates are removed by filtration immediately before the inactivation process. The virus suspension is inactivated by a validated method; the method shall have been shown to be consistently capable of inactivating tick-borne encephalitis virus without destroying the antigenic and immunogenic activity; as part of the validation studies, an inactivation curve is plotted representing residual live virus concentration measured on not fewer than three occasions. If formaldehyde is used for inactivation, the presence of an excess of free formaldehyde is verified at the end of the inactivation process.

Only an inactivated harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Residual infective virus. Inoculate a quantity of the inactivated harvest equivalent to not less than ten human doses of vaccine in the final lot into primary chicken fibroblast cell cultures, or other cells shown to be at least as sensitive to tick-borne encephalitis virus with not less than 3 cm^2 of cell sheet per ml of inoculum. Incubate at 37 ± 1° for 14 days. No cytopathic effect is detected at the end of the incubation period. Collect the culture fluid and inoculate 0.03 ml intracerebrally into each of not fewer than ten mice about 4 weeks old. Observe the mice for 14 days. They show no evidence of tick-borne [illegible] virus infection.

Purification

Several inactivated single harvests may be pooled before concentration and purification by suitable methods, preferably by continuous-flow, sucrose density-gradient centrifugation.

Only a purified, inactivated harvest that complies with the following requirements may be used in the preparation of final bulk vaccine.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11), carried out using 10 ml for each medium.

Specific activity. Determine the antigen content of the purified, inactivated harvest by a suitable immunochemical method (2.2.14). Determine the total protein content by a suitable method. The specific activity, calculated as the antigen content per unit mass of protein, is within the limits approved for the specific product.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more purified, inactivated harvests.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde, bovine serum albumin (where applicable) and pyrogens and the assay have been carried out satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Identification

The vaccine is shown to contain tick-borne encephalitis virus antigen by a suitable immunochemical method using specific antibodies or by the mouse immunogenicity test described under Assay.

Tests

Aluminium (2.3.9). Maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Bovine serum albumin. If bovine serum albumin has been used during production, the vaccine contains not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.2.14).

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject into each rabbit, per kg of body mass, one dose of vaccine or a validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. If used, the content should be within the limits approved by the National Regulatory authority.

Assay

The potency is determined by comparing the dose necessary to protect a given proportion of mice against the effects of a lethal dose of tick-borne encephalitis virus, administered intraperitoneally, with the quantity of a reference preparation of tick-borne encephalitis vaccine necessary to provide the same protection. For this comparison an approved reference preparation and a suitable preparation of tick-borne encephalitis virus from an approved strain for use as the challenge preparation are necessary.

The following is cited as an example of a method that has been found suitable for a given vaccine.

Selection and distribution of test animals. Use healthy mice weighing between 11 and 17 g derived from the same stock. Distribute the mice into not less than six groups of a suitabl size to meet the requirements for validity of the test; for titratio of the challenge suspension, use not fewer than four group of ten mice. Use mice of the same sex or distribute males a females equally between groups.

Determination of potency of the vaccine. Prepare not l than three suitable dilutions of the vaccine under examinati and of the reference preparation; in order to comply w validity criteria four to five dilutions will usually be necessa Prepare dilutions such that the most concentrated suspens is expected to protect more than 50 per cent of the animals the least concentrated suspension less than 50.0 per c Allocate each dilution to a different group of mice and ir subcutaneously into each mouse 0.2 ml of the dilution alloc to its group. Seven days later make a second injection u the same dilution scale. 14 days after the second inje prepare a suspension of the challenge virus containin less than 100 LD_{50} in 0.2 ml. Inject 0.2 ml of this suspension intraperitoneally into each vaccinated mou verify the challenge dose, prepare a series of not fewe three dilutions of the challenge virus suspension at not g than one-hundred fold intervals. Allocate the cha suspension and the four dilutions, one to each of t groups of ten mice, and inject intraperitoneally into each 0.2 ml of the challenge suspension or the dilution alloc its group. Observe the animals for 21 days after the ch and record the number of mice that die in the period b 7 and 21 days after the challenge.

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Calculations. Calculate the results by the usual statistical methods for an assay with quantal responses (5.7).

Validity criteria. The test is not valid unless (1) the concentration of the challenge virus is not less than 100 LD_{50}; (2) for both the vaccine under examination and the reference preparation the 50.0 per cent protective dose (PD_{50}) lies between the largest and smallest doses given to the mice; (3) the statistical analysis shows a significant slope and no significant deviation from linearity and parallelism of the dose-response lines; (4) the fiducial limits (P = 0.95) are not less than 33.0 per cent and not more than 300.0 per cent of the estimated potency.

Potency requirement. Include all valid tests to estimate the mean potency and the fiducial limits (P = 0.95) for the mean potency; compute weighed means with the inverse of the squared standard error as weights. The vaccine complies with the test if the estimated potency is not less than that approved by the competent authority, based on data from clinical efficacy trials.

Labelling. The label states (1) the strain of virus used in preparation; (2) the type of cells used for production of the vaccine.

Tuberculin Purified Protein Derivative

Tuberculin PPD; Tuberculin Purified Protein Derivative for Human Use

Tuberculin Purified Protein Derivative is a preparation made from the heat-treated products of growth and lysis of one or more strains of *Mycobacterium tuberculosis* that reveal delayed hypersensitivity in animals sensitised by a micro-organism of the same species.

Production

It is prepared from the water-soluble fraction obtained by heating in free-flowing steam or in an autoclave and subsequently filtering cultures of the mycobacteria grown in a suitable liquid medium. The active fraction in the filtrate, which is predominantly protein, is separated by precipitation, washed and redissolved. The preparation is free from mycobacteria. An antimicrobial preservative that does not give rise to false positive reactions, such as 0.5 per cent w/v of *phenol*, and a suitable stabiliser may be added. Phenol is not added to preparations that are to be freeze-dried. The final sterile product is distributed into sterile glass containers which are then sealed so as to prevent microbial contamination or alternatively it is freeze-dried and the containers subsequently sealed.*

* *To ensure availability of a preparation of uniform potency, Tuberculin Purified Protein Derivative is produced and issued by the Statens Serum Institute, Denmark as a powder to be reconstituted as stated on the label.*

The preparation may be issued either as a sterile liquid or as a freeze-dried product. If issued as a liquid, it is in a ready-to-use form and 0.1 ml constitutes one intradermal dose containing appropriate number of Units. If issued as a freeze-dried product, it should yield a ready-to-use preparation when reconstituted as per manufacturer's instructions.

Description. A colourless or pale, straw-coloured liquid, or dry cream-coloured powder, or pellet.

The preparation, reconstituted if necessary as stated on the label, complies with the following requirements.

Identification

A. When progressively increasing doses are injected intradermally into specifically sensitised guinea-pigs, reactions occur at the points of injection, varying from erythema to necrosis. When similar injections are administered to non-sensitised guinea pigs no such reactions occur.

B. The potency test described below serves as a test for identity if it is performed on material from the final containers.

Tests

pH (2.4.24). 6.5 to 7.5.

Phenol (*if present*) (2.3.36). Not more than 0.5 per cent w/v.

Sterility (2.2.11). Complies with the tests for sterility.

Potency. Carry out the *biological assay of tuberculin purified protein derivative* described below.

Tuberculin Purified Protein Derivative in freeze-dried form complies with the following additional requirements.

Live mycobacteria

A. Inject 5.0 ml intraperitoneally or subcutaneously into each of two guinea-pigs weighing between 300 and 400 g. Observe the animals for not less than 42 days. Kill the animals and carry out an autopsy. No guinea-pig shows signs of infection with mycobacteria.

B. Carry out tests for live mycobacteria in the preparation under examination using suitable culture media. No growth of mycobacteria should occur.

Sensitising effect. Inject intradermally into each of three guinea-pigs three times, at intervals of 5 days, a dose of the preparation under examination containing about 500 Units in a volume of 0.1 ml. Two to three weeks after the third injection inoculate the same dose intradermally into these animals and into a control group of three guinea-pigs of the same weight but that have not had any previous injections of tuberculin. The reactions of the two groups are not significantly different after 48 to 72 hours.

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Toxicity. Inject subcutaneously into 2 healthy guinea-pigs, weighing not less than 250 g and which have not previously been treated with any material which will interfere with the test, 0.5 ml of a solution containing 1,00,000 Units per ml. No harmful effects are produced within 7 days.

Biological assay

The potency of tuberculin purified protein derivative is determined by comparing the dose necessary to reveal delayed hypersensitivity in guinea-pigs or other animals hypersensitised with mycobacteria of the same type as that used in the preparation of the tuberculin purified protein derivative with the dose of the appropriate Standard Preparation necessary to give the same effect.

The estimated potency is not less 80 per cent and not more than 125 per cent of the stated potency. The fiducial limits of error are not less than 64 per cent and not more than 156 per cent of the stated potency.

Standard Preparation

The Standard Preparation is the 1st International Standard for Tuberculin, purified protein derivative (PPD), mammalian, established in 1951, consisting of PPD derived from cultures of *M. tuberculosis* (supplied in ampoules containing 5,00,000 Units) or another suitable preparation the potency of which has been determined in relation to the International Standard.

Method

Sensitise 12 guinea-pigs each weighing not less than 400 g by the intramuscular injection of a total of about 0.5 ml of a suspension in a suitable mineral oil with or without emulsifier and containing 0.1 mg per ml of heat-inactivated, dried mycobacteria of the same type as that used in the preparation of tuberculin. Use *phosphate-buffered saline pH 7.4* containing 0.005 per cent w/v of *polysorbate 80* to prepare the dilutions of the Standard preparation and of the preparation under examination and to reconstitute freeze-dried preparations containing no stabiliser. Not less than 1 month and not more than 6 months later carry out the following test:

Shave the flanks to provide space for at least three reactions on each side, but not more than a total of 12 injection sites per animal. Use at least three doses of the Standard preparation and at least three doses of the preparation under examination, the highest dose being about 10 times as strong as the lowest. Dilute the preparations so that the lesions produced are 8 to 25 mm in diameter. Allocate the doses to the available sites in a random manner, in a Latin square design. Inject each dose intradermally in the same volume (0.1 or 0.2 ml) at the sites to which it has been allocated. Measure the diameters of the lesions after 24 to 48 hours and calculate the result of the test using standard statistical methods on the basis that the lesion diameters are directly proportional to the logarithm of the concentration of the tuberculin.

Storage. Store in light-resistant containers at a temperature between 2° and 8°. It should not be allowed to freeze.

Labelling. The label states (1) the number of Units per dose of 0.1 ml or per ml or per mg; (2) the total volume in the container (for liquid preparation); (3) the nature and quantity of the reconstituting liquid (for the freeze-dried preparation); (4) the name and proportion of any added substances; (5) the species or strain used; (6) the storage conditions; (7) the date after which the contents are not intended to be used; (8) that care should be taken to avoid inhaling the powder (for the freeze-dried preparation).

Typhoid (Strain Ty 21a) Vaccine, Live (Oral)

Typhoid Vaccine (Live, Oral, strain Ty 21a) is a freeze-dried preparation of the live *Salmonella typhi* strain Ty 21a grown in a suitable medium. When presented in capsules, the vaccine complies with the tests stated under Capsules.

Production

Choice of vaccine strain

The main characteristic of the strains is the defect of the enzyme uridine diphosphate-galactose-4 epimerase. The activities of galactopermease, galactokinase and galactose-1-phosphate uridyl-transferase are reduced by 50 to 90 per cent. Whatever the growth conditions, the strain does not contain Vi antigen. The strain agglutinates to anti-O : 9 antiserum only if grown in medium containing galactose. It contains the flagellar H:d antigen and does not produce hydrogen sulphide on Kligler iron agar. The strain is non-virulent for mice. Cells of strain Ty 21a lyse if grown in the presence of 1.0 per cent of galactose.

SEED LOT

The vaccine is prepared using a seed-lot system. The working seed lots represent not more than one subculture from the master seed lot. The final vaccine represents not more than four subcultures from the original vaccine on which were made; the laboratory and clinical tests showing the strain to be suitable.

Only a master seed lot that complies with the following requirements may be used in the preparation of working seed lots.

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Galactose metabolism

In a spectrophotometric assay, no activity of the enzyme uridine diphosphate-galactose-4-epimerase is found in the cytoplasm of strain Ty 21a compared to strain Ty 2.

Biosynthesis of lipopolysaccharide

Lipopolysaccharides are extracted by the hot-phenol method and examined by size-exclusion chromatography (2.4.16). Strain Ty 21a grown in medium free of galactose shows only the rough (R) type of lipopolysaccharide.

Serological characteristics

Strain Ty 21a grown in a synthetic medium without galactose does not agglutinate to specific anti-O:9 antiserum. Whatever the growth conditions, strain Ty 21a does not agglutinate to Vi antiserum. Strain Ty 21a agglutinates to H:d flagellar antiserum.

Biochemical markers

StrainTy 21a does not produce hydrogen sulphide on Kligler iron agar. This property serves to distinguish Ty 21a from other galactose-epimerase-negative *S. typhi* strains.

Cell growth

Strain Ty 21a cells lyse when grown in the presence of 1.0 per cent of galactose.

PROPAGATION AND HARVEST

The bacteria from the working seed lot are multiplied in a preculture, subcultured once and are then grown in a suitable medium containing 0.001 per cent of galactose at 30° for 13 to 15 hours. The bacteria are harvested. The harvest must be free from contaminating micro-organisms.

Only a single harvest that complies with the following requirements may be used for the preparation of the freeze-dried harvest.

pH (2.4.24). 6.8 to 7.5.

Optical density

The optical density of the culture, measured at 546 nm, is 6.5 to 11.0. Before carrying out the measurement, dilute the culture so that a reading in the range 0.1 to 0.5 is obtained and correct the reading to take account of the dilution.

Identification

Culture bacteria on an agar medium containing 1.0 per cent of *galactose* and *bromothymol blue*. Light blue, concave colonies, transparent due to lysis of cells, should be found. No yellow colonies (galactose-fermenting) should be found.

FREEZE-DRIED HARVEST

The harvest is mixed with a suitable stabilizer and freeze-dried by a process that ensures the survival of at least 10.0 per cent of the bacteria and to a water content shown to be favourable to the stability of the vaccine. No antimicrobial preservative is added to the vaccine.

Only a freeze-dried harvest that complies with the following tests may be used for the preparation of the final bulk.

Identification

Culture bacteria are examined on an agar medium containing 1.0 per cent of *galactose* and *bromoethymol blue*. Light blue, concave colonies, transparent due to lysis of cells, should be found. No yellow colonies (galactose-fermenting) should be found.

Number of live bacteria

Not less than 1×10^{11} live *S. typhi* strain Ty 21a per gram.

Water (2.4.43). 1.5 to 4.0 per cent, determined by the semi-micro determination of water or any other validated method.

FINAL BULK VACCINE

The final bulk vaccine is prepared by aseptically mixing one or more freeze-dried harvests with a suitable sterile excipient.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Number of live bacteria. Not less than 40×10^9 live *S. typhi* strain Ty 21a per gram.

FINAL LOT

The final bulk vaccine is distributed under aseptic conditions into capsules with a gastro-resistant shell or into suitable containers.

Only a final lot that is satisfactory with respect to Identification, Tests and Number of live bacteria will be released for use, except that in the determination of number of live bacteria each dosage unit must contain not less than 4×10^9 live bacteria.

Identification

Culture bacteria from the vaccine under examination on an agar medium containing 1.0 per cent of *galactose* and *bromothymol blue*. Light blue, concave colonies transparent due to lysis of cells, should be found. No yellow colonies (galactose-fermenting) should be found.

Tests

Microbial contamination (2.2.9). Carry out the test using suitable selective media. Determine the total viable count using

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the plate-count method. The number of contaminating micro-organisms per dosage unit is not greater than 10^2 bacteria and 20 fungi. No pathogenic bacterium, particularly *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella* other than strain Ty 21a are found.

Water (2.4.43). 1.5 to 4.0 per cent.

Assay

Number of live bacteria. Carry out the test using not less than five dosage units. Homogenise the contents of the dosage units in a 0.9 per cent w/v solution of *sodium chloride* at 4° using a mixer in a cold room with sufficient glass beads to emerge from the liquid. Immediately after homogenization prepare a suitable dilution of the suspension using cooled diluent and inoculate *brain heart infusion agar*, incubate at 36 ± 1° for 20 to 36 hours. The vaccine contains not less than 2×10^9 live *S. typhi* Ty 21a bacteria per dosage unit.

Labelling. The label states (1) the minimum number of live bacteria per dosage unit; (2) that the vaccine is for oral use only.

Typhoid Polysaccharide Vaccine

Typhoid Polysaccharide Vaccine is a preparation of purified Vi capsular polysaccharide obtained from *Salmonella typhi* Ty2 strain or some other suitable strain of known origin and history that has the capacity to produce Vi polysaccharide, and which have been characterized by suitable biochemical, physicochemical, serological or molecular methods.

Capsular polysaccharide is a partly 3-O-acetylated repeated units of 2-acetylamino-2-deoxy-D-galactopyranuronic acid with α-(1→4)-linkages.

Production

General provisions

The production of Vi polysaccharide is based on a defined seed-lot system. The method of production shall have been shown to yield consistently Vi-polysaccharide typhoid vaccines of adequate immunogenicity and safety in man. The production method is validated to demonstrate that the product, if tested, would comply with the tests for abnormal toxicity.

SEED LOT

The strain of *S. typhi* used for the master seed lot shall be identified by historical records that include information on its origin and by its biochemical and serological characteristics. Cultures from the working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot, shall be demonstrated along with adequate documentation.

Only a strain that has the following characteristics may be used in the preparation of the vaccine: (a) stained smears from a culture are typical of *S. typhi*; (b) the culture utilises glucose without production of gas; (c) colonies on agar are oxidase-negative; (d) a suspension of the culture agglutinates specifically with an appropriate Vi antiserum or colonies form haloes on an agar plate containing a suitable Vi antiserum.

PROPAGATION AND HARVEST

The working seed lot is cultured on a solid medium, which may contain blood-group substances, or a liquid medium; the inoculum obtained is transferred to a liquid medium, which is used to inoculate the final medium. The liquid medium used and the final medium are semi-synthetic, free from substances that are precipitated by *cetrimonium bromide* and do not contain blood-group substances or high-molecular-mass polysaccharides, unless it has been demonstrated that they are removed by the purification process. The bacterial purity of the culture is verified by microscopic examination of Gram-stained smears and by inoculation into appropriate media. The culture is then inactivated at the beginning of the stationary phase by the addition of *formaldehyde*. Bacterial cells are eliminated by centrifugation; the polysaccharide is precipitated from the culture medium by addition of *hexadecyltrimethylammonium bromide* (*cetrimonium bromide*). The precipitate is harvested and may be stored at or below -20° before purification.

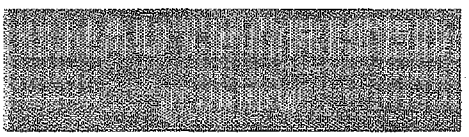
Purified Vi Polysaccharide

The polysaccharide is purified, after dissociation of the polysaccharide/cetrimonium bromide complex, using suitable procedures to eliminate successively nucleic acids, proteins and lipopolysaccharides. The polysaccharide is precipitated in its salt form and dried at 5±3°; the powder obtained constitutes the purified Vi polysaccharide. The loss on drying is determined by thermogravimetry, Karl Fischer or any other suitable method and is used to calculate the results of the chemical tests shown below with reference to the dried substance.

Only those pools of purified Vi-polysaccharide that comply with the following requirements may be used in the preparation of the final bulk:

Protein (2.7.1). Not more than 10 mg per gram of polysaccharide, calculated with reference to the dried substance.

Nucleic acids (2.7.1). Not more than 20 mg per gram of polysaccharide, calculated with reference to the dried substance.



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O-Acetyl groups (2.7.1). Not less than 2 mmol per gram of polysaccharide, calculated with reference to the dried substance.

Molecular size. Examine by gel filtration or size-exclusion chromatography (2.4.16) using cross-linked agarose for chromatography. Use a column 0.9 m long and 16 mm in internal diameter equilibrated with a solvent having an ionic strength of 0.2 mol per kg and a pH of 7.0 to 7.5. Apply about 5 mg of polysaccharide in a volume of 1 ml to the column and elute at about 20 ml per hour. Collect fractions of about 2.5 ml. Determine the point corresponding to $K_0 = 0.25$ and make two pools consisting of fractions eluted before and after this point. Determine O-acetyl groups on the two pools. Not less than 50 per cent of the polysaccharide is found in the pool containing fractions eluted before $K_0 = 0.25$.

Identification

Carry out an identification test using a suitable immunochemical method (2.2.14).

Bacterial endotoxins (2.2.3). Determine by a suitable method, the content should not be more than 150 IU per mcg of polysaccharide.

FINAL BULK VACCINE

One or more batches of purified Vi polysaccharide are dissolved in a suitable solvent, which may contain an antimicrobial preservative, so that the volume corresponding to one dose contains 25 µg of polysaccharide and the solution is isotonic with blood (250 mosm/kg to 350 mosm/kg).

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot;

Sterility (2.2.11). Carry out test for sterility using 10 ml of bulk for each sterility medium.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount. If phenol has been used in the preparation, the content is not more than 2.5 g/l.

FINAL LOT

The final bulk vaccine is distributed and filled aseptically into sterile containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements prescribed below under Identification, Tests and Assay and with the requirements for Bacterial endotoxins may be released for use. Provided the tests for free formaldehyde and antimicrobial preservative have been carried out on the final bulk vaccine, they may be omitted on the final lot.

The vaccine contains minimum of 25 µg purified Vi Polysaccharide per dose of 0.5 ml.

Identification

Carry out an identification test using a suitable immunochemical method (2.2.14).

Tests

Sterility (2.2.11). Complies with the tests for sterility.

pH (2.4.24). 6.5 to 7.5.

O-Acetyl groups (2.7.1). 0.085 (± 25 per cent) µmol per dose (25 µg of polysaccharide).

Test solution. Place 3 ml of the vaccine in each of three tubes (two reaction solutions and one correction solution).

Reference solutions. Dissolve 0.150 g of *acetylcholine chloride* in 10 ml of *water* (*stock solution* containing 15 g/l of *acetylcholine chloride*). Immediately before use, dilute 0.5 ml of the *stock solution* to 50 ml with *water* (working dilution containing 150 µg/ml of *acetylcholine chloride*). In ten tubes, place in duplicate (reaction and correction solutions) 0.1 ml, 0.2 ml, 0.5 ml, 1.0 ml and 1.5 ml of the working dilution.

Prepare a blank using 3 ml of *purified water*.

Make up the volume in each tube to 3 ml with *water*. Add 0.5 ml of a mixture of 1 volume of *water* and 2 volumes of *dilute hydrochloric acid* to each of the correction tubes and to the blank. Add 1.0 ml of *alkaline hydroxylamine solution* to each tube. Allow the reaction to proceed for exactly 2 min and add 0.5 ml of a mixture of 1 volume of *water* and 2 volumes of *dilute hydrochloric acid* to each of the reaction tubes. Add 0.5 ml of a 20 per cent w/v solution of *ferric chloride* in *0.2M hydrochloric* acid to each tube, stopper the tubes and shake vigorously to remove bubbles.

Measure the absorbance of each solution at 540 nm using the blank as the compensation liquid. For each reaction solution, subtract the absorbance of the corresponding correction solution. Draw a calibration curve from the corrected absorbance for the five reference solutions and the corresponding content of acetylcholine chloride and read from the curve the content of acetylcholine chloride in the test solution for each volume tested. Calculate the mean of the two values.

1 mol of acetylcholine chloride (181.7 g) is equivalent to 1 mol of O-acetyl (43.05 g).

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

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If phenol has been used in the preparation, the content is not more than 2.5 g/l.

Assay

Determine Vi polysaccharide content by a suitable immunochemical method (2.2.14) using a reference purified polysaccharide. The estimated amount of polysaccharide per dose is 80.0 per cent to 120.0 per cent of the content stated on the label. The fiducial limits of error (P = 0.95) of the estimated amount of polysaccharide are not less than 80.0 per cent and not more than 120.0 per cent.

Labelling. The label states (1) the number of micrograms of polysaccharide per human dose (25 µg); (2) the total quantity of polysaccharide in the container.

Typhoid Paratyphoid A Vaccine

Typhoid Paratyphoid A Vaccine is a sterile suspension or a freeze-dried solid prepared from one or more strains of *Salmonella typhi* and *S. paratyphi A* that are smooth and have the full complement of O, H and Vi antigens in S. *typhi strain*, and O and H antigens in S. *paratyphi A* strain. The bacteria are killed by heat or by a bactericide such as phenol or formaldehyde or by a chemical such as acetone. The liquid vaccine and any diluent supplied by the manufacturer with the freeze-dried vaccine contains a suitable preservative. The dried vaccine contains no preservative.

Typhoid Paratyphoid A Vaccine for adults contains not less than 1000 million *S. typhi* and 500 million *S. paratyphi A* organisms per ml. The vaccine meant for children contains 333 million *S. typhi* and 167 million of *S. paratyphi A* organisms per ml.

Description. The liquid or the reconstituted vaccine is a white or pale yellow turbid liquid free from clumps.

Identification

Identify by specific agglutination.

Tests

pH (2.2.24). 6.0 to 7.4.

Other tests. Complies with the tests stated under Vaccines with the following modifications.

Phenol (2.3.36) (*if present*). Not more than 0.5 per cent w/v.

Storage. Store at a temperature 2° to 8°. The liquid vaccine must not be allowed to freeze.

Labelling. The label states (1) the number of organisms per ml; (2) whether the vaccine is meant for use in adults or for use in children; (3) the recommended dose; (4) the name and proportion of any added preservative; (5) that in case of liquid preparation, the vaccine should not be allowed to freeze.

Typhoid Vaccine

Typhoid Vaccine is a sterile suspension of inactivated *Salmonella typhi* containing not less than 5 x 10^8 and not more than 1 x 10^9 bacteria per human dose. The human dose does not exceed 1.0 ml.

Production

The vaccine is prepared using a seed-lot system from a suitable strain of *S. typhi* such as, Ty 2. The final vaccine represents not more than 3 subcultures from the strain on which were made the laboratory and clinical tests that showed it to be suitable. The bacteria are inactivated by *acetone*, by *formaldehyde*, by *phenol* or by heating or by a combination of the last two methods.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity (2.2.1) modified to the extent that 0.5 ml of the vaccine is injected subcutaneously or intramuscularly or intraperitoneally into each mouse and 1.0 ml into each guinea-pig.

Identification

It is identified by specific agglutination.

Phenol (2.3.36). If phenol has been used in the preparation, the concentration is not more than 0.5 per cent w/v.

Antigenic power. When injected into susceptible laboratory animals, it elicits anti-O, anti-H and, to a lesser extent, anti-Vi agglutinins.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Labelling. The label states (1) the method used to inactivate the bacteria; (2) the number of bacteria per human dose.

Typhoid Vaccine (Freeze Dried)

Freeze Dried Typhoid Vaccine is a freeze-dried preparation of inactivated *Salmonella typhi*. The vaccine is reconstituted as stated on the label to give a uniform suspension containing not less than 5 x 10^8 and not more than 1 x 10^9 bacteria per human dose. The human dose does not exceed 1.0 ml of the reconstituted vaccine.

Production

The vaccine is prepared from a seed-lot system from a suitable strain of *S. typhi*, such as Ty 2. The final vaccine represents not more than 3 subcultures from the strain on which were made the laboratory and clinical tests that showed it to be

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suitable. The bacteria are inactivated either by *acetone* or by *formaldehyde* or by heat. Phenol is not used in the preparation. The vaccine is distributed into sterile containers and freeze-dried to a moisture content favourable to the stability of the vaccine.

The production method is validated to demonstrate that the product, if tested, would comply with test for abnormal toxicity (2.2.1), modified to the extent that 0.5 ml of the vaccine is injected subcutaneously or intramuscularly or intraperitoneally into each mouse and 1.0 ml into each guinea-pig.

Identification

The vaccine reconstituted as stated on the label is identified by specific agglutination.

Antigenic power. When injected into susceptible laboratory animals, the reconstituted vaccine elicits anti-O, anti-H and, to a lesser extent, anti-Vi agglutinins.

Bacterial and fungal contamination. The reconstituted vaccine complies with the tests for sterility (2.2.11).

Water. Not more than 5.0 percent.

Labelling. The label states (1) the method used to inactivate the bacteria; (2) the number of bacteria per human dose; (3) that the vaccine should be used within 8 hours of reconstitution.

Typhoid Vi Conjugate Vaccine

Typhoid Vi conjugate vaccine is a liquid or freeze-dried preparation of Vi Polysaccharide, derived from a suitable strain of *Salmonella Typhi Ty 2* or *Citrobacter freundii*, covalently bound to a carrier protein.

The capsular Vi polysaccharide consists of partly 3-O-acetylated repeated units of 2-acetylamino-2-deoxy-D-galactopyranuronic acid with α-(1→4) linkages. The carrier protein, when conjugated to Vi polysaccharide is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

Production

General Provisions

The production of Typhoid Vi polysaccharide and of the carrier protein is based on a seed-lot system. The production method shall have been shown to yield consistently Typhoid Vi conjugate vaccines of adequate safety and immunogenicity in man. The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use.

During development studies and wherever validation of the manufacturing process is necessary, it shall be demonstrated by tests in animals that the vaccine consistently induces a T-cell-dependent B-cell immune response.

The stability of the final lot and relevant intermediates is evaluated using one or more indicator tests. Such tests may include determination of molecular size, determination of free Vi polysaccharide in the conjugate and the immunogenicity test in mice. Taking account of the results of the stability testing, release requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

SEED LOT

The strain of S. typhi used for the master seed lot shall be identified by historical records that include information on its origin and by its biochemical and serological characteristics. Cultures from the working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot; shall be demonstrated along with adequate documentation. Strain should be capable of stably producing Vi-Polysaccharide. NMR Spectroscopy and Immunochemical testing are suitable methods to confirm identity of Vi-Ps.

Only a strain that has the following characteristics may be used in the preparation of the vaccine: (a) stained smears from a culture are typical of enterobacteria; (b) the culture utilizes glucose without production of gas; (c) colonies on agar are oxidase-negative; (d) a suspension of the culture agglutinates specifically with a suitable Vi antiserum or colonies form haloes on an agar plate containing a suitable Vi antiserum.

Purity of bacterial strain used for the seed lot is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

No complex products of animal origin are included in the medium used for preservation of strain viability, either for freeze-drying or for frozen storage.

PROPAGATION AND HARVEST

The working seed lot is cultured on a solid medium, which may contain blood-group substances, or a liquid medium; the inoculum obtained is transferred to a liquid medium, which is used to inoculate the final medium. The liquid medium used and the final medium are semi-synthetic, free from substances that are precipitated by *cetrimonium bromide* and do not contain blood-group substances or high-molecular-mass polysaccharides, unless it has been demonstrated that they

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are removed by the purification process. The bacterial purity of the culture is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of Gram-stained smears and culture agglutination with suitable specific antisera.

The culture is then inactivated at the beginning of the stationary phase by the addition of *formaldehyde* or by a suitable inactivating agent or by alternative methods which must be adequately validated. Bacterial cells are eliminated by centrifugation; the polysaccharide is precipitated from the culture medium by addition of *hexadecyltrimethyammonium bromide (cetrimonium bromide)*. The precipitate is harvested and may be stored at -20° before purification. Test for Inactivation must be performed after inactivation process is over.

Purified Vi Polysaccharide

The polysaccharide is purified, after dissociation of the polysaccharide/cetrimonium bromide complex, using suitable procedures to eliminate successively nucleic acids, proteins and lipopolysaccharides. The purified Vi polysaccharide may be stored at -20° or below in aqueous form. A portion of this has to be dried and used for analysis.

Only those pools of purified Vi polysaccharide that comply with the following requirements may be used in the preparation of the final bulk.

Protein (2.7.1). Not more than 1 per cent of polysaccharide, calculated with reference to the dried substance.

Nucleic acids (2.7.1). Not more than 2 per cent of polysaccharide, calculated with reference to the dried substance.

O-Acetyl groups (2.7.1). Not less than 2 mmol per gram of polysaccharide (52 per cent o-acetylation), calculated with reference to the dried substance.

Molecular size. Examine by size-exclusion chromatography (2.4.16) using cross-linked agarose for chromatography. The molecular size or mass distribution of each lot of purified polysaccharide should be estimated to assess the consistency of each batch. The distribution constant (kd) should be determined by measuring the molecular size distribution at the main peak of the elution curve obtained by a suitable chromatographic method.

Not less than 50 per cent of the polysaccharide is eluted before a distribution coefficient (k_d) of 0.25 is reached.

Polysaccharide content. Carry out test using Hestrin's method or any other suitable method. Calculate the polysaccharide content from the O-acetyl values obtained by applying conversion factor.

Identification and serological specificity

The identification and serological specificity are determined by a suitable immunochemical method or other suitable method, for example ^{1}H nuclear magnetic resonance.

Bacterial Endotoxins (2.2.3). Not more than 150 EU per µg of polysaccharide.

Residual reagents. Where applicable, tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established for the particular product and each batch of Vi polysaccharide must be shown to comply with this limit. Where validation studies have demonstrated removal of a residual reagent, the test on purified Vi polysaccharide may be omitted.

CARRIER PROTEIN

The carrier protein is chosen so that when the Vi polysaccharide is conjugated it is able to induce T-cell-dependent B-cell immune response. The carrier proteins are produced by culture of suitable micro-organisms; the bacterial purity of the culture is verified; the culture may be inactivated; the carrier protein is purified by a suitable method.

Only a carrier protein that complies with the following requirements may be used in the preparation of the conjugate.

Identification

The carrier protein is identified by a suitable immunochemical method (2.2.14).

Diphtheria toxoid. Where diphtheria toxin-like protein or recombinant form is used as the carrier, it contains not less than 90 per cent of CRM197, determined by a suitable method. Suitable tests are carried out, for validation or routinely, to demonstrate that the product is non-toxic.

Tetanus toxoid. Where tetanus toxoid is used as the carrier, it is produced as described in Tetanus vaccine (adsorbed) and complies with the requirements prescribed therein for bulk purified toxoid, except that the antigenic purity is not less than 1500 Lf per milligram of protein nitrogen.

Diphtheria protein CRM 197. Suitable tests are carried out, for validation or routinely, to demonstrate that the product is non toxic. The protein obtained contains not less than 90.0 per cent of Diphtheria CRM 197 protein, when tested by suitable method.

OMP (Meningococcal group B outer membrane protein complex)

OMP complies with the following requirements for lipopolysaccharide and pyrogens.

Lipopolysaccharide. Not more than 8.0 per cent of lipopolysaccharide, determined by a suitable method.

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Pyrogens (2.2.8). Inject into each rabbit 0.25 µg OMP per kg of body weight, for determining the pyrogenic effect.

NOTE — Recombinant Pseudomonas aeroginosa Exoprotein A (r-EPA) may also be used as carrier protein.

Degree of Activation of modified carrier protein should be assessed.

BULK CONJUGATE

Vi Polysaccharide is chemically modified to enable conjugation; it is usually partly depolymerized either before or during this procedure. Reactive functional groups or spacers may be introduced into the carrier protein or Vi Polysaccharide prior to conjugation. As a measure of consistency, the extent of derivatisation is monitored. The conjugate is obtained by the covalent binding of Vi Polysaccharide and carrier protein. Where applicable, unreacted but potentially reactogenic functional groups are made unreactive by means of capping agents; the conjugate is purified to remove reagents.

Only a Vi Polysaccharide bulk conjugate that complies with the following requirements may be used in the preparation of the final bulk vaccine.

For each test and for each particular product, limits of acceptance are established and each batch of conjugate must be shown to comply with the limits of acceptance as approved by NRA. For a freeze-dried vaccine, some of the tests may be carried out on the final lot rather than on the bulk conjugate where the freeze-drying process may affect the component being tested.

Identification

Carry out the test for identification by a suitable immunochemical method or NMR Spectroscopy for identification of bulk conjugate.

Vi Polysaccharide

Vi Polysaccharide content is Vi Polysaccharide content is determined by Hestrin method or HPAEC-PAD or Acridine orange dye based UV-Vis spectrophotometry method or Immunochemical method.

Protein content (2.7.1). The protein content in the bulk conjugate should be determined by means of an appropriate validated assay and comply with limits for the particular product. Each batch of bulk conjugate must be shown to comply with this limit.

Vi Polysaccharide to protein ratio. Determine the ratio by calculation.

Molecular-size distribution. Molecular size distribution is determined by size exclusion chromatography (2.4.16).

The relative molecular size of the polysaccharide-protein conjugate should be determined for each purified bulk conjugate using a gel matrix appropriate to the size of the conjugate. The method should be validated and should have the specificity to distinguish the polysaccharide-protein conjugate from other components that may be present. An acceptable value is established for the particular product and each batch of Vi polysaccharide must be shown to comply with this limit.

Typically the size of the polysaccharide – protein conjugate may be examined by methods such as gel filtration using HPSEC (High Performance Size Exclusion Chromatography) on an appropriate column.

Free Vi polysaccharide. Unbound saccharide is determined after removal of the conjugate, for example by DOC precipitation, Gel filtration, size-exclusion, anion exchange and hydrophobic chromatography and ultracentrifugation. The free Vi Polysaccharide can then be quantified by a range of techniques, including high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and immunoassays with anti-Vi Polysaccharide antibodies.

An acceptable value is established for the particular product and each batch of bulk conjugate must be shown to comply with this limit.

Free carrier protein. Determine the content, either directly by a suitable method or by deriving the content by calculation from the results of other tests. An acceptable value is established for the particular product and each batch of bulk conjugate must be shown to comply with this limit.

Residual reagents. Where applicable, tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established for the particular product and each batch of Vi polysaccharide must be shown to comply with this limit. Where validation studies have demonstrated removal of a residual reagent, the test on purified Vi polysaccharide may be omitted.

Sterility (2.2.11). Complies the test for sterility using for each medium 10 ml or the equivalent of 100 doses, whichever is less.

Specific Toxicity. The bulk conjugate should be tested for absence of specific toxicity of the carrier protein where appropriate

FINAL BULK VACCINE

An adjuvant, an antimicrobial preservative and a stabilizer may be added to the bulk conjugate before dilution to the final concentration with a suitable diluent so that the volume corresponding to 1 dose contains established amount of

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polysaccharide and the solution is isotonic with blood (250 mOsmol/kg to 350 mOsmol/kg).

Only a final bulk vaccine that complies with the following requirements may be used in preparation of the final lot.

pH (2.4.24). 6.5 to 7.5

Osmolality (2.4.23). The osmolality should be between 250 mOsmol per kg to 350 mOsmol per kg.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physio-chemical method. The content is not less than 85.0 per cent and not greater than 115.0 per cent of the intended amount.

Sterility (2.2.11). It complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

Only a final lot that is satisfactory with respect to each of the following requirements and the requirements given below under Identification and Tests may be released for use. Provided the test for antimicrobial preservative has been carried out on the final bulk vaccine, it may be omitted on the final lot.

Identification

Carry out identification test by a suitable immunochemical method.

Tests

pH (2.4.24). 6.5 to 7.5

Osmolality (2.4.23). The osmolality should be between 250 mOsmol/kg to 350 mOmol/kg.

Aluminium (2.3.9). Maximum 1.25 mg of aluminium per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Also, carry out test for degree of adsorption for the carrier protein being used.

Water (2.3.43). Not more than 3.0 per cent, as determined by thermogravimetry, Karl fischer or any other suitable method for freeze-dried vaccines.

O-Acetyl content (2.7.1). 0.0034 (± 25 per cent) µmole per µg of polysaccharide [0.085 (± 25 per cent) µmole per dose for vaccine having label claim of 25 µg of polysaccharide per dose and 0.017 (± 25 per cent) µmole per dose for vaccine having 5 µg of polysaccharide per dose]. O-acetyl content can be quantified by either HPAEC-CD (High-Performance Anion Exchange Chromatography with Conductivity Detection), by NMR or by by method as described below.

Test solution. Take 3 ml of the finished product sample in each of the 3 tubes (2 reaction solutions and 1 correction solution).

Reference solutions. Dissolve 0.150 g of *acetylcholine chloride* in 10 ml of *water* (stock solution containing 15 g/l of acetylcholine chloride). Immediately before use, dilute the stock solution with *water* to prepare working dilution containing 150 µg per ml of acetylcholine chloride. In 10 tubes, place in duplicate (reaction and correction solutions) 0.1 ml, 0.2 ml, 0.5 ml, 1.0 ml and 1.5 ml of the working dilution and make up the volume to 3 ml with water for injection which corresponds to give O-acetyl concentration of 0.0825, 0.1650, 0.4125, 0.8250 and 1.2375 µ moles per ml.

Add 0.5 ml of a mixture of 1 volumes of water and 2 volumes of *dilute hydrochloric acid* in each correction tube. Add 1.0 ml alkaline hydroxylamine solution (Freshly prepared by mixing equal volumes of 2 M Hydroxylamine Hydrochloride and 3.5 M sodium Hydroxide) to all the correction, reaction tubes. Allow the reaction to proceed for exactly 2 min at room temperature. Add 0.5 ml of a mixture of 1 volumes of water and 2 volumes of *dilute hydrochloric acid* in each of the reaction tubes exactly after 2 min. Add 0.5 ml of a 20 per cent solution of *ferric chloride* in *0.2 M hydrochloric acid* to each tube, stopper the tubes and shake vigorously to remove bubbles.

Measure the absorbance of each solution of correction tube against its corresponding solution of reaction tube at 540 nm. Draw a calibration curve from the corrected absorbance's for the 5 reference solutions and the corresponding content of acetylcholine chloride and read from the curve the content of acetylcholine chloride in the test solution for each volume tested. Calculate the mean of the 2 values.

1 mole of acetylcholine chloride (181.7 g) is equivalent to 1 mole of O-acetyl (43.05 g).

Routine release testing of each lot for o-acetyl content in the final product may be omitted if the o- acetyl content is measured at the level of conjugate bulk and the data validating the process that were obtained during the product's development confirmed that formulation and filling do not alter the integrity of the functional groups.

Free formaldehyde (2.3.20). Not more than 0.02 per cent w/v.

Free Vi Polysaccharide

Unbound saccharide is determined after removal of the conjugate, for example by DOC precipitation, Gel filtration, size-exclusion, anion exchange and hydrophobic chromatography and ultracentrifugation. The free Vi Polysaccharide can then be quantified by a range of techniques, including high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and immunoassays with anti-Vi Polysaccharide antibodies.

An acceptable value is established for the particular product and each batch of bulk conjugate must be shown to comply with this limit.

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Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable physiochemical method. The content is not less than 85.0 per cent and not more than 115.0 per cent of the content stated on the label. If phenol has been used in the preparation, the content is not more than 2.5 g per l.

Sterility (2.2.11). It complies with the test for sterility.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inject per kilogram of the rabbit's weight a quantity of 0.025 µg of Vi Polysaccharide for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein or tetanus toxoid or OMP as carrier.

OR

A validated test for bacterial endotoxins (2.2.3) may be used instead of the test for pyrogens. The content of bacterial endotoxins determined by a suitable method is within the limit approved for the specific product by the National Regulatory authority.

Assay

Determine Vi polysaccharide by a range of techniques, including High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) and a suitable immunochemical method, using a reference purified polysaccharide. The estimated amount of polysaccharide per dose is 80.0 per cent to 120.0 per cent of the content stated on the label. The confidence limits ($P = 0.95$) of the estimated amount of polysaccharide are not less than 80.0 per cent and not more than 120.0 per cent.

Labelling. The label states (1) the number of micrograms of Vi polysaccharide per human dose (2); the type and number of micrograms of carrier protein per human dose (3); nature and amount of preservative if added (4); nature and quantity of adjuvant if added (5); storage temperature (6); Route of administration.

Typhus Vaccine

Typhus Vaccine is a sterile suspension of killed rickettsiae of a strain or strains of epidemic typhus rickettsiae *(Rickettsia prowazeki)* selected for antigenic efficiency.

The vaccine is prepared by injecting virulent rickettsiae into the yolk sacs of fertile eggs which have been incubated for 7 days. Within 9 to 13 days after the injection, the yolk sacs are collected under aseptic conditions and subjected to suitable treatment to liberate the maximum number of rickettsiae. The material is suspended in *saline solution* or other suitable solution isotonic with blood to which *formaldehyde solution* has been added so that the concentration of formaldehyde is 0.2 per cent to 0.5 per cent. The suspension so formed contains 10 per cent to 15 per cent w/w of yolk sac tissue. It is purified by treatment with ether. The aqueous middle layer of the resultant mixture is collected and distributed under aseptic conditions into sterile containers which are then sealed so as to exclude micro-organisms.

The vaccine may also be prepared from the lungs of small rodents in which rickettsial pneumonias have been caused by inhalation of massive doses of virulent rickettsiae, or from the peritoneal cavities of gerbils which have received intraperitoneal injections of rickettsiae.

Description. A slightly turbid liquid; a white deposit of the rickettsiae may separate on standing which can be readily redistributed on shaking.

Identification

The vaccine specifically protects laboratory animals against epidemic typhus.

Tests

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Potency. Carry out the biological assay of typhus vaccine described below. The vaccine passes the test if the serum of immunised guinea-pigs, when diluted 32-fold, protects mice against the toxin of epidemic typhus rickettsiae.

Biological Assay of Typhus Vaccine

Inject subcutaneously 0.5 ml of the vaccine under examination into each of 10 or more healthy guinea-pigs, each weighing between 400 and 500 g. After 7 days inject once again 0.5 ml of the vaccine into the animals. Fourteen days after the second injection bleed the animals and pool aliquots of the sera. Test the pools against a toxic substance prepared from injected yolk sacs of living fertile eggs as described below.

Incubate for 4 to 10 days after inoculation with epidemic typhus rickettsiae. Harvest the yolk sac and stain a smear by the Macchiavello technique. Use only yolk sac of living embryos showing 4+ or more rickettsiae. Determine the wet weight of the yolk sac, grind the yolk sac with a suitable grade of *aluminium oxide* and suspend in sterile milk (pH adjusted to 7.6) or in a mixture of one part of fresh egg yolk and 99 parts of *sodium chloride injection* using, for each g of the yolk sac, 10 ml of the vehicle. Centrifuge this suspension for 5 minutes at low speed, decant the supernatant liquid and distribute in ampoules for freezing. Dilute a small amount of this suspension 8-, 16-, 32- and 64-fold with *saline solution*. Inject intravenously doses of 0.5 ml of each dilution into 8 white mice, each weighing between 11 and 15 g. 18 hours later, estimate the toxicity of the preparation as the LD_{50} per g of yolk sac, calculated from the numbers of mice killed by each

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dilution. Those toxic suspensions showing 160 or more LD_{50} per g of yolk sac are satisfactory for use in the neutralisation test. The remainder of the suspension may be sealed in ampoules and preserved for 18 months in liquid nitrogen.

Prepare mixtures of the toxic substance and the sera under test for the neutralisation test in mice and allow to stand for 2 hours before injection. Inject intravenously into mice, each weighing between 11 and 15 g, 0.5 ml containing 2 LD_{50} of the toxic substance together with serial 1 to 2 dilutions of serum. Keep the mice in an incubator at a temperature between 28° and 30° until the results of the test are recorded. Record the deaths at the end of 24 hours and express the results in terms of the highest dilution giving complete protection.

Storage. Store at a temperature 2° to 8°. When stored under the prescribed conditions, the vaccine may be expected to retain its potency for at least 1 year.

Labelling. The label states (1) the nature of the preparation, i.e., whether it has been prepared in eggs, rodent's lungs or otherwise and whether it has been purified; (2) the name and proportion of any added preservative.

Varicella Vaccine, Live

Varicella Vaccine (Live) is a freeze-dried preparation of a suitable attenuated strain of *Herpesvirus varicellae*.

Production

General provisions

The production of vaccine is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to yield consistently live varicella vaccines of adequate immunogenicity and safety in man. The virus in the final vaccine shall not have been passaged in cell cultures beyond the 38th passage from the original isolated virus.

The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy.

Substrate for virus propagation

The virus is propagated in human diploid cells (2.7.2).

SEED LOT

The strain of varicella virus shall be identified as being suitable by historical records which shall include information on the origin of the strain and its subsequent manipulation. The virus shall at no time have been passaged in continuous cell lines. Seed lots are prepared in the same kind of cells as those used for the production of the final vaccine. To avoid the unnecessary use of monkeys in the test for neurovirulence, virus seed lots are prepared in large quantities and stored at temperatures below -20°, if freeze-dried, or below -60°, if not freeze-dried.

Only a virus seed lot that complies with the following requirements may be used for virus propagation.

Identification

The master and working seed lots are identified as varicella virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The virus concentration of the master and working seed lots is determined as prescribed under Assay to monitor consistency of production.

Extraneous agents (2.7.3). Complies with the requirements for seed lots for live virus vaccines; a sample of 50 ml is taken for the test in cell cultures.

Neurovirulence (2.7.5). Complies with the test for neurovirulence of live virus vaccines.

Mycoplasma (2.7.4).Complies with the test for absence of mycoplasma.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled. Approved animal (but not human) serum may be used in the media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. 5.0 per cent, but not less than 50.0 ml, of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells). The infected cells constituting a single harvest are washed, released from the support surface and pooled. The cell suspension is disrupted by sonication.

Only a virus harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification

The virus harvest contains virus that is identified as varicella virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The concentration of infective virus in virus harvests is determined as prescribed under assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

Extraneous agents (2.7.3). Use 50 ml for the test in cell cultures.

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Control cells. The control cells of the production cell culture from which the single harvest is derived comply with a test for identity and with the requirements for extraneous agents (2.7.3).

Mycoplasma (2.7.4).Complies with the test for absence of mycoplasma.

FINAL BULK VACCINE

Virus harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. Carry out test for sterility (2.2.11) using 10 ml of bulk for each sterility medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers and freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to prevent contamination and the introduction of moisture.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Identification

When the vaccine reconstituted as stated on the label is mixed with specific *Herpesvirus varicellae* antibodies, it is no longer able to infect susceptible cell cultures.

Tests

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Bovine serum albumin. Not more than 0.5 µg per human dose, determined by a suitable immunochemical method (2.2.14).

Water (2.3.43). Not more than 3.0 per cent, determined by the semi-micro determination of water.

Mycoplasma (2.7.4).Complies with the test for absence of mycoplasma.

Assay

Titrate for infective virus, using at least ten cell cultures for each fourfold dilution or by a technique of equal precision. Use a suitable virus reference preparation to validate each assay. The virus concentration is not less than the minimum stated on the label.

Labelling. The label states (1) the strain of virus used for the preparation of the vaccine; (2) the type and origin of the cells used for the preparation of the vaccine; (3) that contact with disinfectants is to be avoided; (4) the minimum virus concentration; (5) that the vaccine is not to be administered to pregnant women; (6) the time within which the vaccine must be used after reconstitution.

Viper Venom

Daboia Venom

Viper Venom is the dried secretion obtained from the poison glands of *Viperae russelli* and other species of *Viperae* (Fam. Viperidae).

Viper Venom contains not less than 50 Mouse Units per mg.

Production

Immediately after extraction, the poisonous secretion is dried from the frozen state. The dried venom is pooled, mixed, dissolved in ice-cold *water for injection* and then filtered through a bacteria-proof filter to give a stock solution. Further dilutions of the stock solution are made with *water for injection* under aseptic conditions to give solutions with the required number of Mouse Units per ml. These solutions are then distributed in single dose sterile glass containers, dried from the frozen state and sealed at a pressure not exceeding 2.75 kPa.

Description. An almost white or very light yellow, dry powder which when mixed with *water* yields a clear solution with some insoluble residue.

Identification

A. Produces almost immediate coagulation of blood and citrated human plasma.

B. Mix the soluble fraction from at least 0.6 mg with 1 ml of *polyvalent antisnake venom serum* and incubate the mixture at 37° for 30 minutes. Inject 0.5 ml of the mixture intravenously into a group of mice weighing between 18 and 20 g. Observe the animals for 24 hours; no animal dies.

Tests

Sterility (2.2.11). Complies with tests for sterility.

Assay. Carry out the biological assay of snake venom described below:

Biological assay of snake venom

Dissolve a quantity of the freeze-dried venom equivalent to 50 Mouse Units in 25 ml of *saline solution*. Inject 0.5 ml intravenously into each of 10 mice weighing between 18 and

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20 g and observe the animals for 24 hours. Not less than 3 and not more than 8 of the mice die in 2 to 24 hours. If the number of deaths is not within this range, change the dilution of the venom suitably.

Express the result in terms of number of Mouse Units per mg.

NOTE — The quantity in mg of the venom which will kill in 2 to 24 hours not less than 3 and not more than 8 mice represents one Mouse Unit.

Storage. Store in single dose, light-resistant containers.

Labelling. The label states (1) the number of Mouse Units per container; (2) the volume of *water for injection* to be used for reconstitution.

Yellow Fever Vaccine

Yellow Fever Vaccine (Live) is a freeze-dried preparation of the 17D strain of yellow fever virus grown in fertilised hen eggs.

Production

General provisions

The production of vaccine is based on a virus seed-lot system. The production method shall have been shown to yield consistently the yellow fever vaccine (live) of acceptable immunogenicity and safety for man.

The production method is validated to demonstrate that the product, if tested, would comply with the test for safety and efficacy.

Reference preparation. In the test for neurotropism, a suitable batch of vaccine known to have satisfactory properties in man is used as the reference preparation.

Substrate for virus propagation

Virus for the preparation of master and working seed lots and for all vaccine batches is grown in the tissues of chick embryos from a flock free from specified pathogens (2.7.7).

SEED LOT

The 17D strain shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are prepared in large quantities and stored at a temperature below -60°. Master and working seed lots shall not contain any human protein or added serum.

Unless otherwise justified and authorised, the virus in the final vaccine shall be between passage levels 204 and 240 from the original isolate of strain 17D. A working seed lot shall be only one passage from a master seed lot.

A working seed lot shall be used without intervening passage as the inoculums for infecting the tissues used in the production of a vaccine lot, so that no vaccine virus is more than one passage from a seed lot that has passed all the safety tests.

Only a virus seed lot that complies with the following requirements may be used for virus propagation.

Identification

The master and working seed lots are identified as containing yellow fever virus by serum neutralisation in cell culture, using specific antibodies or by suitable molecular methods (e.g. Nucleic acid amplification techniques (NAT), sequencing).

Extraneous agents (2.7.3). Each working seed lot complies with the test for extraneous agents.

Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

PROPAGATION AND HARVEST

All processing of the fertilised eggs is done under aseptic conditions in an area where no other infectious agents or cells are handled at the same time. Two per cent but not less than twenty and not more than eighty eggs are set aside as uninfected control eggs. After inoculation and incubation at a controlled temperature, only living and typical chick embryos are harvested. The age of the embryo at the time of virus harvest is reckoned from the initial introduction of the egg into the incubator and shall not be more than 12 days. After homogenisation and clarification by centrifugation, the extract of embryonic pulp is tested as described below and kept at -70° or colder until further processing. Virus harvests that comply with the prescribed tests may be pooled. No human protein is added to the virus suspension at any stage during production. If stabilisers are added, they shall have been shown to have no antigenic or sensitising properties for man.

Only a single harvest that complies with the following tests may be used in the preparation of the final bulk vaccine.

Identification

The single harvest contains virus that is identified as yellow fever virus by serum neutralisation in cell culture, using specific antibodies or by suitable molecular methods (e.g. Nucleic acid amplification techniques (NAT), sequencing).

Control eggs. Complies with the tests for extraneous agents (2.7.3).

Virus concentration. In order to calculate the dilution for formulation of the final bulk, each single harvest is titrated as described under Assay.



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Mycoplasma (2.7.4). Complies with the test for absence of mycoplasma.

Mycobacteria (2.7.14). Complies with the test for absence of mycobacteria.

FINAL BULK VACCINE

Single harvests that comply with the tests prescribed above are pooled and clarified again. A test for protein nitrogen content is carried out. A suitable stabiliser may be added and the pooled harvests diluted as appropriate. Only a final bulk vaccine that complies with the following tests may be used in the preparation of the final lot.

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11), carried out using 10 ml of bulk for each sterility medium.

Protein nitrogen content (2.3.30). The protein nitrogen content, before the addition of any stabiliser, is not more than 0.25 mg per human dose.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers and freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to prevent contamination and the introduction of moisture.

Only a final lot that is satisfactory with respect to thermal stability and each of the tests given under Identification, Tests and Assay may be released for use. Provided that the test for ovalbumin has been performed with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Thermal stability. Maintain samples of the final lot of freeze-dried vaccine in the dry state at 37° for 14 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for unheated vaccine. The virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

Identification

When the vaccine reconstituted as stated on the label is mixed with specific yellow fever virus antibodies, there is a significant reduction in its ability to infect susceptible cell cultures or any other validated molecular method with the approval of National Regulatory Authority.

Tests

Bacterial and fungal contamination. Complies with the test for sterility (2.2.11).

Ovalbumin. Not more than 5 µg of ovalbumin per human dose, determined by a suitable immunochemical method (2.2.14).

Bacterial endotoxins (2.2.3). Not more than 5 IU of bacterial endotoxin per human dose.

Water (2.3.43). Not more than 3.0 per cent, determined by the semi-micro determination of water.

Assay

Titrate for infective virus in cell cultures using at least 3 separate containers of vaccine. Titrate 1 container of an appropriate virus reference preparation in triplicate to validate each assay.

The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. Calculate the individual virus concentration for each container of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations using the usual statistical methods. The combined virus concentration for the 3 containers of vaccine is compared to the results of the reference preparation titrated in parallel, to obtain results in International Units.

The assay is not valid if:

- the confidence interval (P = 0.95) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ IU;
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ IU from the established value.

The assay is repeated if the confidence interval (P = 0.95) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ IU; data obtained from valid assays only are combined by the usual statistical methods to calculate the virus concentration of the sample. The confidence interval (P = 0.95) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ IU.

The virus concentration is not less than the equivalent in $TCID_{50}$ or PFU of 1×10^3 mouse LD_{50} per human dose or 1×10^3 IU per human dose or 3.0 $\log_{10}$ IU per human dose. If the virus concentration is determined in $TCID_{50}$ or PFU, the relationship between mouse LD_{50} and $TCID_{50}$ or PFU is established by each laboratory and approved by the competent authority. The method shown below, or another suitable technique, may be used to determine the mouse LD_{50} or IU per human dose or 3.0 $\log_{10}$ per human dose.

Mouse LD_{50}. The statistically calculated quantity of virus suspension that is expected to produce fatal specific encephalitis in 50 per cent of mice of a highly susceptible strain, 4 to 6 weeks of age, after intracerebral inoculation.

Appropriate serial dilutions of the reconstituted vaccine are made in diluent for yellow fever virus (0.75 per cent solution of *bovine albumin* in *phosphate-buffered saline pH 7.4*, or

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any other diluent that has been shown to be equivalent for maintaining the infectivity of the virus).

Mice of a highly susceptible strain, 4 to 6 weeks of age, are injected intracerebrally under anesthesia with 0.03 ml of the vaccine dilution. Groups of not less than eight mice are used for each dilution; the series of dilutions is chosen so as to cover the range 0 to 100.0 per cent mortality of the mice. Injection of the mice is performed immediately after the dilutions have been made. The mice are observed for 21 days and all deaths are recorded. Only survivors and deaths caused by typical yellow fever infections are counted in the computations. Mice paralyzed on the twenty-first day of observation are counted as survivors. The assay is not valid unless the confidence interval (P = 0.95) of the estimated virus concentration of the reference preparation for the 3 replicates

Tests in monkeys for Yellow Fever Vaccine

Each master and working seed lot complies with the following tests in monkeys for viraemia (viscerotropism), immunogenicity and neurotropism. The monkeys shall be *Macaca spp.* susceptible to yellow fever virus and shall have been shown to be non-immune to yellow fever at the time of injecting the seed virus. They shall be healthy and shall not have received previously intracerebralor intraspinal inoculation. Furthermore, they shall not have been inoculated by other routes with neurotropic viruses or with antigens related to yellow fever virus. Not fewer than ten monkeys are used for each test.

Use a test dose of 0.25 ml containing the equivalent of not less than 5000 (3.7 log10) IU and not more than 50,000 (4.7 log10) IU, determined by a titration for infectious virus and using the established equivalence between virus concentration and mouse LD_{50} (see under Assay) determined by an in vitro titration for infectious virus in cell culture. Inject the test dose into one frontal lobe of each monkey under anesthesia and observe the monkeys for not less than 30 days.

Viraemia (Viscerotropism). Viscerotropism is indicated by the amount of virus present in serum. Take blood from each of the test monkeys on the second, fourth and sixth days after inoculation and prepare serum from each sample. Prepare 1:10, 1:100 and 1:1000 dilutions from each serum and inoculate each dilution into a group of at least four cell culture vessels used for the determination of the virus concentration. The seed lot complies with the test if none of the sera contains more than the equivalent of 500 (2.7 $\log_{10}$) IU in 0.03 ml and at most one serum contains more than the equivalent of 100 (2.0 $\log_{10}$) IU in 0.03 ml.

Immunogenicity. Take blood from each monkey 30 days after the injection of the test dose and prepare serum from each sample. The seed lot complies with the test if at least 90.0 per cent of the test monkeys are shown to be immune, as determined by examining their sera in the test for neutralization of yellow fever virus described below.

It has been shown that a low dilution of serum (for example, 1:10) may contain non-specific inhibitors that influence this test; such serum shall be treated to remove inhibitors. Mix dilutions of at least 1: 10, 1:40 and 1: 160 of serum from each monkey with an equal volume of 17D vaccine virus at a dilution that will yield an optimum number of plaques with the titration method used. Incubate the serum-virus mixtures in a water bath at 37° for 1 h and then cool in iced water; add 0.2 ml of each serum-virus mixture to each of four cell-culture plates and proceed as for the determination of virus concentration. Inoculate similarly ten plates with the same amount of virus plus an equal volume of a 1:10 dilution of monkey serum known to contain no neutralizing antibodies to yellow fever virus. At the end of the observation period, compare the mean number of plaques in the plates receiving virus plus non-immune serum with the mean number of plaques in the plates receiving virus plus dilutions of each monkey serum. Not more than 10 per cent of the test monkeys have serum that fails to reduce the number of plaques by 50.0 per cent at the 1:10 dilution.

Neurotropism. Neurotropism is assessed from clinical evidence of encephalitis, from incidence of clinical manifestations and by evaluation of histological lesions, in comparison with ten monkeys injected with the reference preparation. The seed lot is not acceptable if either the onset or duration of the febrile reaction or the clinical signs of encephalitis and pathological findings are such as to indicate a change in the properties of the virus.

Clinical evaluation. The monkeys are examined daily for 30 days by personnel familiar with clinical signs of encephalitis in primates (if necessary, the monkeys are removed from their cage and examined for signs of motor weakness or spasticity).

The seed lot is not acceptable if in the monkeys injected with it the incidence of severe signs of encephalitis, such as paralysis or inability to stand when stimulated, or mortality is greater than for the reference vaccine. These and other signs of encephalitis, such as paresis, in-coordination, lethargy, tremors or spasticity are assigned numerical values for the severity of symptoms by a grading method. Each day each monkey in the test is given a score based on the scale:

Grade 1 — rough coat, not eating,

Grade 2 — high-pitched voice, inactive, slow moving,

Grade 3 — shaky, tremors, unco-ordinated, limb weakness,

Grade 4 — inability to stand, limb paralysis or death (a dead monkey receives a daily score of 4 from the day of death until day 30).

A clinical score for a particular monkey is the average of its daily scores; the clinical score for the seed lot is the mean of

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the individual monkey scores. The seed lot is not acceptable if the mean of the clinical severity scores for the group of monkeys inoculated with it is significantly greater (P = 0.95) than the mean for the group of monkeys injected with the reference preparation. In addition, special consideration is given to any animal showing unusually severe signs when deciding on the acceptability of the seed lot.

Histological evaluation. Five levels of the brain are examined including:

Block I — the corpus striatum at the level of the optic chiasma,

Block II — the thalamus at the level of the mamillary bodies,

Block III — the mesencephalon at the level of the superior colliculi,

Block IV — the pons and cerebellum at the level of the superior olives,

Block V — the medulla oblongata and cerebellum at the level of the mid-inferior olivary nuclei.

Cervical and lumbar enlargements of the spinal cord are each divided equally into six blocks; 15 µm sections are cut from the tissue blocks embedded in paraffin wax and stained with gallocyanin. Numerical scores are given to each hemisection of the cord and to structures in each hemisection of the brain as listed below. Lesions are scored as follows:

Grade 1 — Minimal: 1 to 3 small focal inflammatory infiltrates. Degeneration or loss of a few neurons.

Grade 2 — Moderate: 4 or more focal inflammatory infiltrates. Degeneration or loss of neurons affecting not more than one third of cells.

Grade 3 — Severe: moderate focal or diffuse inflammatory infiltration. Degeneration or loss of 33 to 90 per cent of the neurons.

Grade 4 — Overwhelming: variable but often severe inflammatory reaction. Degeneration or loss of more than 90.0 per cent of neurons.

It has been found that inoculation of yellow fever vaccine into the monkey brain causes histological lesions in different anatomical formations of the central nervous system with varying frequency and severity (I. S. Levenboo k*et al., Journal of Biological Standardization*, 1987, 15, 305-313). Based on these two indicators, the anatomical structures can be divided into target, spared and discriminator areas. Target areas are those which show more severe specific lesions in a majority of monkeys irrespective of the degree of neurovirulence of the seed lot. Spared areas are those which show only minimal specific lesions and in a minority of monkeys. Discriminator areas are those where there is a significant increase in the frequency of more severe specific lesions with seed lots having a higher degree of neurovirulence. Discriminator and target areas for *Macaca cynomolgus* and *Macaca rhesus* monkeys are shown in the table 1.

Scores for discriminator and target areas are used for the final evaluation of the seed lot. The individual monkey score is calculated from the sum of individual target area scores in each hemisection divided by the number of areas examined. A separate score is calculated similarly for the discriminator areas.

Mean scores for the test group are calculated in two ways: (1) by dividing the sum of the individual monkey discriminator scores by the number of monkeys and (2) by dividing the sum of the individual monkey target and discriminator scores by the number of monkeys. These two mean scores are taken into account when deciding on the acceptability of the seed lot. The seed lot is not acceptable if either of the mean lesion scores is significantly greater (P = 0.95) than for the reference preparation.

Table 1 - The discriminator and target areas for monkey

Type of monkey	Discriminator areas	Target areas
Macaca cynomolgus	Globus pallidus	Substantia nigra
	Putamen	
	Anterior/median thalamic nucleus	
	Lateral thalamic nucleus	
Macaca rhesus	Caudate nucleus	Substantia nigra
	Globus pallidus	Cervical enlargement
	Putamen	Lumbar enlargement
	Anterior/ median thalamic nucleus	
	Lateral thalamic nucleus	
	Cervical enlargement	
	Lumbar enlargement	

Labelling. The label states (1) the strain of virus used in preparation; (2) that the vaccine has been prepared in chick embryos; (3) the minimum virus concentration; (4) that contact with disinfectants is to be avoided; (5) the period of time within which the vaccine is to be used after reconstitution.

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BLOOD AND BLOOD-RELATED PRODUCTS

Anti-A Blood Grouping Serum 4499
Anti-B Blood Grouping Serum 4500
Anti-AB Blood Grouping Reagent 4502
Anticoagulant Heparin Solution 4504
Anti-D (Rh_o) Immunoglobulin 4504
Anti-D Immunoglobulin for Intravenous Use 4505
Anti-D (IgM) Monoclonal Blood Grouping Reagent 4506
Anti-D Blend (IgM + IgG) Monoclonal Reagent 4507
Anti-D (IgG) Monoclonal Reagent 4509
Anti-Human Globulin (AHG) Serum 4509
Antithrombin III Concentrate 4511
Blood Grouping Lectins Anti-A_1 4512
Blood Grouping Lectins Anti-H 4513
Blood Grouping Reagents Anti-Fy^a, Anti-Fy^b 4515
Blood Grouping Reagents Anti-Jk^a, Anti-Jk^b 4516
Blood Grouping Reagents Anti-K, Anti-k 4518
Blood Grouping Reagents Anti-Le^a, Anti-Le^b 4519
Blood Grouping Reagents Anti-M, Anti-N 4520
Blood Grouping Reagents Anti-P_1 4522
Blood Grouping Reagents Anti-S, Anti-s 4523
Blood Grouping Serums 4524
Blood Grouping Serums Anti-D, Anti-C, Anti-E, Anti-c, Anti-e 4525
Concentrated Human Red Blood Corpuscles 4526
Cryoprecipitated Antihaemophilic Factor 4527
Dried Human Antihaemophilic Fraction 4528
Fibrin Sealant Kit 4533
Hepatitis B Immunoglobulin 4534
Human Albumin 4535
Human Coagulation Factor VII 4537
Human Coagulation Factor VIII (rDNA) 4539

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and yields positive reaction and red cell suspension which lacks the antigen corresponding to reagent antibody yields negative reaction.

For this 1 volume of Anti-A Monoclonal Blood Grouping Reagent is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension which exhibits the antigen corresponding to the reagent antibody and red cell suspension which lacks the antigen corresponding to reagent antibody. Centrifuge the tube at 1000 rpm for 1 minute. Tube with corresponding antigen gives positive reaction and the tube which lacks the antigen gives negative reaction.

Type of Reagent	*Test Red Blood Cells*	*Specificity*
Anti-A (Monoclonal)	A_1	Positive
	A_2	Positive
	A_2B	Positive
	B	Negative
	O	Negative

Reactivity

Haemolysis. It is defined as the release of hemoglobin from the red cells as a result of osmotic effect or due to the breaking up of the red blood cells. No immune haemolysis should be observed with the red blood cells used for specificity.

Type of Reagent	*Test Red Blood Cells*	*Haemolysis*
Anti-A (Monoclonal)	A_1	Absent
	A_2	Absent
	A_2B	Absent
	B	Absent
	O	Absent

Rouleaux. It is defined as a form of pseudo agglutination in which the red blood cells look like pile of coins. The red blood cells lacking the antigen corresponding to the reagent antibody should not show rouleaux formation. For this 1 volume of Anti-A Monoclonal Blood Grouping Reagent is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension which lacks the antigen corresponding to reagent antibody and the tube is centrifuged at 1000 rpm for 1 minute and observed microscopically for rouleaux formation.

Type of Reagent	*Test Red Blood Cells*	*Rouleaux*
Anti-A (Monoclonal)	B	Absent
	O	Absent

Prozone. It is defined as a phenomenon in which negative reactions are obtained with low dilutions of an antibody, while a positive reaction is obtained with higher dilutions. For this 1 volume of Anti-A Monoclonal Blood Grouping Reagent is mixed with 1 volume of 2-3 per cent red cell suspension in test tubes which exhibit the antigen corresponding to the reagent antibody for "15 minutes", "30 minutes" and "60 minutes". If the reaction grades are the same or increase as the incubation time increases, no prozone is present and if the reaction grades decrease as the incubation time increases, a prozone is present.

Type of Reagent	*Test Red Blood Cells*	*Prozone*
Anti-A (Monoclonal)	A_1	Absent
	A_2	Absent
	A_2B	Absent

Expiration date. The expiration date for Anti-A blood grouping reagent is not less than 1 year at 2° to 8°.

Storage. Store at a temperature between 2° and 8°.

Labelling. The label states that the source material was not reactive for hepatitis B surface antigen, but that no known test method offers assurance that products derived from human blood will not transmit hepatitis in case of polyclonal. Label also states that it is for *in vitro* diagnostic use.

NOTE—The labeling is in black lettering imprinted on paper that is white or is coloured completely or in part to match the specified blue colour standard.

Anti-B Blood Grouping Serum

Anti-B Blood Grouping Serum is a sterile, liquid or dried preparation containing the particular blood group antibodies derived from high-titered blood plasma or serum of human subjects, with or without stimulation by the injection of Blood Group Specific Substance B (or AB). It contains a suitable antimicrobial preservative.

Production

The monoclonal antibody technique devised by Kohler and Milsten has proved useful in producing high titre and specific antibodies. Laboratory animals, usually mice are immunized for the production of monoclonal antibodies. After suitable immune response, mouse spleen cells containing antibody secreting lymphocytes are fused to neoplastic plasma cells of infinite reproductive capacity from a mouse (that is myeloma cells). The resulting hybridomas are screened for antibody with the required specificity and affinity. The antibody secreting clones may then be propagated in tissue culture or by inoculation into mice in which case the antibodies are collected as ascites. The clonal line produces a single antibody, there is no need for absorption or to remove heterospecific antibodies. All antibody molecules produced by a clone of hybridoma cells are identical in terms of antibody structure and antigen specificity. Once one antibody secreting clone of cells has been established, antibody with same specificity and reaction characteristics will be available indefinitely.

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Identification

It agglutinates human red cells containing B-antigens, that is blood groups B and AB (including subgroups A_1B and A_2B).

Tests

It complies with the test for potency, in parallel with, and not less than equivalent to, the Reference Blood Grouping Serum Anti-B, in agglutinating red blood cells from Group B donors. It complies with the tests for specificity with Group A_1, B, and O cells and confirms the absence of contaminating antibodies reactive with M^s, Wr^a antigens as well as other antigens having an incidence of 1.0 per cent or greater in the general population (see under *Blood Grouping Serums Anti-D, Anti-C, Anti-E, Anti-c, Anti-e*). It complies with the test for avidity with Group B cells. All fresh or frozen red blood cell suspensions used for these tests are prepared under specified conditions and meet specified criteria. Anti-B Blood Grouping Serum may be artificially coloured yellow.

Anti-B Monoclonal Blood Grouping Reagents are also tested for their physical appearance, potency, avidity, intensity, specificity, rouleaux, haemolysis and prozone parameters and have values greater than or equal to the appropriate International Standards or suitable reference preparation as approved by National Regulatory Authority.

Physical Appearance and Colour

Anti-B Monoclonal Blood Grouping reagent should be visibly clear with no turbidity, precipitate, particles or gel formation. Anti-B Monoclonal blood grouping Reagents may be artificially colored yellow.

Potency. It is defined as the reciprocal of the greatest reagent dilution for which the reaction is graded at 1+. Anti-B Monoclonal Blood Grouping Reagent should have an average potency value greater than or equal to that of the reference preparation.

Type of Reagent	*Test Red Blood Cells*	*Titre*
Anti-B (Monoclonal)	B	1 : ≥ 256
	A_1B	1 : ≥ 128

Avidity. It is defined as the time taken for agglutination to appear first to the unaided eye when 1 volume of the Anti-B monoclonal Blood Grouping Reagent is mixed with 1 volume of a 40 per cent v/v suspension of human red corpuscles of specific group or sub group and compared with the time taken by that of the reference preparation. Rapid mixing is performed by spreading with use of agitation, with a centrifugal spiral movement to form a circular reaction surface about 3 cm in diameter. The value for avidity is the mean of three measurements.

Type of Reagent	*Test Red Blood Cells*	*Avidity*
Anti-B (Monoclonal)	B	≤ 4 seconds
	A_1B	≤ 6 seconds

Intensity. It is defined by the appearance of agglutination obtained after mixing undiluted Anti-B Monoclonal Blood Grouping Reagent with washed red blood cell suspension. For this 1 volume of the reagent is added to 1 volume of 40 per cent red cell suspension on a slide and mixed for 2 minutes. At the end of 2 minutes, reaction grade is compared with the strength of reaction obtained with the reference preparation.

Type of Reagent	*Test Red Blood Cells*	*Intensity*
Anti-B (Monoclonal)	B	4+
	A_1B	2+ to 3+

Specificity. Reagent is tested with red cell suspension which exhibits the antigen corresponding to the reagent antibody and yields positive reaction and red cell suspension which lacks the antigen corresponding to reagent antibody yields negative reaction.

For this 1 volume of Anti-B Monoclonal Blood Grouping Reagent is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension which exhibits the antigen corresponding to the reagent antibody and red cell suspension which lacks the antigen corresponding to reagent antibody. Centrifuge the tube at 1000 rpm for 1 minute. Tube with corresponding antigen gives positive reaction and the tube which lacks the antigen gives negative reaction.

Type of Reagent	*Test Red Blood Cells*	*Specificity*
Anti-B (Monoclonal)	A_1	Negative
	A_1B	Positive
	B	Positive
	O	Negative

Reactivity

Haemolysis. It is defined as the release of hemoglobin from the red cells as a result of osmotic effect or due to the breaking up of the red blood cells. No immune haemolysis should be observed with the red blood cells used for specificity.

Type of Reagent	*Test Red Blood Cells*	*Haemolysis*
Anti-B (Monoclonal)	A_1	Absent
	A_1B	Absent
	B	Absent
	O	Absent

Rouleaux. It is defined as a form of pseudo agglutination in which the red blood cells look like pile of coins. The red blood cells lacking the antigen corresponding to the reagent antibody should not show rouleaux formation. For this 1 volume of

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Anti-B Monoclonal Blood Grouping Reagent is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension which lacks the antigen corresponding to reagent antibody and the tube is centrifuged at 1000 rpm for 1 minute and observed microscopically for rouleaux formation.

Type of Reagent	*Test Red Blood Cells*	*Rouleaux*
Anti-B	A_1	Absent
(Monoclonal)	O	Absent

Prozone. It is defined as a phenomenon in which negative reactions are obtained with low dilutions of an antibody, while a positive reaction is obtained with higher dilutions. For this 1 volume of Anti-B Monoclonal Blood Grouping Reagent is mixed with 1 volume of 2-3 per cent red cell suspension in test tubes which exhibit the antigen corresponding to the reagent antibody for "15 minutes", "30 minutes" and "60 minutes". If the reaction grades are the same or increase as the incubation time increases, no prozone is present and if the reaction grades decrease as the incubation time increases, a prozone is present.

Type of Reagent	*Test Red Blood Cells*	*Prozone*
Anti-B	A_1B	Absent
(Monoclonal)	B	Absent

Expiration date. The expiration date for Anti-B blood grouping reagent is not less than 1 year at 2° to 8°.

Storage. Store at a temperature between 2° and 8°.

Labelling. The label states that the source material was not reactive for hepatitis B surface antigen, but that no known test method offers assurance that products derived from human blood will not transmit hepatitis in case of polyclonal. Label also states that it is for *in vitro* diagnostic use.

NOTE—The labelling is in black lettering imprinted on paper that is white or is coloured completely or in part to match the specified yellow colour standard.

Anti-AB Blood Grouping Reagent

Anti-AB Blood Grouping reagent is a sterile, liquid containing blood group antibodies which agglutinates human red cells containing A and B antigens that is of blood groups A, B and AB. It contains a suitable antimicrobial preservative. It is of two types polyclonal and monoclonal.

Production

The monoclonal antibody technique devised by Kohler and Milsten has proved useful in producing high titre and specific antibodies. Laboratory animals, usually mice are immunized for the production of monoclonal antibodies. After suitable immune response, mouse spleen cells containing antibody secreting lymphocytes are fused to neoplastic plasma cells of infinite reproductive capacity from a mouse (that is myeloma cells). The resulting hybridomas are screened for antibody with the required specificity and affinity. The antibody secreting clones may then be propagated in tissue culture or by inoculation into mice in which case the antibodies are collected as ascites. The clonal line produces a single antibody, there is no need for absorption or to remove heterospecific antibodies. All antibody molecules produced by a clone of hybridoma cells are identical in terms of antibody structure and antigen specificity. Once an antibody secreting clone of cells has been established, antibody with same specificity and reaction characteristics will be available indefinitely.

Identification

It agglutinates human red cells containing A and B antigens that is blood groups A, B and AB (including subgroups A_1, A_2, A_1B, A_2B, A_x).

Tests

Anti-AB Monoclonal Blood Grouping Reagents

Anti-AB Monoclonal Blood Grouping Reagents are tested for their physical appearance, potency, avidity, intensity, specificity, rouleaux, haemolysis and prozone parameters and have values greater than or equal to the appropriate International Standards or suitable reference preparation as approved by National Regulatory Authority.

Physical Appearance and Color

Anti-AB Monoclonal Blood Grouping reagent should be visibly clear with no turbidity, precipitate, particles or gel formation. Anti-AB Monoclonal blood grouping Reagents may be colorless or cherry colored.

Potency. It is defined as the reciprocal of the greatest reagent dilution for which the reaction is graded at 1+. Anti-AB Monoclonal Blood Grouping Reagent should have an average potency value greater than or equal to that of the reference.

Type of Reagent	*Test Red Blood Cells*	*Titre*
Anti-AB	A_1	$1 : \geq 256$
(Monoclonal)	A_2	$1 : \geq 128$
	B	$1 : \geq 256$

Avidity. It is defined as the time taken for agglutination to appear first to the unaided eye when 1 volume of the Anti-AB monoclonal Blood Grouping Reagent is mixed with 1 volume of a 40 per cent v/v suspension of human red corpuscles of specific group or sub group and compared with the time taken

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by that of the reference preparation. Rapid mixing is performed by spreading with use of agitation, with a centrifugal spiral movement to form a circular reaction surface about 3 cm in diameter. The value for avidity is the mean of three measurements.

Type of Reagent	*Test Red Blood Cells*	*Avidity*
Anti-AB (Monoclonal)	A_1	≤ 4 seconds
	A_2	≤ 6 seconds
	B	≤ 4 seconds

Intensity. It is defined by the appearance of agglutination obtained after mixing undiluted Anti-AB Monoclonal Blood Grouping Reagent with washed red blood cell suspension. For this 1 volume of the reagent is added to 1 volume of 40 per cent red cell suspension on a slide and mixed for 2 minutes. At the end of 2 minutes, reaction grade is compared with the strength of reaction obtained with the reference preparation.

Type of Reagent	*Test Red Blood Cells*	*Intensity*
Anti-AB (Monoclonal)	A_1	4+
	A_2	3+
	B	4+

Specificity. Reagent is tested with red cell suspension which exhibits the antigen corresponding to the reagent antibody and yields positive reaction and red cell suspension which lacks the antigen corresponding to reagent antibody yields negative reaction.

For this 1 volume of Anti-AB Monoclonal Blood Grouping Reagent is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension which exhibits the antigen corresponding to the reagent antibody and red cell suspension which lacks the antigen corresponding to reagent antibody. Centrifuge the tube at 1000 rpm for 1 minute. Tube with corresponding antigen gives positive reaction and the tube which lacks the antigen gives negative reaction.

Type of Reagent	*Test Red Blood Cells*	*Specificity*
Anti-AB (Monoclonal)	A_1	Positive
	A_2	Positive
	A_X	Positive
	B	Positive
	O	Negative

Reactivity

Haemolysis. It is defined as the release of hemoglobin from the red cells as a result of osmotic effect or due to the breaking up of the red blood cells. No immune haemolysis should be observed with the red blood cells used for specificity.

Type of Reagent	*Test Red Blood Cells*	*Haemolysis*
Anti-AB (Monoclonal)	A_1	Absent
	A_2	Absent
	A_X	Absent
	B	Absent
	O	Absent

Rouleaux. It is defined as a form of pseudo agglutination in which the red blood cells look like pile of coins. The red blood cells lacking the antigen corresponding to the reagent antibody should not show rouleaux formation. For this 1 volume of Anti-AB Monoclonal Blood Grouping Reagent is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension which lacks the antigen corresponding to reagent antibody and the tube is centrifuged at 1000 rpm for 1 minute and observed microscopically for rouleaux formation.

Type of Reagent	*Test Red Blood Cells*	*Rouleaux*
Anti-AB (Monoclonal)	O	Absent

Prozone. It is defined as a phenomenon in which negative reactions are obtained with low dilutions of an antibody, while a positive reaction is obtained with higher dilutions. For this 1 volume of Anti-AB Monoclonal Blood Grouping Reagent is mixed with 1 volume of 2-3 per cent red cell suspension in test tubes which exhibit the antigen corresponding to the reagent antibody for "15 minutes", "30 minutes" and "60 minutes". If the reaction grades are the same or increase as the incubation time increases, no prozone is present and if the reaction grades decrease as the incubation time increases, a prozone is present.

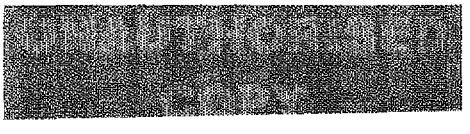
Type of Reagent	*Test Red Blood Cells*	*Prozone*
Anti-AB (Monoclonal)	A_1	Absent
	A_2	Absent
	B	Absent

Expiration date. The expiration date for Anti-AB blood grouping reagent is not less than 1 year at 2° to 8°.

Storage. Store at a temperature between 2° and 8°.

Labelling. The label states that the source material was not reactive for hepatitis B surface antigen, but that no known test method offers assurance that products derived from human blood will not transmit hepatitis in case of polyclonal. Label also states that it is for *in vitro* diagnostic use.

NOTE—The labelling is in black lettering imprinted on paper that is white or is coloured completely or in part to match the specified yellow colour standard.



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Anticoagulant Heparin Solution

Category. Anticoagulant.

Anticoagulant Heparin Solution is a sterile solution of Heparin Sodium in Sodium Chloride injection.

Anticoagulant Heparin Solution contains not less than 90.0 per cent and not more than 110.0 per cent of the stated potency in terms of Units per ml. It contains not less than 0.85 per cent and not more than 0.95 per cent of Sodium Chloride. It may be buffered. It contains no antimicrobial agents.

Heparin Sodium	75,000 units
Sodium Chloride Injection, a suficient quantity to make	1000 ml

Add the Heparin Sodium, in solid form or in solution, to the Sodium Chloride Injection, mix, filter if necessary, and sterilize.

Tests

pH (2.4.24). 5.0 to 7.5.

Bacterial endotoxins (2.2.3). Not more than 2.5 Endotoxin Units per ml.

Other tests. Complies with the tests stated under Parenteral Preparations (Injections).

Assay for heparin sodium. Proceed as directed in the Assay under Heparin Sodium Injection, substituting Anticoagulant Heparin Solution for the injection.

Assay for sodium chloride. Pipet 10 ml of solution in to a suitable container, add 2 ml of *potassium chromate*, and titrate with *0.1 M silver nitrate*.

1 ml of *0.1 M silver nitrate* is equivalent to 0.005844 g of NaCl.

Storage. Store in colourless, transparent single dose containers of type I or type II glass, or of a suitable plastic material.

Labelling. The label states (1) the number of heparin units; (2) the number of ml of solution required per 100 ml of whole blood.

Anti-D (Rh_0) Immunoglobulin

Human Anti-D Immunoglobulin

Human anti-D immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It contains specific antibodies against erythrocyte D-antigen and may also contain small quantities of other blood-group antibodies. Human normal immunoglobulin may be added.

It complies with the monograph on Human Normal Immunoglobulin, except for the minimum number of donors and the minimum total protein content. For products prepared by a method that eliminates immunoglobulins with specificities other than anti-D, where authorised, the test for antibodies to hepatitis B surface antigen is not required.

Production

Human anti-D immunoglobulin is preferably obtained from the plasma of donors with a sufficient titre of previously acquired anti-D antibodies. Where necessary, in order to ensure an adequate supply of human anti-D immunoglobulin, it is obtained from plasma derived from donors immunised with D-positive erythrocytes that are compatible in relevant blood group systems in order to avoid formation of undesirable antibodies.

Erythrocyte donors

Erythrocyte donors comply with the requirements for donors prescribed in the monograph on Human Plasma for Fractionation.

Immunisation

Immunisation of the plasma donor is carried out under proper medical supervision. Recommendations concerning donor immunisation, including testing of erythrocyte donors, have been formulated by the World Health Organisation (*Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives*, WHO Technical Report Series, No. 840, 1994 or subsequent revision).

Pooled plasma

To limit the potential B19 virus burden in plasma pools used for the manufacture of anti-D immunoglobulin, the plasma pool is tested for B19 virus using validated nucleic acid amplification techniques (2.8.1).

Tests

B19 virus DNA. Maximum 104 IU per ml. A positive control with 104 IU of B19 virus DNA per ml and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is nonreactive or if the result obtained with the internal control indicates the presence of inhibitors.

B19 virus DNA for NAT testing reference preparation is suitable for use as a positive control. If Human Normal Immunoglobulin is added to the preparation, the plasma pool from which it is derived complies with the above requirement for B19 virus DNA.

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Potency. Determine the assay of human anti-D immunoglobulin by any of the suitable method (2.8.2). The estimated potency is not less than 90.0 percent of the stated potency. The confidence limits (P 0.95) are not less than 80.0 percent and not more than 120.0 percent of the estimated potency.

Storage. For the liquid preparation, store protected from light, in a plastic container. For the freeze-dried preparation, store protected from light, in a plastic container.

Labelling. The label states (1) for liquid preparations, the volume of the preparation in the container and the protein content expressed in grams per litre; (2) for freeze-dried preparations, the quantity of protein in the container; (3) the route of administration; (4) for freeze-dried preparations, the name or composition and the volume of the reconstituting liquid to be added; (5) where applicable, that the preparation is suitable for use in the prophylaxis of hepatitis A infection; (6) where applicable, the anti-hepatitis A virus activity in International Units per ml; (7) where applicable, the name and amount of antimicrobial preservative in the preparation; (8) the number of International Units per container.

Anti-D Immunoglobulin for Intravenous Use

Anti-D Immunoglobulin Human for Intravenous Use

Anti-D Immunoglobulin for intravenous administration is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. It contains specific antibodies against erythrocyte D-antigen and may also contain small quantities of other blood-group antibodies. Human normal immunoglobulin for intravenous use may be added.

It complies with the monograph on Human Normal Immunoglobulin for Intravenous Administration, except for the minimum number of donors, the minimum total protein content, the limit for osmolality and the limit for prekallikrein activator. For products prepared by a method that eliminates immunoglobulins with specificities other than anti-D where authorised, the test for antibodies to hepatitis B surface antigen is not required; a suitable test for Fc function is carried out.

Production

Polyclonal (human) anti Rh (D) serum

Human anti-D immunoglobulin is preferably obtained from the plasma of donors with a sufficient titre of previously acquired anti-D antibodies. Where necessary, in order to ensure an adequate supply of human anti-D immunoglobulin, it is obtained from plasma derived from donors immunised with D-positive erythrocytes that are compatible in relevant blood group systems in order to avoid formation of undesirable antibodies.

Erythrocyte donors

Laboratory tests are carried out for each donation to detect the following viral markers:

1. Antibodies against human immunodeficiency virus 1 (anti-HIV-1),
2. Antibodies against human immunodeficiency virus 2 (anti-HIV-2),
3. Antibodies against hepatitis C virus (anti-HCV),
4. Hepatitis B surface antigen (HBsAg),

Pending complete harmonisation of the laboratory tests to be carried out, the competent authority may require that a test for alanine aminotransferase (ALT) also be carried out.

The test methods used are of suitable sensitivity and specificity and comply with the regulations in force. If a repeat-reactive result is found in any of these tests, the donation is not accepted.

Immunisation

Immunisation of the plasma donor is carried out under proper medical supervision. Recommendations concerning donor immunisation, including testing of erythrocyte donors, have been formulated by the World Health Organisation (*Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives*, WHO Technical Report Series, No. 840, 1994 or subsequent revision).

Pooled plasma

To limit the potential B19 virus burden in plasma pools used for the manufacture of anti-D immunoglobulin, the plasma pool is tested for B19 virus using validated nucleic acid amplification techniques (2.8.1).

Identification

Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and sample under examination in a dilution of 10 g per litre of protein. The main component of the sample corresponds to the IgG component of normal human serum.

Tests

B19 virus DNA. Maximum 10^4 IU per ml.

A positive control with 10^4 IU of B19 virus DNA per ml and, to test for inhibitors, an internal control prepared by addition of

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a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors.

B19 virus DNA for NAT testing RP is suitable for use as a positive control.

If Human normal immunoglobulin for intravenous administration is added to the preparation, the plasma pool from which it is derived complies with the above test for B19 virus DNA.

Assay. Determine the assay of human anti-D immunoglobulin by any of the suitable method (2.8.2). The estimated potency is not less than 90.0 percent of the stated potency. The confidence limits (P 0.95) are not less than 80.0 percent and not more than 120.0 percent of the estimated potency.

Storage. For the liquid preparation, store protected from light, in a colourless glass container, at the temperature stated on the label. For the freeze-dried preparation, store protected from light, in colourless glass container, at a temperature not exceeding 25°.

Labelling. The label states (1) for liquid preparations, the volume of the preparation in the container and the protein content expressed in gram per litre; (2) for freeze-dried preparations, the quantity of protein in the container; (3) the amount of immunoglobulin in the container; (4) the route of administration, (5) for freeze-dried preparations, the name or composition and the volume of the reconstituting liquid to be added; (6) the distribution of subclasses of immunoglobulin G present in the preparation; (7) where applicable, the amount of albumin added as a stabilizer; (8) the maximum content of immunoglobulin A; (9) the number of International Units per container.

Anti-D (IgM) Monoclonal Blood Grouping Reagent

Monoclonal antibodies are derived from hybridoma cell lines produced by fusing mouse antibody produced by B lymphocyte with mouse myeloma cells or are derived from a human B cell line through Epstein-Barr Virus (EBV) transformation. Each hybridoma cell line produces homogenous antibodies of only one immunoglobulin class, which are identical in their chemical structure and immunological activity.

The types of monoclonal anti-D reagents are:

1. IgM anti-D monoclonal reagent,
2. Blend of IgM and IgG monoclonal antibodies reagent,
3. Blend of monoclonal IgM and polyclonal (human)Ig Ganti-D.

IgM anti-D monoclonal antibodies are highly specific and saline reacting equally well at room temperature and at 37°. They are good for slide test or immediate spin tube tests as well as routine Rh(D) typing in tube. IgM anti D are unreliable for detection of weak D (Du) by AHG test. Blend of IgM and IgG (monoclonal)anti-D or blend of IgM (monoclonal) and polyclonal (human) IgG anti-D can be used for testing weak D (Du) antigen by AHG test. Mostly blended IgM and IgG (monoclonal) anti- Rh(D) or blended monoclonal IgM and polyclonal (human) IgG anti Rh (D) antibodies are used now in routine.

Tests

Anti-D (IgM) Monoclonal blood grouping reagents are tested for their Physical Appearance, Potency, Avidity, Intensity, Specificity, Rouleaux, Haemolysis and Prozone parameters and have values greater than or equal to the appropriate International Reference Standards or suitable National reference or in house preparation used.

Physical Appearance and Color

Anti-D (IgM) Monoclonal blood grouping reagent should be visibly clear with no turbidity, precipitate, particles or gel formation. Anti-D (IgM) Monoclonal blood grouping reagent is colorless.

Potency. It is defined as the reciprocal of the greatest reagent dilution for which the reaction is graded at 1+. Anti-D (IgM) Monoclonal blood grouping reagent should have an average potency value greater than or equal to that of suitable reference standard.

Type of Reagent	*Test Red Blood Cells*	*Titre*	
		Immediate spin	*At 37° for 30 minutes*
Anti-D (IgM) (Monoclonal)	O+ve (R_1r or R_1R_2)	1:≥64	1:≥128

Avidity. It is defined as the time taken for agglutination to appear first to the unaided eye when 1 volume of the Anti-D (IgM) Monoclonal blood grouping reagent is mixed with 1 volume of a 40 per cent v/v suspension of human red corpuscles of specific group and compared with the time taken by that of the reference standard. Rapid mixing is performed by spreading with use of agitation, with a centrifugal spiral movement to form a circular reaction surface about 3 cm in diameter. The value for avidity is the mean of three measurements.

Type of Reagent	*Test Red Blood Cells*	*Avidity*
Anti-D (IgM) (Monoclonal)	O+ve (R_1r or R_1R_2)	≤ 10 seconds

Intensity. It is defined by the appearance of agglutination obtained after mixing undiluted Anti-D (IgM) Monoclonal blood grouping reagent with washed red blood cell suspension. For this 1 volume of the reagent is added to 1 volume of 40 per cent v/v red cell suspension on a slide and mixed for 2 minutes. At the end of 2 minutes, reaction grade is compared with the strength of reaction obtained with the reference preparation.

Type of Reagent	*Test Red Blood Cells*	*Intensity*
Anti-D (IgM) (Monoclonal)	O+ve (R_1r or R_1R_2)	3+

Specificity. Reagent is tested with red cell suspension which exhibits the antigen corresponding to the reagent antibody and yields positive reaction and red cell suspension which lacks the antigen corresponding to reagent antibody yields negative reaction.

For this 1 volume of Anti-D (IgM) Monoclonal blood grouping reagent is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension which exhibits the antigen corresponding to the reagent antibody and red cell suspension which lacks the antigen corresponding to reagent antibody. Centrifuge the tube at 1000 rpm for 1 minute. Tube with corresponding antigen gives positive reaction and the tube which lacks the antigen gives negative reaction.

Type of Reagent	*Test Red Blood Cells*	*Specificity*
Anti-D (IgM) (Monoclonal)	O +ve (R_1r or R_1R_2)	Positive
	O-ve (rr or r'r or r"r)	Negative

Reactivity

Haemolysis. It is defined as the release of hemoglobin from the red cells as a result of osmotic effect or due to the breaking up of the red blood cells. No immune haemolysis should be observed with the red blood cells used for specificity.

Type of Reagent	*Test Red Blood Cells*	*Haemolysis*
Anti-D (IgM) (Monoclonal)	O +ve (R_1r or R_1R_2)	Absent
	O-ve (rr or r'r or r"r)	Absent

Rouleaux. It is defined as a form of pseudo agglutination in which the red blood cells look like pile of coins. The red blood cells lacking the antigen corresponding to the reagent antibody should not show rouleaux formation. For this 1 volume of Anti- D (IgM) Monoclonal blood grouping reagent is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension which lacks the antigen corresponding to reagent antibody and the tube is centrifuged at 1000 rpm for 1 minute and observed microscopically for rouleaux formation.

Type of Reagent	*Test Red Blood Cells*	*Rouleaux*
Anti-D (IgM) (Monoclonal)	O-ve (rr or r'r or r"r)	Absent

Prozone. It is defined as a phenomenon in which negative reactions are obtained with low dilutions of an antibody, while a positive reaction is obtained with higher dilutions. Prozone is observed when an excess amount of antibody is present in the reagent. For this 1 volume of Anti-D (IgM) Monoclonal blood grouping reagent is mixed with 1 volume of 2-3 per cent red cell suspension in test tubes which exhibit the antigen corresponding to the reagent antibody for "15 minutes", "30 minutes" and "60 minutes". If the reaction grades are the same or increase as the incubation time increases, no prozone is present and if the reaction grades decrease as the incubation time increases, a prozone is present.

Type of Reagent	*Test Red Blood Cells*	*Prozone*
Anti-D (IgM) (Monoclonal)	O+ve (R_1r or R_1R_2)	Absent

Expiration date. The expiration date for Anti-D (IgM) blood grouping reagent is not less than 1 year at 2° to 8°.

Storage. Storage at a temperature between 2° and 8°.

Labelling. Label states (1) Source of product; (2) Test method (s) recommended; (3) Storage temperature; (4) Preservative used and its concentration; (5) Statement "FOR IN VITRO DIAGNOSTIC USE".

Anti-D Blend (IgM + IgG) Monoclonal Reagent

Anti-D Blend or (IgM + IgG) Monoclonal blood grouping reagents are tested for their Physical Appearance, Color, Potency, Avidity, Intensity, Specificity, Rouleaux, Haemolysis and Prozone parameters and have values greater than or equal to the appropriate International Standards or suitable reference preparation used.

Tests

Physical Appearance and Color

Anti-D Blend or (IgM + IgG) Monoclonal blood grouping reagent should be visibly clear with no turbidity, precipitate, particles or gel formation. Anti-D Blend or (IgM + IgG) Monoclonal blood grouping reagents are colorless.

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Potency. It is defined as the reciprocal of the greatest reagent dilution for which the reaction is graded at 1+. Anti-D Blend or (IgM + IgG) Monoclonal blood grouping reagent should have an average potency value greater than or equal to that of the reference preparation.

Type of Reagent	*Test Red Blood Cells*	*Titre*	
		Immediate spin	*At 37° for 30 minutes*
Anti-D Blend or (IgM + IgG) Monoclonal	O+ve (R_1r or R_1R_2)	1:≥32	1:≥128

Avidity. It is defined as the time taken for agglutination to appear first to the unaided eye when 1 volume of the Anti-D Blend or (IgM + IgG) Monoclonal blood grouping reagent is mixed with 1 volume of a 40 per cent v/v suspension of human red corpuscles in normal saline of specific group and compared with the time taken by that of the reference preparation. Rapid mixing is performed by spreading with use of agitation, with a centrifugal spiral movement to form a circular reaction surface about 3 cm in diameter. The value for avidity is the mean of three measurements.

Type of Reagent	*Test Red Blood Cells*	*Avidity*
Anti-D Blend or (IgM + IgG) Monoclonal	O+ve (R_1r or R_1R_2)	≤ 20 seconds

Intensity. It is defined by the appearance of agglutination obtained after mixing undiluted Anti-D (IgM + IgG) Monoclonal blood grouping reagent with washed red blood cell suspension. For this 1 volume of the reagent is added to 1 volume of 40 per cent red cell suspension in normal saline on a slide and mixed for 2 minutes. At the end of 2 minutes, reaction grade is compared with the strength of reaction obtained with the reference preparation.

Type of Reagent	*Test Red Blood Cells*	*Intensity*
Anti-D Blend or (IgM + IgG) Monoclonal	O+ve (R_1r or R_1R_2)	3+

Specificity. Reagent is tested with red cell suspension which exhibits the antigen corresponding to the reagent antibody and yields positive reaction and red cell suspension which lacks the antigen corresponding to reagent antibody yields negative reaction.

For this 1 volume of Anti-D (IgM + IgG) Monoclonal blood grouping reagent is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension in normal saline which exhibits the antigen corresponding to the reagent antibody and red cell suspension which lacks the antigen corresponding to reagent antibody. Centrifuge the tube at 1000 rpm for 1 minute. Tube containing RBC with corresponding antigen gives positive reaction and the tube containing RBC which lacks the antigen gives negative reaction.

Type of Reagent	*Test Red Blood Cells*	*Specificity*
Anti-D Blend or (IgM + IgG) Monoclonal	O +ve (R_1r or R_1R_2)	Positive
	O –ve (rr or r'r or r"r)	Negative
	D^u antigen (Weak D) IAT-Indirect antiglobulin test	Positive

Reactivity

Haemolysis. It is defined as the release of hemoglobin from the red cells as a result of osmotic effect or due to the breaking up of the red blood cells. No immune haemolysis should be observed with the red blood cells used for specificity.

Type of Reagent	*Test Red Blood Cells*	*Haemolysis*
Anti-D Blend or (IgM + IgG) Monoclonal	O +ve (R_1r or R_1R_2)	Absent
	O –ve (rr or r'r or r"r)	Absent
	D^u antigen (Weak D)	Absent

Rouleaux. It is defined as a form of pseudo agglutination in which the red blood cells look like pile of coins. The red blood cells lacking the antigen corresponding to the reagent antibody should not show rouleaux formation. For this 1 volume of Anti- D (IgM + IgG) Monoclonal blood grouping reagent is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension in normal saline which lacks the antigen corresponding to reagent antibody and the tube is centrifuged at 1000 rpm for 1 minute and observed microscopically for rouleaux formation.

Type of Reagent	*Test Red Blood Cells*	*Rouleaux*
Anti-D Blend or (IgM + IgG) Monoclonal	O –ve (rr or r'r or r"r)	Absent

Prozone. It is defined as a phenomenon in which negative reactions are obtained with low dilutions of an antibody, while a positive reaction is obtained with higher dilutions. For this 1 volume of Anti-D Blend or (IgM + IgG) Monoclonal blood grouping reagent is mixed with 1 volume of 2-3 per cent red cell suspension in normal saline in test tubes which exhibit the antigen corresponding to the reagent antibody for "15 minutes", "30 minutes", and "60 minutes". If the reaction grades are the same or increase as the incubation time increases, no prozone is present and if the reaction grades decrease as the incubation time increases, a prozone is present.

Type of Reagent	*Test Red Blood Cells*	*Prozone*
Anti-D Blend or (IgM + IgG) Monoclonal	O+ve (R_1r or R_1R_2)	Absent

Expiration date. The expiration date for Anti-D Blend or (IgM + IgG)Monoclonal blood grouping reagent is not less than 1 year at 2° to 8°.

Storage. Storage at a temperature between 2° to 8°.

Labeling. Label states (1) Source of Product; (2) Test method (s) recommended; (3) Storage temperature; (4) Preservative used and its concentration; (5) Statement "FOR IN VITRO DIAGNOSTIC USE".

Anti-D (IgG) Monoclonal Reagent

Anti-D (IgG) Monoclonal blood grouping reagents are tested for their Physical Appearance, Color, Potency, Specificity, Rouleaux and Haemolysis parameters and have values greater than or equal to the appropriate International Standards or suitable reference preparation used.

Tests

Physical Appearance and Color

Anti-D (IgG) Monoclonal blood grouping reagent should be visibly clear with no turbidity, precipitate, particles or gel formation. Anti-D (IgG) Monoclonal blood grouping reagents should be amber colored.

Potency

It is defined as the reciprocal of the greatest reagent dilution for which the reaction is graded at 1+. Anti-D (IgG) Monoclonal blood grouping reagent should have an average potency value greater than or equal to that of the reference preparation.

Type of Reagent	*Test Red Blood Cells*	*Potency*
		At 37° for 30 minutes
Anti-D (IgG)	O+ve (R_1r or R_0r)	1:≥32

Specificity

Reagent is tested with red cell suspension which exhibits the antigen corresponding to the reagent antibody and yields positive reaction and red cell suspension which lacks the antigen corresponding to reagent antibody yields negative reaction.

For this Anti-D (IgG) Monoclonal blood grouping reagent is mixed with 1 volume of 2-3 per cent red cell suspension in a test tube and incubated at 37° for 30 minutes. Wash the tubes 4 times with large volumes of Normal Saline. To the final cell button add 100 µl of Anti Human Globulin. Centrifuge the tube at 1000 rpm for 1 minute. Red blood cells with (R_1r and R_0r) antigen gives positive reaction and the tube which lacks the antigen gives negative reaction.

Type of Reagent	*Test Red Blood Cells*	*Specificity*
Anti-D (IgG)	O +ve (R_1r or R_0r)	Positive
	O –ve (rr or r'r or r"r)	Negative

Haemolysis

It is defined as the release of haemoglobin from the red cells as a result of osmotic effect or due to the breaking up of the red blood cells. No immune haemolysis should be observed with the red blood cells used for specificity.

Type of Reagent	*Test Red Blood Cells*	*Haemolysis*
Anti-D (IgG)	O +ve (R_1r or R_0r)	Absent
	O –ve (rr or r'r or r"r)	Absent

Rouleaux

It is defined as a form of pseudo agglutination in which the red blood cells look like pile of coins. The red blood cells lacking the antigen corresponding to the reagent antibody should not show rouleaux formation. For this 1 volume of Anti- D (IgG) Monoclonal blood grouping reagent is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension in normal saline which lacks the antigen corresponding to reagent antibody and the tube is centrifuged at 1000 rpm for 1 minute and observed microscopically on a slide for rouleaux formation.

Type of Reagent	*Test Red Blood Cells*	*Rouleaux*
Anti-D (IgG)	O –ve (rr or r'r or r"r)	Absent

Expiration date. The expiration date for Anti-D (IgG) Monoclonal blood grouping reagent is not less than 1 year at 2° to 8°.

Storage. Storage at a temperature between 2° to 8°.

Labeling. Label states (1) Source of Product; (2) Test method (s) recommended; (3) Storage temperature; (4) Preservative used and its concentration; (5) Statement "FOR IN VITRO DIAGNOSTIC USE".

Anti Human Globulin (AHG) Serum

Anti Human Globulin (AHG) reagent

Anti-Human Globulin Serum is a sterile, liquid preparation of serum produced by immunizing lower animals such as rabbits or goats with human serum or plasma, or with selected human

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plasma proteins. It is free from agglutinins and from hemolysins to nonsensitized human red cells of all blood groups. It contains a suitable antimicrobial preservative.

Three varieties of Anti-Human Globulin Serum are recognized: (1) a general-purpose polyspecific reagent which, as a minimum, contains antibodies specific for immunoglobulin (IgG) and at least the C3d component of human complement (for use in the direct antiglobulin test, it contains this Anti-C3d and Anti-IgG activity) and which may be artificially colored green; (2) a reagent containing antibodies only against immunoglobulin IgG (not heavy chain specific) intended for use in the indirect antiglobulin test, and which may be artificially coloured green; and (3) reagents containing antibodies specific for individual or selected components of human complement, such as Anti-C3, and Anti-C3b-C3d-C4, or a single class of immunoglobulins, such as Anti-IgG (heavy chain specific), used only to identify plasma components coated on the surface of red blood cells.

Tests

Anti Human Globulin (AHG) reagent is tested for their Physical Appearance, Color, Potency, Intensity, Specificity, Rouleaux, Haemolysis, Prozone, Heterospecific antibodies, Reaction with enzyme treated cells parameters and have values greater than or equal to the appropriate International Standards or suitable reference preparation used.

Physical Appearance and Color

Anti Human Globulin (AHG) reagent should be visibly clear with no turbidity, precipitate, particles or gel formation. Anti Human Globulin (AHG) reagent can be green or colorless.

Potency

It is defined as the reciprocal of the greatest reagent dilution for which the reaction is graded at 1+. Anti Human Globulin (AHG) reagent should have an average potency value greater than or equal to that of the reference preparation.

Type of Reagent	*Test Red Blood Cells*	*Potency*	
		Anti-D IgG	*Anti C3d*
Anti Human Globulin	O +ve (R_1r or R_1R_2)	1: ≥ 32	1: ≥ 4

Intensity

It is defined by the appearance of agglutination obtained after mixing undiluted Anti Human Globulin (AHG) reagent with 2-3 per cent C3d sensitized red blood cell suspension in normal saline. For this 1 volume of the reagent is added to 1 volume of 2-3 per cent C3d sensitized red cell suspension in normal saline in a test tube, mixed and incubated at 20° to 30° for 5 minutes, then the tube is centrifuged at 1000 rpm for 1 minute.

Type of Reagent	*Test Red Blood Cells*	*Intensity*
		Anti C3d
Anti Human Globulin	O +ve	2+ with undiluted reagent

Specificity

Reagent is tested with red cell suspension which exhibits the antigen corresponding to the reagent antibody and yields positive reaction and red cell suspension which lacks the antigen corresponding to reagent antibody yields negative reaction.

For this Anti Human Globulin (AHG) reagent is mixed with 1 volume of 2-3 per cent sensitized red cell suspension in a test tube and in another tube Anti Human Globulin (AHG) reagent is mixed with 1 volume of 2-3 per cent unsensitized red cell suspension in normal saline. Centrifuge the tubes at 1000 rpm for 1 minute. Red blood cells with (R_1r and R_0r) antigen gives positive reaction and the tube which lacks the antigen gives negative reaction.

Type of Reagent	*Test Red Blood Cells*	*Specificity*
Anti Human Globulin	O +ve (R_1r and R_0r) - sensitized with IgG	Positive
	O +ve (R_1r and R_0r) - unsensitized	Negative

Reactivity

Haemolysis

It is defined as the release of haemoglobin from the red cells as a result of osmotic effect or due to the breaking up of the red blood cells. No immune haemolysis should be observed with the red blood cells used for specificity.

Type of Reagent	*Test Red Blood Cells*	*Haemolysis*
Anti Human Globulin	O +ve (R_1r and R_0r) - sensitized with IgG	Absent
	O +ve (R_1r and R_0r) - unsensitized	Absent

Rouleaux

It is defined as a form of pseudo agglutination in which the red blood cells look like pile of coins. The red blood cells lacking the antigen corresponding to the reagent antibody should not show rouleaux formation. For this 1 volume of Anti Human Globulin (AHG) reagent is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension in normal saline which lacks the antigen corresponding to reagent antibody and the tube is centrifuged at 1000 rpm for 1 minute and observed microscopically for rouleaux formation.

Type of Reagent	*Test Red Blood Cells*	*Rouleaux*
Anti Human Globulin	O +ve (R_1r and R_0r) - unsensitized	Absent

Test for Heterospecific Antibodies

Anti Human Globulin (AHG) reagent should be free of antibodies capable of agglutinating or hemolyzing untreated human red blood cells.

Type of Reagent	*Enzyme treated Red Blood Cells*	*Rouleaux*
Anti Human Globulin	O	Absent
	A	Absent
	B	Absent

Test with Enzyme treated Red Blood Cells

Anti Human Globulin (AHG) reagent should not agglutinate 0.1 per cent enzyme (trypsin or papain or ficin) treated red blood cells.

Type of Reagent	*Enzyme treated Red Blood Cells*	*Rouleaux*
Anti Human Globulin	O	Absent
	A	Absent
	B	Absent

Expiration date. Its expiration date is not later than 1 year after the date of issue from manufacturer's cold storage (5°, 1 year; or 0°, 2 years).

Storage. Storage at a temperature between 2° to 8°.

Labeling. Label states (1) Source of Product; (2) Test method (s) recommended; (3) Storage temperature; (4) Preservative used and its concentration; (5) Statement "FOR IN VITRO DIAGNOSTIC USE"

NOTE— The lettering on the label of the general-purpose polyspecific reagent is black on a white background. The label of all other Anti-Human Globulin Serum containers is in white lettering on a black background.

Antithrombin III Concentrate

Human Antithrombin III Concentrate

Category. Anticoagulant factor.

Antithrombin III concentrate is a preparation of a glycoprotein fraction obtained from human plasma that inactivates thrombin in the presence of an excess of heparin. It is obtained from plasma that complies with the requirements of the monograph on Plasma for Fractionation.

When reconstituted in the volume of solvent stated on the label, the potency is not less than 25 IU of antithrombin III per ml.

Production

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and any residues are such as not to compromise the safety of the preparation for patients.

The antithrombin III is purified and concentrated and a suitable stabiliser may be added. The specific activity is not less than 3 IU of antithrombin III per mg of total protein, excluding albumin. The antithrombin III concentrate is passed through a bacteria-retentive filter, distributed aseptically into its final, sterile containers and immediately frozen. It is then freeze-dried and the containers are closed under vacuum or in an atmosphere of inert gas. No antimicrobial preservative is added at any stage of production.

It shall be demonstrated that the manufacturing process yields a product that consistently complies with the following test.

Heparin-binding fraction. Examine by agarose gel electrophoresis (2.4.12). Prepare a 1.0 per cent w/v solution of agarose for electrophoresis containing 15 IU of *heparin* per ml in *barbital buffer solution pH 8.4*. Pour 5 ml of this solution onto a glass plate 5 cm square. Cool at 4° for 30 minutes. Cut 2 wells, 2 mm in diameter, 1 cm and 4 cm from the side of the plate and 1 cm from the cathode. Introduce into one well 5 µl of the preparation under examination, diluted to an activity of about 1 IU of antithrombin III per ml. Introduce into the other well 5 µl of a solution of a marker dye such as *bromophenol blue*. Allow the electrophoresis to proceed at 4°, using a constant electric field of 7 V/cm, until the dye reaches the anode.

Cut across the agarose gel, 1.5 cm from that side of the plate on which the preparation under examination was applied and remove the larger portion of the gel leaving a band 1.5 cm wide containing the material under examination. Replace the removed portion with an even layer consisting of 3.5 ml of a 1.0 per cent w/v solution of *agarose for electrophoresis* in *barbital buffer solution pH 8.4*, containing a rabbit anti-human antithrombin III antiserum at a suitable concentration, previously determined, to give adequate peak heights of atleast 1.5 cm. Place the plate with the original gel at the cathode so that a second electrophoretic migration can occur at right angles to the first. Allow this second electrophoresis to proceed using a constant electric field of 2 V/cm for 16 hours.

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Cover the plates with filter paper and several layers of thick lint soaked in a 0.9 per cent w/v solution of *sodium chloride* and compress for 2 hours, renewing the *saline* several times. Rinse with *water,* dry the plates and stain with *acid blue 92 solution.*

Calculate the fraction of antithrombin III bound to heparin, which is the peak closest to the anode, with respect to the total amount of antithrombin III, by measuring the area defined by the 2 precipitation peaks.

The fraction of antithrombin III able to bind to heparin is not less than 60 per cent.

Description. A white or almost white, hygroscopic, friable solid or a powder.

Reconstitute the preparation under examination as stated on the label immediately before carrying out the Identification, the Tests (except those for solubility, total protein and water), and the Assay.

Identification

It complies with the limits of the Assay.

Tests

pH (2.4.24). 6.0 to 7.5.

Solubility. It dissolves completely under gentle swirling within 10 minutes in the volume of the solvent stated on the label, forming a clear or slightly turbid, colourless solution.

Osmolality (2.4.23). Minimum 240 mosmol per kg.

Total protein. If necessary, dilute an accurately measured volume of the preparation under examination with *water* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of the solution in a round-bottomed centrifuge tube add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid* and 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.3.30) and calculate the content of protein by multiplying the result by 6.25.

Heparin (2.8.9). Not more than 0.1 IU of heparin activity per International Unit of antithrombin III activity. It is necessary to validate the method for assay of heparin for each specific preparation under examination to allow for interference by antithrombin III.

Water. Determined by a suitable method, such as the semi-micro determination of water (2.3.43), loss on drying (2.4.19) or near infrared spectrophotometry (2.4.6), the water content is within the limits approved by the competent authority.

Pyrogens (2.2.8). Complies with the test for pyrogens. Inj per kg of the rabit's mass a volume of the preparation un examination equivalent to 50 IU of antithrombin III, caluca from the activity stated on the label.

Sterility (2.2.11). Complies with the tests for sterility.

Assay. Assay of human antithrombin III (2.8.12).

The estimated potency is not less than 80.0 per cent and more than 120.0 per cent of the potency stated on the lab The confidence limits (P = 0.95) are not less than 90.0 per cen and not more than 110.0 per cent of the estimated potency.

Storage. Store protected from light in an airtight containe

Labelling. The label states (1) the content of antithrombin III expressed in International Units per container; (2) the name and volume of solvent to be used to reconstitute the preparation; (3) where applicable, the amount of albumin present as a stabiliser.

Blood Grouping Lectins Anti-A_1

Anti-A_1 (Lectin) is an extract of *Dolichos biflorus* seeds prepared in a saline diluent.

Among various subdivisions of A antigen (i.e., A_1, A_2, A_3, A_m, A_x, etc), A_1 and A_2 are encountered most frequently. Group A red blood cells which are agglutinated with Anti-A_1 Lectin are said to be sub group A_1 and those which are not agglutinated by Anti-A_1 Lectin fall into subgroups weaker than A_1, the majority being classified as A_2. Approximately 80 per cent of the population of blood group A is A_1 while the remaining 20 per cent are A_2 or weaker subgroup.

In the undiluted state, the lectin extract of *Dolichos biflorus* reacts as Anti-A, since it agglutinates both A_1 and A_2 cells When prepared at an appropriate dilution, however, the lecti reacts directly with A_1 and A_1B but not A_2 or A_2B red cells.

Direct agglutination of group A and AB red blood cells b Anti-A_1 (Lectin) indicates the presence of the A_1 or A_1 antigens. No agglutination, indicates that the red blood cel carry a weaker form of the A antigen.

Anti-A_1 (Lectin) reagents are tested for their Physical appearance, color, potency, intensity, specificity, rouleaux, haemolysis, prozone parameters and have values at least equal to the appropriate International Standards or suitable reference preparation as approved by National Regulatory Authority.

Physical Appearance and Color

Anti-A_1 (Lectin) reagent should be clear with no turbidity, precipitate, particles or gel formation by visual

inspection. Anti-A_1 (Lectin) blood grouping reagents are colorless liquids.

Potency. It is defined as the reciprocal of the greatest reagent dilution for which the reaction is graded at 1+. Anti-A_1 (Lectin) reagent should have an average potency value at least equal to that of the reference preparation.

Type of Reagent	*Test Red Blood Cells*	*Titre*
Anti-A_1 (Lectin)	A_1 (2 cells)	≥ 1:8
	A_1B (2 cells)	≥ 1:4

Intensity. It is defined by the appearance of agglutination obtained after mixing undiluted Anti-A_1 (Lectin) reagent with washed red blood cell suspension. For this 1 volume of the reagent is added to 1 volume of 40 per cent red cell suspension on a slide and mixed for 2 minutes. At the end of 2 minutes, reaction grade is compared with the strength of reaction obtained with the reference preparation.

Type of Reagent	*Test Red Blood Cells*	*Intensity*
Anti-A_1 (Lectin)	A_1 (2 cells)	2+ to 3+

Specificity. Reagent is tested with red cell suspension which exhibits the antigen corresponding to the reagent antibody and yields positive reaction and red cell suspension which lacks the antigen corresponding to reagent antibody yields negative reaction.

For this 1 volume of Anti-A_1 (Lectin) reagent is mixed in a test tube with 1 volume of 2 to 3 per cent red cell suspension which exhibits the antigen corresponding to the reagent antibody and red cell suspension which lacks the antigen corresponding to reagent antibody. Centrifuge the tube at 1000 rpm for 1 minute. Tube with corresponding antigen gives positive reaction and the tube which lacks the antigen gives negative reaction.

Type of Reagent	*Test Red Blood Cells*	*Specificity*
Anti-A_1 (Lectin)	A_1 (2 cells)	Positive
	A_1B (2 cells)	Positive
	A_2 (2 cells)	Negative
	A_2B (2 cells)	Negative
	B (2 cells)	Negative
	O (2 cells)	Negative

Reactivity

Haemolysis. It is defined as the release of hemoglobin from the red cells as a result either of osmotic effect or of the breaking up of the red blood cells. No immune hemolysis should be observed with the red blood cells used for specificity.

Type of Reagent	*Test Red Blood Cells*	*Haemolysis*
Anti-A_1 (Lectin)	A_1 (2 cells)	Absent
	A_1B (2 cells)	Absent
	A_2 (2 cells)	Absent
	A_2B (2 cells)	Absent
	B (2 cells)	Absent
	O (2 cells)	Absent

Rouleaux. It is defined as a form of pseudo agglutination in which the red blood cells look like pile of coins. The red blood cells lacking the antigen corresponding to the reagent antibody should not show rouleaux formation. For this 1 volume of Anti-A_1 (Lectin) is mixed in a test tube with 1 volume of 2 to 3 per cent red cell suspension which lacks the antigen corresponding to reagent antibody and the tube is centrifuged at 1000 rpm for 1 minute and observed microscopically for rouleaux formation.

Type of Reagent	*Test Red Blood Cells*	*Rouleaux*
Anti-A_1 (Lectin)	A_2 (2 cells)	Absent
	A_2B (2 cells)	Absent
	B (2 cells)	Absent
	O (2 cells)	Absent

Prozone. It is defined as a phenomenon in which negative reactions are obtained with low dilutions of an antibody, while a positive reaction is obtained with higher dilutions. For this 1 volume of Anti-A_1 (Lectin) is mixed with 1 volume of 2-3 per cent red cell suspension in test tubes which exhibit the antigen corresponding to the reagent antibody for "15 minutes", "30 minutes" and "60 minutes". If the reaction grades are the same or increase as the incubation time increases, no prozone is present and if the reaction grades decrease as the incubation time increases, prozone is present.

Type of Reagent	*Test Red Blood Cells*	*Prozone*
Anti-A_1 (Lectin)	A_1 (2 cells)	Absent
	A_1B (2 cells)	Absent

Expiration date. The expiration date for Anti-A_1 (Lectin) reagent is not less than 1 year at 2° to 8°.

Storage. Store at a temperature between 2° and 8°.

Blood Grouping Lectins Anti-H

Anti- H (Lectin) is an extract of *(Ulex europaeus)* seeds prepared in a saline diluent.

The H antigen is present in variable quantity on human red blood cells, depending on the ABO blood group of the individual. The greatest amount is present on red blood cells of group O, intermediate amounts occur on red blood cells of

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groups A_2 and certain other A- subgroups, A_2B and B, least amounts are present on red cells of A_1 and A_1B. Bombay group (O_h) does not contain H antigen and does not react with Anti-H lectin.

For resolving problems related to ABO subgroups by direct agglutination tests, wherein agglutination of the test red blood cells indicates the presence of the H antigen, and variability in the strength of the reaction may be a reflection of variable expression of the antigen. No agglutination indicates that the H antigen is absent, or possibly that its expression on the test red blood cells is too weak to be detected.

Anti- H reagents are tested for their physical appearance, colour, potency, intensity, specificity, rouleaux, haemolysis, prozone parameters and have values at least equal to the appropriate International Standards or suitable reference preparation as approved by National Regulatory Authority.

Physical Appearance and Color

Anti-H reagent should be clear with no turbidity, precipitate, particles or gel formation by visual inspection. Anti-H (Lectin) blood grouping Reagents are colorless to mild amber color liquid.

Potency. It is defined as the reciprocal of the greatest reagent dilution for which the reaction is graded at 1+. Anti-H (Lectin) reagent should have an average potency value at least equal to that of the reference preparation.

Type of Reagent	*Test Red Blood Cells*	*Titre*
Anti-H (Lectin)	O (2 cells)	≥ 1:8
	A_2 (2 cells)	≥ 1:4
	A_1* (2 cells)	≥ 1:1

*In rare cases negative reaction is observed

Intensity. It is defined by the appearance of agglutination obtained after mixing undiluted Anti-H (Lectin) reagent with washed red blood cell suspension. For this 1 volume of the reagent is added to 1 volume of 40 per cent red cell suspension on a slide and mixed for 3 minutes. At the end of 3 minutes, reaction grade is compared with the strength of reaction obtained with the reference preparation.

Type of Reagent	*Test Red Blood Cells*	*Intensity*
Anti-H (Lectin)	O (2 cells)	Strong 2+ to 4+ (within 3 minutes)
	A_2 (2 cells)	2+ (within 3 minutes)
	A_1 (2 cells)	<2+ (within 3 minutes)

Specificity. Reagent is tested with red cell suspension which exhibits the antigen corresponding to the reagent antibody and yields positive reaction and red cell suspension which lacks the antigen corresponding to reagent antibody yields negative reaction.

For this 1 volume of Anti-H (Lectin) reagent is mixed in a test tube with 1 volume of 2 to 3 per cent red cell suspension which exhibits the antigen corresponding to the reagent antibody and red cell suspension which lacks the antigen corresponding to reagent antibody. Centrifuge the tube at 1000 rpm for 1 minute. Tube with corresponding antigen gives positive reaction and the tube which lacks the antigen gives negative reaction.

Type of Reagent	*Test Red Blood Cells*	*Specificity*
Anti-H (Lectin)	O (2 cells)	Positive
	A_2 (2 cells)	Positive
	A_1 (2 cells)	Positive
	O_h (2 cells)	Negative

Reactivity

Haemolysis. It is defined as the release of hemoglobin from the red cells as a result either of osmotic effect or of the breaking up of the red blood cells. No immune hemolysis should be observed with the red blood cells used for specificity.

Type of Reagent	*Test Red Blood Cells*	*Haemolysis*
Anti-H (Lectin)	O (2 cells)	Absent
	A_2 (2 cells)	Absent
	A_1 (2 cells)	Absent
	O_h (2 cells)	Absent

Rouleaux. It is defined as a form of pseudo agglutination in which the red blood cells look like pile of coins. The red blood cells lacking the antigen corresponding to the reagent antibody should not show rouleaux formation. For this 1 volume of Anti-A_1 (Lectin) is mixed in a test tube with 1 volume of 2-3 per cent red cell suspension which lacks the antigen corresponding to reagent antibody and the tube is centrifuged at 1000 rpm for 1 minute and observed microscopically for rouleaux formation.

Type of Reagent	*Test Red Blood Cells*	*Rouleaux*
Anti-H (Lectin)	O_h (2 cells)	Absent

Prozone. It is defined as a phenomenon in which negative reactions are obtained with low dilutions of an antibody, while a positive reaction is obtained with higher dilutions. For this 1 volume of Anti-A_1 (Lectin) is mixed with 1 volume of 2-3 per cent red cell suspension in test tubes which exhibit the antigen corresponding to the reagent antibody for "15 minutes", "30 minutes" and "60 minutes". If the reaction grades are the same or increase as the incubation time increases, no prozone is present and if the reaction grades decrease as the incubation time increases, prozone is present.

Type of Reagent	*Test Red Blood Cells*	*Prozone*
Anti-H (Lectin)	O (2 cells)	Absent
	A_2 (2 cells)	Absent
	A_1 (2 cells)	Absent

Expiration date. The expiration date for Anti-H (Lectin) reagent is not less than 1 year at 2° to 8°.

Storage. Store at a temperature between 2° and 8°.

Blood Grouping Reagent Anti-Fy^a, Anti-Fy^b

Duffy Blood Grouping Serum. The Duffy blood grouping system is composed of two antigens - Fy^a and Fy^b. Fy^a antigen is found on the erythrocytes of approximately 85 to 90 per cent of Indian population, while Fy^b antigen is found in 55 to 60 per cent of Indian population. Agglutination of red blood cells with Anti-Fy^a or Anti-Fy^b blood grouping reagent, indicates the presence of Fy^a antigen and Fy^b antigen respectively.

Production

Anti Fy blood grouping reagent is a polyclonal preparation obtained from the pooled human plasma / serum who have developed specific Anti-Fy^a or Anti-Fy^b antibodies, as a result of alloimmunization. They are free from agglutinins for A and B antigens and from alloantibodies other than Anti-Fy^a /Anti-Fy^b as mentioned in the label. They are IgG in characteristic.

The procedure used with these reagents is based on the principle of agglutination. Normal human red blood cells, possessing Fy^a or Fy^b antigens, will agglutinate in the presence of antibody directed towards the antigens. The Duffy blood grouping antisera contains incomplete or irregular antibodies (IgG in nature and non-saline reacting), indirect antiglobulin tests are applied to detect the presence or absence of an antigen, either Fy^a or Fy^b antigen as shown in Table 1.

Table -1

Serum	Antigen(s)	Phenotype of cells
Anti-Fy^a	Fy^a	Fy ($a^+ b^-$), Fy ($a^+ b^+$)
Anti-Fy^b	Fy^b	Fy ($a^- b^+$), Fy ($a^+ b^+$)

Processing

Processing method

(i) The processing method shall be one that has been shown to yield consistently a specific, potent final product, free of properties that would affect adversely the intended use of the product throughout its dating period. Stability testing shall be performed on an adequate number of representative samples of each group of products manufactured.

(ii) A lot may be subdivided into sublots. If lots are to be subdivided, the manufacturer shall include this information in the biologics license application. The manufacturer shall describe the test specifications to verify that each sublot is identical to other sublots of the lot.

(iii) Each lot of blood grouping reagent shall be identified by a lot number. Each sub-lot shall be identified by that lot number to which a distinctive prefix or suffix shall be added. Final container and package labels shall bear the lot number and all distinctive prefixes and suffixes that have been applied to identify the sub-lot from which filling was accomplished.

Color coding of reagents. Anti-Duffy blood grouping reagents should be colourless with no added colorant as approved by National Regulatory Authority.

Preservative and other constituents. The reagents should contain suitable anti-microbial preservative, preferably 0.1 per cent *sodium azide*. A warning information about the hazards of the preservative should be mentioned on the label. Additional constituents like sodium chloride (normal saline), bovine albumin or others should be mentioned.

Potency requirements with reference preparations

Each antiserum for which Reference Blood Grouping Reagents are available shall comply with the potency titer value at least equal to that of the reference preparation.

Potency tests without reference preparations

Products for which Reference Blood Grouping Reagents are not available shall be tested for potency by a method approved by the National Regulatory Authority.

Products recommended for test tube techniques. Polyclonal Anti-Fy^a reagents recommended for the test tube methods by indirect anti-globulin tests, should have the potency titer values of not less than 1+ reaction with a 1:8 dilution of the reagent.

Polyclonal Anti-Fy^b reagents recommended for the test tube methods by indirect anti-globulin tests should have the potency titer values of not less than 2+ reaction with undiluted reagent.

Products recommended for test tube or microplate techniques. Blood Grouping Reagent recommended for test tube methods or microplate techniques shall produce clearly positive

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macroscopic results when both undiluted reagent and reagent diluted with an equal volume of diluent are tested by all methods recommended in the manufacturer's package insert using red blood cells showing heterozygous or diminished expression of the corresponding antigen. The dilution shall be made with an equal volume of compatible serum or approved diluent.

Products recommended for use in an automated system. The manufacturer of Anti-Fy^a and Anti-Fy^b reagents that is recommended for use in an automated system shall demonstrate that its product when used both undiluted and diluted with an equal volume of diluent satisfactorily performs when tested with cells representing heterozygous or diminished expression of the corresponding antigen.

Specificity and avidity tests shall be performed using test procedures approved by National Regulatory Authority. Each serum (Anti-Fy^a or Anti-Fy^b) should comply with the tests for avidity with the cells as indicated in Table 2.

Table -2

Serum	Phenotype of testing cells
Anti-Fy^a	Fy ($a^+ b^-$), Fy ($a^+ b^+$)
Anti-Fy^b	Fy ($a^- b^+$), Fy ($a^+ b^+$)
No agglutination	Fy ($a^- b^-$)

Each serum complies with the tests for specificity by the most sensitive methods recommended by the manufacturer and approved by National Regulatory Authority, in which not less than 4 positive control samples (homozygous and heterozygous form for each antigen) and 4 negative controls (red cells lacking the corresponding antigen) are included. Attention should be given to confirm the absence of contaminating antibodies.

Red blood cells used for these tests should be either fresh or frozen red blood cell suspension which are prepared under specific conditions and meet specified criteria.

Labelling. The label states (1) name of the preparation; (2) name of the antibody or antibodies present; (3) the volume of the preparation; (4) source of the preparation (whether human serum or plasma or other source); (5) storage condition; (6) date of expiry; (7) name and concentration of the preservative used; (8) the statement: "FOR IN VITRO DIAGNOSTIC USE"; (9) if human blood was used in manufacturing the preparation, a cautionary statement "All blood products should be treated as potentially infectious. Source material from which this product was derived was found negative when tested in accordance with current required tests. No known test methods can offer assurance that products derived from human blood will not transmit infectious agents."

Blood Grouping Reagent Anti-Jk^a, Anti-Jk^b

The Kidd blood grouping reagent is composed of two antigen - Jk^a and Jk^b. Jk^a antigen is found on the erythrocytes o approximately 80 to 85 per cent of Indian population, while J antigen is found in 60 to 65 per cent of Indian populatio Agglutination of red blood cells with Anti-Jk^a or Anti-J antiserum, indicates the presence of Jk^a antigen and Jk^b antige respectively.

Production

Anti-Jk blood grouping reagent is either prepared from (i) human / mouse heterohybridoma cell lines secreting huma IgM antibodies producing monoclonal preparation for tub and microplate test. Clone MS 15 (for Anti-Jk^a) and Clone M 8 (for Anti-Jk^b) are designed for recognition of the corresponding antigens on the human red blood cells. (ii) from pooled human plasma/ serum who have developed specif Anti-Jk^a or Anti-Jk^b antibodies, as a result of alloimmunizatio They are free from agglutinins for A and B antigens and fro alloantibodies other than Anti-Jk^a /Anti-Jk^b as mentioned i the label. They are IgG in characteristic.

The procedure used with these reagents is based on th principle of agglutination. Normal human red blood cell possessing Jk^a or Jk^b antigens, will agglutinate in the presenc of antibody directed towards the antigens.The monoclona type of Kidd blood group antisera contain saline agglutinatin complete antibodies which specifically agglutinate human re blood cells suspended in saline and at room temperature. Th Polyclonal type of Kidd blood grouping antisera contai incomplete or irregular antibodies (IgG in nature and no saline reacting), indirect antiglobulin test are applied to dete the presence or absence of an antigen, either Jk^a or Jk^b antige as shown in Table 1.

Table -1

Serum	Antigen(s)	Phenotype of cells
Anti-Jk^a	Jk^a	Jk ($a^+ b^-$), Jk ($a^+ b^+$)
Anti-Jk^b	Jk^b	Jk ($a^- b^+$), Jk ($a^+ b^+$)

Processing

Processing method

(i) The processing method shall be one that has been show to yield consistently a specific, potent final product, fre of properties that would affect adversely the intende use of the product throughout its dating period. Stabili testing shall be performed on an adequate number o representative samples of each group of product manufactured.

(ii) A lot may be subdivided into sublots. If lots are to be subdivided, the manufacturer shall include this information in the biologics license application. The manufacturer shall describe the test specifications to verify that each sublot is identical to other sublots of the lot.

(iii) Each lot of Blood Grouping Reagent shall be identified by a lot number. Each sub-lot shall be identified by that lot number to which a distinctive prefix or suffix shall be added. Final container and package labels shall bear the lot number and all distinctive prefixes and suffixes that have been applied to identify the sub-lot from which filling was accomplished.

Color coding of reagents. Anti-Kidd Blood Grouping Reagents should be colourless with no added colorant as approved by National Regulatory Authority.

Preservative and other constituents. The reagents should contain suitable anti-microbial preservative, preferably 0.1 per cent *sodium azide*. A warning information about the hazards of the preservative should be mentioned on the label. Additional constituents like sodium chloride (normal saline), bovine albumin or others should be mentioned.

Potency requirements with reference preparations

Each antiserum for which Reference Blood Grouping Reagents are available shall comply with the potency titer value at least equal to that of the reference preparation.

Reference preparations. National Regulatory Authority approved Reference Blood Grouping Reagents shall be used as described in the accompanying package insert for determining the potency of Blood Grouping Reagents.

Potency tests without reference preparations

Products for which Reference Blood Grouping Reagents are not available shall be tested for potency by a method approved by National Regulatory Authority.

Products recommended for test tube techniques. Polyclonal Anti-Jk^a reagents recommended for the test tube methods by indirect anti-globulin tests shall have the potency titer values of not less than 1+ reaction with a 1:8 dilution of the reagent.

Polyclonal Anti-Jk^b reagents recommended for the test tube methods by indirect anti-globulin tests shall have the potency titer values of not less than 2+ reaction with undiluted reagent.

Products recommended for test tube or microplate techniques. Blood Grouping Reagent recommended for test tube methods or microplate techniques shall produce clearly positive macroscopic results when both undiluted reagent and reagent diluted with an equal volume of diluent are tested by all methods recommended in the manufacturer's package insert using red blood cells showing heterozygous or diminished expression of the corresponding antigen. The dilution shall be made with an equal volume of compatible serum or approved diluent.

Products recommended for use in an automated system. The manufacturer of Anti-Jk^a and Anti-Jk^b reagents that is recommended for use in an automated system shall demonstrate that its product when used both undiluted and diluted with an equal volume of diluent satisfactorily performs when tested with cells representing heterozygous or diminished expression of the corresponding antigen.

Specificity tests and avidity tests

Specificity and avidity tests shall be performed using test procedures approved by National Regulatory Authority. Each serum (Anti-Jk^a or Anti-Jk^b) should comply with the tests for avidity with the cells as indicated in Table 2.

Table -2

Serum	Phenotype of testing cells
Anti-Jk^a	Jk ($a^+ b^-$), Jk ($a^+ b^+$)
Anti-Jk^b	Jk ($a^- b^+$), Jk ($a^+ b^+$)

Each serum complies with the tests for specificity by the most sensitive methods recommended by the manufacturer and approved by National Regulatory Authority, in which not less than 4 positive control samples (homozygous and heterozygous form for each antigen) and 4 negative controls (red cells lacking the corresponding antigen) are included. Attention should be given to confirm the absence of contaminating antibodies.

Red blood cells used for these tests should be either fresh or frozen red blood cell suspension which are prepared under specific conditions and meet specified criteria.

Labelling. The label states (1) name of the preparation; (2) name of the antibody or antibodies present; (3) the volume of the preparation; (4) source of the preparation (whether human serum or plasma or other source); (5) storage condition; (6) date of expiry; (7) name and concentration of the preservative used; (8) the statement: "FOR IN VITRO DIAGNOSTIC USE"; (9) if human blood was used in manufacturing the preparation, a cautionary statement "All blood products should be treated as potentially infectious. Source material from which this product was derived was found negative when tested in accordance with current required tests. No known test methods can offer assurance that products derived from human blood will not transmit infectious agents."

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Blood Grouping Reagent Anti-K, Anti-k

Anti-Kell and Anti-Cellano Blood Grouping Reagent. The K antigen is found on the erythrocytes of approximately 4 per cent of Indian population, while k antigen is found in more than 99 per cent of Indian population. Agglutination of red blood cells with Anti-K or Anti-k antiserum, indicates the presence of K antigen and k antigen respectively. The phenotype frequency of K and k antigens are given in Table1.

Production

Anti-K, Anti-k blood grouping reagent is obtained from the pool of human plasma / serum that has developed specific Anti-K or Anti-k antibodies, as a result of alloimmunization. They are free from agglutinins for A and B antigens and from alloantibodies other than Anti-K /Anti-k as mentioned in the label.

The procedure used with these reagents is based on the principle of agglutination. Normal human red blood cells, possessing K or k antigens, will agglutinate in the presence of antibody directed towards the antigens. These blood grouping antisera contains incomplete or irregular antibodies (IgG in nature and non-saline reacting), indirect antiglobulin tests are applied to detect the presence or absence of an antigen, either K or k antigen as shown in Table-1.

Table -1

Serum	Antigen(s)	Phenotype of cells
Anti-K	K	KK (Rare), Kk (4 per cent)
Anti-k	k	kk (96 per cent) Kk (4 per cent)

Processing

Processing method

(i) The processing method shall be one that has been shown to yield consistently a specific, potent final product, free of properties that would affect adversely the intended use of the product throughout its dating period. Stability testing shall be performed on an adequate number of representative samples of each group of products manufactured.

(ii) A lot may be subdivided into sublots. If lots are to be subdivided, the manufacturer shall include this information in the biologics license application. The manufacturer shall describe the test specifications to verify that each sublot is identical to other sublots of the lot.

(iii) Each lot of Blood Grouping Reagent shall be identified by a lot number. Each sub-lot shall be identified by that lot number to which a distinctive prefix or suffix shall be added. Final container and package labels shall bear the lot number and all distinctive prefixes and suffixes that have been applied to identify the sub-lot from which filling was accomplished.

Color coding of reagents. Blood Grouping Reagents may be colored provided the added colorant does not adversely affect the safety, purity, or potency of the product and the colorant is approved by National Regulatory Authority.

Preservative and other constituents. The reagents should contain suitable anti-microbial preservative, preferably 0.1 per cent *sodium azide*. A warning information about the hazards of the preservative should be mentioned on the label. Additional constituents like sodium chloride (normal saline), bovine albumin or others should be mentioned

Potency requirements with reference preparations

Each antiserum for which Reference Blood Grouping Reagents are available shall comply with the potency titer value at least equal to that of the reference preparation.

Potency tests without reference preparations

Products recommended for test tube techniques. Anti-K and Anti-k Reagents recommended for the test tube methods, including the indirect antiglobulin tests, shall have the potency titer values of not less than 1+ reaction with a 1:8 dilution of the reagent.

Products recommended for slide tests or microplate techniques. Blood Grouping Reagents recommended for slide test methods or microplate techniques shall produce clearly positive macroscopic results when both undiluted reagent and reagent diluted with an equal volume of diluent are tested by all methods recommended in the manufacturer's package insert using red blood cells showing heterozygous or diminished expression of the corresponding antigen. The dilution shall be made with an equal volume of compatible serum or approved diluent.

Products recommended for use in an automated system. The manufacturer of Anti-K and Anti-k Reagents that is recommended for use in an automated system shall demonstrate that its product when used both undiluted and diluted with an equal volume of diluent satisfactorily performs when tested with cells representing heterozygous or diminished expression of the corresponding antigen.

Specificity tests and avidity tests

Specificity and avidity tests shall be performed using test procedures approved by the National Regulatory Authority.

Each serum (Anti-K or Anti-k) should comply with the tests for avidity with the cells as indicated in Table-2.

Table -2

Serum	Phenotype of testing cells
Anti-K	KK and Kk
Anti-k	kk and Kk

Each serum complies with the tests for specificity by the most sensitive methods recommended by the manufacturer and approved by National Regulatory Authority, in which not less than 4 positive (homozygous and heterozygous form for each antigen) and 4 negative phenotype are included. Attention should be given to confirm the absence of contaminating antibodies.

Red blood cells used for these tests should be either fresh or frozen red blood cell suspension which are prepared under specific conditions and should meet specified criteria.

Labelling. The label states (1) name of the preparation; (2) name of the antibody or antibodies present; (3) the volume of the preparation; (4) source of the preparation (whether human serum or plasma or other source); (5) storage condition; (6) date of expiry; (7) name and concentration of the preservative used; (8) the statement: "FOR IN VITRO DIAGNOSTIC USE"; (9) if human blood was used in manufacturing the preparation, a cautionary statement "All blood products should be treated as potentially infectious. Source material from which this product was derived was found negative when tested in accordance with current required tests. No known test methods can offer assurance that products derived from human blood will not transmit infectious agents."

Blood Grouping Reagent Anti-Le^a, Anti-Le^b

Lewis Blood Grouping Reagent. The Lewis blood grouping reagent is composed of two antigens- Le^a and Le^b. Le^a antigen is found on the erythrocytes of approximately 80 to 85 per cent of Indian population, while Le^b antigen is found in 60 to 65 per cent of Indian population. Agglutination of red blood cells with Anti-Le^a or Anti-Le^b blood grouping reagent, indicates the presence of Le^a antigen and Le^b antigen respectively. Absence of agglutination of red blood cells with both Anti-Le^a or Anti-Le^b blood grouping reagent indicates absence of both Lewis antigens.

Production

Anti-Le blood grouping reagent is produced from murine hybridoma cell lines secreting human IgM antibodies. Clone 17A5G8 (for Anti-Le^a) and Clone 4C4C7 (for Anti-Le^b) are designed for recognition of their corresponding antigens on the human red blood cells.

The procedure used with these reagents is based on the principle of agglutination. Normal human red blood cells, possessing Le^a or Le^b antigens, will agglutinate in the presence of antibody directed towards the antigens. The monoclonal type of Lewis blood group antisera contains saline agglutinating complete antibodies which specifically agglutinate human red blood cells suspended in saline and at room temperature, to detect the presence or absence of an antigen, either Le^a or Le^b antigen as shown in Table-1.

Table -1

Serum	Antigen(s)	Phenotype of cells
Anti-Le^a	Le^a	Le ($a^+ b^-$), Le ($a^+ b^+$)
Anti-Le^b	Le^b	Le ($a^- b^+$), Le ($a^+ b^+$)

Processing

Processing method

(i) The processing method shall be one that has been shown to yield consistently a specific, potent final product, free of properties that would affect adversely the intended use of the product throughout its dating period. Stability testing shall be performed on an adequate number of representative samples of each group of products manufactured.

(ii) A lot may be subdivided into sublots. If lots are to be subdivided, the manufacturer shall include this information in the biologics license application. The manufacturer shall describe the test specifications to verify that each sublot is identical to other sublots of the lot.

(iii) Each lot of Blood Grouping Reagent shall be identified by a lot number. Each sub-lot shall be identified by that lot number to which a distinctive prefix or suffix shall be added. Final container and package labels shall bear the lot number and all distinctive prefixes and suffixes that have been applied to identify the sub-lot from which filling was accomplished.

Color coding of reagents. Anti-Lewis Blood Grouping Reagents should be colourless with no added colorant as approved by National Regulatory Authority.

Preservative and other constituents. The reagents should contain suitable anti-microbial preservative, preferably 0.1 per cent *sodium azide*/ 1 per cent *2-chloroacetamide*. A warning information about the hazards of the preservative should be mentioned on the label. Additional constituents like EDTA,

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sodium chloride (normal saline), bovine albumin or others should be mentioned.

Potency requirements with reference preparations

Each antiserum for which Reference Blood Grouping Reagents are available shall comply with the potency titer value at least equal to that of the reference preparation.

Potency tests without reference preparations

Products for which Reference Blood Grouping Reagents are not available shall be tested for potency by a method approved by the National Regulatory Authority.

Products recommended for test tube techniques. Anti-Le^a and Anti-Le^b reagents recommended for the test tube methods shall have the potency titer values of not less than 2+ reaction with undiluted reagent.

Products recommended for test tube or microplate techniques. Blood Grouping Reagents recommended for test tube methods or microplate techniques shall produce clearly positive macroscopic results when both undiluted reagent and reagent diluted with an equal volume of diluent are tested by all methods recommended in the manufacturer's package insert using red blood cells showing heterozygous or diminished expression of the corresponding antigen. The dilution shall be made with an equal volume of compatible serum or approved diluent.

Products recommended for use in an automated system. The manufacturer of Anti-Le^a nd Anti-Le^b reagents that is recommended for use in an automated system shall demonstrate that its product when used both undiluted and diluted with an equal volume of diluent satisfactorily performs when tested with cells representing heterozygous or diminished expression of the corresponding antigen.

Specificity tests and avidity tests

Specificity and avidity tests shall be performed using test procedures approved by National Regulatory Authority. Each serum (Anti-Le^a or Anti-Le^b) should comply with the tests for avidity with the cells as indicated in Table-2.

Table -2

Serum	Phenotype of testing cells
Anti-Le^a	Le $(a^+ b^-)$, Le $(a^+ b^+)$
Anti-Le^b	Le $(a^- b^+)$, Le $(a^+ b^-)$
No agglutination	Le $(a^- b^-)$

Each serum complies with the tests for specificity by the most sensitive methods recommended by the manufacturer and approved by National Regulatory Authority, in which not less than 4 positive control samples (homozygous and heterozygous form for each antigen) and 4 negative control (red cells lacking the corresponding antigen) are included. Attention should be given to confirm the absence of contaminating antibodies.

Red blood cells used for these tests should be either fresh or frozen red blood cell suspension which are prepared under specific conditions and meet specified criteria.

Labelling. The label states (1) name of the preparation; (2) name of the antibody or antibodies present; (3) the volume of the preparation; (4) source of the preparation (whether human serum or plasma or other source); (5) storage condition; (6) date of expiry; (7) name and concentration of the preservative used; (8) the statement: "FOR IN VITRO DIAGNOSTIC USE"; (9) if human blood was used in manufacturing the preparation, a cautionary statement "All blood products should be treated as potentially infectious. Source material from which this product was derived was found negative when tested in accordance with current required tests. No known test methods can offer assurance that products derived from human blood will not transmit infectious agents".

Blood Grouping Reagent Anti-M, Anti-N

MN Blood Grouping Serum is composed of two antigens - M and N. M antigen (also called MNS1) is found on the erythrocytes of approximately 80 to 90 per cent of Indian population, while N antigen (also called MNS2) is found in 67 to 68 per cent of Indian population. Agglutination of red blood cells with Anti-M or Anti-N blood grouping reagent, indicates the presence of M antigen and N antigen respectively. Absence of agglutination of red blood cells with Anti-M or Anti-N blood grouping reagent indicates absence of M and N antigens.

Production

Anti M, Anti N blood grouping reagent is produced either from (i) mouse hybridoma cell lines secreting human IgM antibodies. Clone M-11H2 (for Anti-M) and Clone 1422-C7 (for Anti-N) are designed for recognition of their corresponding antigens on the human red blood cells. (ii) or from pooled plasma/ serum by immunizing rabbits with specific antigens, who have developed specific Anti-M or Anti-N antibodies, as a result of alloimmunization. They are free from agglutinins for A and B antigens and from alloantibodies other than Anti-M/ Anti-N as mentioned in the label. Anti-M and Anti-N are mostly IgM (saline and cold reacting), do not bind complement and do not react with enzyme treated RBCs. They exhibit dosage

effects. Both Anti-M and Anti-N are rarely IgG and reactive at 37° and are haemolytic in nature.

The procedure used with these reagents is based on the principle of agglutination. Normal human red blood cells, possessing M or N antigens, will agglutinate in the presence of antibody directed towards the antigens. The MN blood group reagent contains saline agglutinating complete antibodies which specifically agglutinate human red blood cells suspended in saline at room temperature to detect the presence or absence of an antigen, either M or N antigen as shown in Table-1.

Table -1

Serum	Antigen(s)	Phenotype of cells
Anti-M	M	$M^+ N^-$, $M^+ N^+$
Anti-N	N	$M^- N^+$, $M^+ N^+$

Processing

Processing method

(i) The processing method shall be one that has been shown to yield consistently a specific, potent final product, free of properties that would affect adversely the intended use of the product throughout its dating period. Stability testing shall be performed on an adequate number of representative samples of each group of products manufactured.

(ii) A lot may be subdivided into sub-lots. If lots are to be subdivided, the manufacturer shall include this information in the biologics license application. The manufacturer shall describe the test specifications to verify that each sub-lot is identical to other sub-lots of the lot. Each lot of Blood Grouping Reagent shall be identified by a lot number. Each sub-lot shall be identified by that lot number to which a distinctive prefix or suffix shall be added. Final container and package labels shall bear the lot number and all distinctive prefixes and suffixes that have been applied to identify the sub-lot from which filling was accomplished.

Color coding of reagents. Anti-MN Blood Grouping Reagents should be colourless with no added colorant as approved by National Regulatory Authority.

Preservative and other constituents. The reagents should contain suitable anti-microbial preservative, preferably 0.1 per cent *sodium azide*/ 1 per cent *2-chloroacetamide*. A warning information about the hazards of the preservative should be mentioned on the label. Additional constituents like EDTA, sodium chloride (normal saline), bovine albumin or others should be mentioned.

Potency requirements with reference preparations

Each antiserum for which Reference Blood Grouping Reagents are available shall comply with the potency titre value at least equal to that of the reference preparation.

Potency tests without reference preparations

Products for which Reference Blood Grouping Reagents are not available shall be tested for potency by a method approved by the National Regulatory Authority.

Products recommended for test tube techniques. Anti-M reagents recommended for the test tube methods should have potency titre values of not less than 1+ reaction with a 1:4 dilution of the reagent.

Anti-N reagents recommended for the test tube methods should have the potency titre values of not less than 2+ reaction with undiluted reagent.

Products recommended for test tube or microplate techniques. Blood Grouping Reagent recommended for test tube methods or microplate techniques shall produce clearly positive macroscopic results when both undiluted reagent and reagent diluted with an equal volume of diluent are tested by all methods recommended in the manufacturer's package insert using red blood cells showing heterozygous or diminished expression of the corresponding antigen. The dilution shall be made with an equal volume of compatible serum or approved diluent.

Products recommended for use in an automated system. The manufacturer of Anti-M and Anti-N reagents that is recommended for use in an automated system shall demonstrate that its product when used both undiluted and diluted with an equal volume of diluent satisfactorily performs when tested with cells representing heterozygous or diminished expression of the corresponding antigen.

Specificity tests and avidity tests

Specificity and avidity tests shall be performed using test procedures approved by National Regulatory Authority. Each serum (Anti-M or Anti-N) should comply with the tests for avidity with the cells as indicated in Table-2.

Table -2

Serum	Phenotype of testing cells
Anti-M	$M^+ N^-$, $M^+ N^+$
Anti-N	$M^- N^+$, $M^+ N^+$

Each serum complies with the tests for specificity by the most sensitive methods recommended by the manufacturer and approved by National Regulatory Authority, in which not less than 4 positive control samples (homozygous and

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heterozygous form for each antigen) and 4 negative control (red cells lacking the corresponding antigen) are included. Attention should be given to confirm the absence of contaminating antibodies.

Red blood cells used for these tests should be either fresh or frozen red blood cell suspension which are prepared under specific conditions and meet specified criteria.

Labelling. The label states (1) name of the preparation; (2) name of the antibody or antibodies present; (3) the volume of the preparation; (4) source of the preparation (whether human serum or plasma or other source); (5) storage condition; (6) date of expiry; (7) name and concentration of the preservative used; (8) the statement: "FOR IN VITRO DIAGNOSTIC USE"; (9) if human blood was used in manufacturing the preparation, a cautionary statement "All blood products should be treated as potentially infectious. Source material from which this product was derived was found negative when tested in accordance with current required tests. No known test methods can offer assurance that products derived from human blood will not transmit infectious agents".

Blood Grouping Reagent Anti-P_1

The P blood grouping reagent is composed of three antigens -P_1, P and P^k. P antigen is found on the erythrocytes of approximately 94 per cent of Indian population. Agglutination of red blood cells with Anti-P_1 blood grouping reagent, indicates the presence of P_1 antigen. Absence of agglutination of red blood cells with Anti-P_1 blood grouping reagent indicates absence of P_1 antigen.

Production

Anti-P_1 blood grouping reagent is a polyclonal preparation obtained from the goats immunized with P_1 antigens.

The procedure used with these reagents is based on the principle of agglutination. Normal human red blood cells, possessing P_1 antigens, will agglutinate in the presence of antibody directed towards the antigens. The Polyclonal type of P blood group antisera contains saline agglutinating complete antibodies which specifically agglutinate human red blood cells suspended in saline and at room temperature. To detect the presence or absence of an antigen, either P, P_1 or P^k antigen as shown in Table-1.

Table -1

Serum	Antigen(s)	Phenotype of cells
Anti-P_1	P_1, P, P^k	P_1 (P^{1+})
Anti-P	P, P^k	P_2 (P^{1-})
Anti-P^k	P_1, P_2	P_1, P_2

Processing

Processing method

(i) The processing method shall be one that has been shown to yield consistently a specific, potent final product, free of properties that would affect adversely the intended use of the product throughout its dating period. Stability testing shall be performed on an adequate number of representative samples of each group of products manufactured.

(ii) A lot may be subdivided into sublots. If lots are to be subdivided, the manufacturer shall include this information in the biologics license application. The manufacturer shall describe the test specifications to verify that each sublot is identical to other sublots of the lot.

(iii) Each lot of Blood Grouping Reagent shall be identified by a lot number. Each sub-lot shall be identified by that lot number to which a distinctive prefix or suffix shall be added. Final container and package labels shall bear the lot number and all distinctive prefixes and suffixes that have been applied to identify the sub-lot from which filling was accomplished.

Color coding of reagents. Anti-Lewis Blood Grouping Reagents should be colourless with no added colorant as approved by National Regulatory Authority.

Preservative and other constituents. The reagents should contain suitable anti-microbial preservative, preferably 0.1 per cent *sodium azide*/ 1 per cent *2-chloroacetamide*. A warning information about the hazards of the preservative should be mentioned on the label. Additional constituents like EDTA, sodium chloride (normal saline), bovine albumin or others should be mentioned.

Potency requirements with reference preparations

Each antiserum for which Reference Blood Grouping Reagents are available shall comply with the potency titer value at least equal to that of the reference preparation.

Potency tests without reference preparations

Products for which Reference Blood Grouping Reagents are not available shall be tested for potency by a method approved by the National Regulatory Authority.

Products recommended for test tube techniques. Anti-P_1 reagent recommended for the test tube methods shall have the potency titer values of not less than 1+ reaction with 1:4 dilution of the reagent.

Products recommended for test tube or microplate techniques. Blood Grouping Reagent recommended for test tube methods or microplate techniques shall produce clearly positive macroscopic results when both undiluted reagent and reagent diluted with an equal volume of diluent are tested by all

methods recommended in the manufacturer's package insert using red blood cells showing heterozygous or diminished expression of the corresponding antigen. The dilution shall be made with an equal volume of compatible serum or approved diluent.

Products recommended for use in an automated system. The manufacturer of Anti-P_1 reagents that is recommended for use in an automated system shall demonstrate that its product when used both undiluted and diluted with an equal volume of diluent satisfactorily performs when tested with cells representing diminished expression of the corresponding antigen.

Specificity tests and avidity tests

Specificity and avidity tests shall be performed using test procedures approved by National Regulatory Authority. Each serum should comply with the tests for avidity with the cells as indicated in Table-2.

Table -2

Pheneotype	Reactivity
P1	Anti-P_1 (+), Anti-P (+), Anti-PP_1P^k (+), Anti-P^k (-)
P2	Anti-P_1 (-), Anti-P (+), Anti-PP_1P^k (+), Anti-P^k (-)
Absence of P antigens (rare)	Anti-P_1 (-), Anti-P (-), Anti-PP_1P^k (-), Anti-P^k (-)

Each serum complies with the tests for specificity by the most sensitive methods recommended by the manufacturer and approved by National Regulatory Authority, in which not less than 4 positive control samples (homozygous and heterozygous form for each antigen) and 4 negative control (red cells lacking the corresponding antigen) are included. Attention should be given to confirm the absence of contaminating antibodies.

Red blood cells used for these tests should be either fresh or frozen red blood cell suspension which are prepared under specific conditions and meet specified criteria.

Labelling. The label states (1) name of the preparation; (2) name of the antibody or antibodies present; (3) the volume of the preparation; (4) source of the preparation; (5) storage condition; (6) date of expiry; (7) name and concentration of the preservative used; (8) the statement: "FOR IN VITRO DIAGNOSTIC USE"; (9) if human blood was used in manufacturing the preparation, a cautionary statement "All blood products should be treated as potentially infectious. Source material from which this product was derived was found negative when tested in accordance with current required tests. No known test methods can offer assurance that products derived from human blood will not transmit infectious agents."

Blood Grouping Reagent Anti-S, Anti-s

The Ss blood grouping reagent is composed of two antigens - S and s. S-antigen (also called MNS 3) is found on the erythrocytes of approximately 52 to 58 per cent of Indian population, while s-antigen (also called MNS 4) is found in 85 to 90 per cent of Indian population. Agglutination of red blood cells with Anti-S or Anti-s blood grouping reagent, indicates the presence of S-antigen and s-antigen respectively. Absence of agglutination of red blood cells with both Anti-S or Anti-s blood grouping reagent indicates absence of both Ss antigens and is very rare.

Production

Anti-S and Anti-s blood grouping reagents are polyclonal preparation pooled from human plasma / serum who have developed specific Anti-S or Anti-s antibodies, as a result of alloimmunization. They are free from agglutinins for A and B antigens and from alloantibodies other than Anti-S /Anti-s as mentioned in the label. They are IgG in characteristic, exhibit minimal dosage effect and may or may not react with enzyme treated RBCs.

The procedure used with these reagents is based on the principle of agglutination. Normal human red blood cells, possessing S or s antigens, will agglutinate in the presence of antibody directed towards the antigens. The Ss blood grouping antisera contains incomplete or irregular antibodies (IgG in nature and non-saline reacting), indirect antiglobulin tests are applied to detect the presence or absence of an antigen, either S or s antigen as shown in Table-1.

Table -1

Serum	Antigen(s)	Phenotype of cells
Anti-S	S	$S^+ s^-$, $S^+ s^+$
Anti-s	s	$S^- s^+$, $S^+ s^+$

Processing

Processing method

(i) The processing method shall be one that has been shown to yield consistently a specific, potent final product, free of properties that would affect adversely the intended use of the product throughout its dating period. Stability testing shall be performed on an adequate number of representative samples of each group of products manufactured.

(ii) A lot may be subdivided into sublots. If lots are to be subdivided, the manufacturer shall include this information in the biologics license application. The manufacturer shall describe the test specifications to

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verify that each sublot is identical to other sublots of the lot.

(iii) Each lot of Blood Grouping Reagent shall be identified by a lot number. Each sub-lot shall be identified by that lot number to which a distinctive prefix or suffix shall be added. Final container and package labels shall bear the lot number and all distinctive prefixes and suffixes that have been applied to identify the sub-lot from which filling was accomplished.

Color coding of reagents. Anti-Ss Blood Grouping Reagents should be colourless with no added colorant as approved by National Regulatory Authority.

Preservative and other constituents. The reagents should contain suitable anti-microbial preservative, preferably 0.1 per cent *sodium azide*/ 1 per cent *2-chloroacetamide*. A warning information about the hazards of the preservative should be mentioned on the label. Additional constituents like EDTA, sodium chloride (normal saline), bovine albumin or others should be mentioned.

Potency requirements with reference preparations

Each antiserum for which Reference Blood Grouping Reagents are availabfe shall comply with the potency titer value at least equal to that of the reference preparation.

Potency tests without reference preparations

Products for which Reference Blood Grouping Reagents are not available shall be tested for potency by a method approved by the National Regulatory Authority.

Products recommended for test tube techniques. Anti-S and Anti-s reagents recommended for the test tube methods shall have the potency titer values of not less than 1+ reaction with a 1:4 dilution of the reagent.

Products recommended for test tube or microplate techniques. Blood Grouping Reagents recommended for test tube methods or microplate techniques shall produce clearly positive macroscopic results when both undiluted reagent and reagent diluted with an equal volume of diluent are tested by all methods recommended in the manufacturer's package insert using red blood cells showing heterozygous or diminished expression of the corresponding antigen. The dilution shall be made with an equal volume of compatible serum or approved diluent.

Products recommended for use in an automated system. The manufacturer of Anti-S and Anti-s reagents that is recommended for use in an automated system shall demonstrate that its product when used both undiluted and diluted with an equal volume of diluent satisfactorily performs when tested with cells representing heterozygous or diminished expression of the corresponding antigen.

Specificity tests and avidity tests

Specificity and avidity tests shall be performed using test procedures approved by National Regulatory Authority. Each serum (Anti-S or Anti-s) should comply with the tests for avidity with the cells as indicated in Table-2.

Table -2

Serum	Phenotype of testing cells
Anti-S	$S^+ s^-$, $S^+ s^+$
Anti-s	$S^- s^+$, $S^+ s^+$

Each serum complies with the tests for specificity by the most sensitive methods recommended by the manufacturer and approved by National Regulatory Authority, in which not less than 4 positive control samples (homozygous and heterozygous form for each antigen) and 4 negative control (red cells lacking the corresponding antigen) are included. Attention should be given to confirm the absence of contaminating antibodies.

Red blood cells used for these tests should be either fresh or frozen red blood cell suspension which are prepared under specific conditions and meet specified criteria.

Labelling. The label states (1) name of the preparation; (2) name of the antibody or antibodies present; (3) the volume of the preparation; (4) source of the preparation (whether human serum or plasma or other source); (5) storage condition; (6) date of expiry; (7) name and concentration of the preservative used; (8) the statement: "FOR IN VITRO DIAGNOSTIC USE"; (9) if human blood was used in manufacturing the preparation, a cautionary statement "All blood products should be treated as potentially infectious. Source material from which this product was derived was found negative when tested in accordance with current required tests. No known test methods can offer assurance that products derived from human blood will not transmit infectious agents".

Blood Grouping Serums

Category. Blood product.

NOTE—This monograph deals with those blood grouping serums for which there are no individual monographs and which are not routinely used or required for the testing of blood or blood products for transfusion.

Each is a sterile, liquid or dried preparation containing one or more of the particular blood group antibodies derived from high-titered blood plasma or serum of human subjects, with or without stimulation by the injection of red cells or other

substances, or of animals after stimulation by substances that cause such antibody production.

Tests

Antiglobulin test. It causes either directly, or indirectly the visible agglutination of human red cells containing the particular antigen(s) for which it is specific.

Potency

In the case of tube test reagents, when tested by the specified method, of agglutinating red blood cells containing the specified antigens with the specified degree of reactivity, as; not less than a 1 + reaction (i.e., agglutinated cells dislodged in to finely granular, but definite, small clumps)with a 1:8 dilution of serum for Anti-K, Anti-k, Anti-Jk^a, Anti-Fy^a, Anti-C^w not less than a 1 + reaction with a 1:4 dilution of serum for Anti-S, Anti-s, Anti-P_1, Anti-M, anti-I, Anti-e (saline), Anti-c (saline), Anti-A_1, and not less than a 2+ reaction (i.e., agglutinated cells dislodged in to many small clumps of equal size) with undiluted serum for Anti-U, Anti-Kp^a, Anti-Kp^b, Anti-Js^a, Anti-Fy^b, Anti-N, Anti-Le^a, Anti-Le^b, Anti-Di^a, Anti-M^g, Anti-Jk^b and Anti-Xg^a.

In the case of reagents recommended for slide test methods, agglutinating red blood cells, with the specified degree of reactivity, as; with both undiluted serum and with a 1:2 dilution of serum, when tested by the manufacturer's recommended method(s) using cells heterozygous for the corresponding antigens (s). It meets the requirements of the test for specificity by the most sensitive method recommended by the manufacturers, in which not less than 4 positive and 4 negative phenotypes are included, and confirms the absence of contaminating antibodies reactive with M^g, Wr^a antigens as well as other antigens having an incidence of 1 per cent or greater in the general population.

It meets the requirements of the test for avidity with the manufacturer's recommended method, red bloods cells heterozygous for the corresponding antigen(s) being used. All fresh or frozen red blood cells suspension used for these test are prepared under specified conditions and meet specified criteria.

Antimicrobial preservatives. It contains a suitable antimicrobial preservative.

Storage. Store at a temperature between 2° to 8°.

Labelling. The label states (1) the source of the product if other than human and if of human origin, the source material was not reactive for hepatitis B surface antigen; (2) the products derived from human blood will not transmit hepatitis; (3) it is for *in vitro* diagnostic use.

Blood Grouping Serums Anti-D, Anti-C, Anti-E, Anti-c, Anti-e

Anti-Rh Blood Grouping Serums

Blood Grouping Serums Anti-D, Anti-C, Anti-E, Anti-c, Anti-e (Anti-Rh Group) are sterile, liquid or dried preparations derived from the blood plasma or serum of human subjects who have developed specific Rh antibodies. They are free from agglutinins for the A or B antigens and from alloantibodies other than those for which claims are made in the labelling. They contain a suitable antimicrobial preservative. Liquid serums are not artificially coloured. Two varieties of Anti-Rh Blood Grouping Serums are recognized, i.e., (1) saline agglutinating "complete" antiserums, which specifically agglutinate human red blood cells suspended in saline and (2) "blocking or incomplete" antiserums, which contain protein or other macromolecular substances, usually require the cells to be suspended in serum or plasma, and generally are for slide or rapid tube tests. The most commonly used of these blood grouping serums are listed in Table 1 each reacting with the antigen(s) designated by the corresponding letter(s) with the alternative nomenclature indicated parenthetically.

Table - 1

Serum	Antigen(s) Reacting
Anti-D (Anti-Rh_o)	D (Rh_o)
Anti-C (Anti-rh′)	C (rh′)
Anti-E (Anti-rh″)	E (rh″)
Anti-CD (Anti-Rh_o′)	D (Rh_o) and C (rh′)
Anti-DE (Anti-Rh_o″)	D (Rh_o) and E (rh″)
Anti-CDE (Anti-Rh_o‴)	D (Rh_o), C (rh′), and E (rh′)
Anti-c (Anti-hr′)	c (hr′)
Anti-e (Anti-hr″)	e (hr″)

Each serum complies with the test for potency in the case of serums for saline tube test in parallel with, and not less than equivalent to, the Reference Blood Grouping Serum for Anti-D, Anti-C, or Anti-E, whichever is applicable, or, in the case of Anti-c and Anti-e for saline tube test which have no reference preparations, the test for minimum agglutination reactivity at a specified dilution; and in the case of serums for slide or rapid tube test in parallel with, and not less than equivalent to, the Reference Blood Grouping Serum for Anti-D, Anti-C, Anti-E, Anti-c, or Anti-e, whichever is applicable, in agglutinating as a minimum red blood cells from the donors indicated in Table 2 (which may be from Group A, B, AB, or O).

Each serum for slide or rapid tube test complies with the tests for avidity with the cells as indicated under tests for potency above.

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Table - 2

Serum	Phenotype of Cells
Anti-D	cDe
Anti-C	Ccde
Anti-E	cdEe
Anti-CD	cDe, Ccde
Anti-DE	cDe, cdEe
Anti-CDE	cdEe, cDe, Ccde
Anti-c	CcDEe
Anti-e	cdEe

Each serum complies with the tests for specificity by the most sensitive method recommended by the manufacturer, in which not less than 4 positive and 4 negative phenotypes are included (see Table 3), and confirms the absence of contaminating antibodies reactive with M^g, Wr^a antigens as well as other antigens having an incidence of 1.0 per cent or greater in the general population, except where some of these confirmatory tests can not be done by the manufacturer, in which event such omissions are noted.

Table - 3

Serum	Cells
Anti-D	CcDe, cDe, Ccde, cdEe, A_1 cde, B cde, O cde, and where recommended for use by indirect antiglobulin technique, cde Bg(a+) cells from 3 different donors
Anti-C	cDe, Ccde, cdEe, C + rh_i neg. cells, A_1 cde, B cde, O cde
Anti-E	cDe, Ccde, cdEe, A_1 cde, B cde, O cde
Anti-CD	cDe, Ccde, cdEe, A_1 cde, B cde, O cde, and where recommended for detection of the G antigen, r^Gr
Anti-DE	cDe, Ccde, cdEe, A_1 cde, B cde, O cde
Anti-CDE	cDe, Ccde, cdEe, A_1 cde, B cde, O cde, and where recommended for detection of the G antigen, r^Gr
Anti-c	Ccde, A_1 CDe, B CDe, O CDe, and CDEe or CDE or CdE
Anti-e	cdEe, A_1 cDE, B cDE, O cDE, and CcDE or CDE or CdE

All fresh or frozen red blood cell suspensions used for these tests are prepared under specified conditions and meet specified criteria.

Monoclonal Rh (D) antibodies. Monoclonal antibodies are derived from hybridoma cell lines produced by fusing mouse antibody produced by B lymphocyte with mouse myeloma cells or are derived from a human B cell line through Epstein-Barr Virus (EBV) transformation. Each hybridoma cell line produces homogenous antibodies of only one immunoglobulin class, which are identical in their chemical structure and immunological activity.

The type of monoclonal anti-D reagents are:

1. IgM anti-D monoclonal reagent,
2. Blend of IgM and IgG monoclonal antibodies reagent,
3. Blend of monoclonal IgM and polyclonal (human) IgG anti-D.

IgM anti-D monoclonal antibodies are highly specific and saline reacting equally well at room temperature and at 37°. They are good for slide test or immediate spin tube tests as well as routine Rh(D) typing in tube. IgM anti D are unreliable for detection of weak D (Du) by AHG test. Blend of IgM and IgG (monoclonal) anti-D or blend of IgM (monoclonal) and polyclonal (human) IgG anti-D can be used for testing weak D (Du) antigen by AHG test. Mostly blended IgM and IgG (monoclonal) anti- Rh(D) or blended monoclonal IgM and polyclonal (human) IgG anti Rh (D) antibodies are used now in routine.

Expiration date. The expiration date for liquid serums is not later than 1 year and for dried serums not later than 5 years after date of issue from manufacturer's cold storage (5°, 1 year; or 0°, 2 years), provided that the expiration date for dried serums is not later than 1 year after constitution.

Storage. Store at a temperature between 2° and 8°.

Labelling. The label each to state that the source material was not reactive for hepatitis B surface antigen, but that no known test method offers assurance that products derived from human blood will not transmit hepatitis. Label each to state that it is for *in vitro* diagnostic use.

Concentrated Human Red Blood Corpuscles

Concentrate of Human Red Blood Cells; Packed Red Cells

Concentrated Red Blood Cells (RBCs) are units of whole blood with most of the plasma removed. Additive red cell preservative systems consist of a primary collection bag containing an anticoagulant preservative with at least two satellite bags integrally attached; one is empty and one contains an additive solution (AS).

Each 100 ml citrate phosphate dextrose (CPD) contains

Citric acid (monohydrate)	0.327 g
Sodium citrate (dihydrate)	2.63 g
Sodium acid phosphate (dihydrate)	0.251 g
Dextrose (monohydate)	2.55 g
Water for injection	100 ml

AS contains sodium chloride, dextrose, adenine and other substances that support red cell survival and function up to 42 days. The volume of AS in 350 ml is 49 ml and in 450 ml is 63 ml. AS is added to the red cells remaining in the primary bag after most of the plasma has been removed. This allows blood centres to use or recover a maximum amount of plasma, yet still prepare a red cell component with a final haematocrit between 55.0 per cent and 65.0 per cent, a level that facilitates excellent flow rates and allows easy administration.

Production

It may be prepared by centrifugation or undisturbed sedimentation for the separation of plasma and anticoagulant solution equivalent to not less than 40.0 per cent of the total volume of Whole Human Blood. A portion of the plasma, sufficient to ensure optimal cell preservation, shall be left. All surfaces that come in contact with the red cells and plasma shall be sterile and pyrogen-free and the entire processing of the blood shall be conducted in a sterile system or in a closed system by use of satellite bags. The final containers used for the Concentrated Human Red Blood Corpuscles shall be the original blood containers unless the method of processing requires a different container. Immediately after processing, the containers are stored at a temperature between 2° and 8° and not opened until immediately before transfusion. Human Red Blood Corpuscles may be stored for a period not longer than that for which the Whole Human Blood from which it is prepared. However, if the hermetic seal is broken during processing, the product must be used within 24 hours. From the tube of the blood bag sample is taken for compatibility and infections markers testing.

Concentrated Human Red Blood Corpuscles should be administered only with suitable equipment meant for the transfusion of blood and blood components.

Concentrated Human Red Blood Corpuscles contains not less than 15.5 per cent w/v of haemoglobin.

Description. A dark red fluid when prepared; after standing, the red corpuscles may form a sediment, leaving a small supernatant layer of yellowish plasma.

Tests

Sterility (2.2.11). Complies with the tests for sterility, determined by Method B.

Assay. Determine the haemoglobin content by photometric haemoglobinometry (2.8.12).

Storage. Store in containers which are made of colourless and transparent glass, or of a suitable plastic material, are sterile and sealed so as to exclude micro-organisms. Store at a temperature between 2° and 8°.

Labelling. The label states (1) the reference number of the Whole Human Blood from which the preparation was made; (2) the ABO and Rh groups of the Whole Human Blood; (3) the date of collection of the Whole Human Blood from which the preparation was made; (4) the storage conditions; (5) the date after which the preparation is not suitable for transfusion; (6) that the preparation should not be used if there is any visible evidence of haemolysis or other deterioration.

Cryoprecipitated Antihaemophilic Factor

Cryoprecipitated Antihaemophilic Factor is a sterile, frozen concentrate of human antihaemophilic factor prepared from the Factor VIII-rich cryoprotein fraction of human venous plasma obtained from suitable whole-blood donors from a single unit of plasma derived from whole blood or by plasmapheresis, collected and processed in a closed system. It contains no preservative. It complies with the test for potency by comparison with the Standard Antihaemophilic Factor (Factor VIII) or with a working reference that has been calibrated with it, in having an average potency of not less than 80 Antihaemophilic Factor Units per container, made at intervals of not more than 1 month during the dating period.

Expiration date. The expiration date is not later than 1 year from the date of collection of source material.

Storage. Store in hermetic containers at a temperature of - 18° or lower.

Labelling. Label it to indicate (1) the ABO blood group designation and the identification number of the donor from whom the source material was obtained; (2) with the type and result of a serologic test for syphilis, or to indicate that it was non-reactive in such test; with the type and result of a test for hepatitis B surface antigen, or to indicate that it was non-reactive in such test; with a warning not to use it if there is evidence of breakage or thawing; with instructions to thaw it before use to a temperature between 20° and 37°, after which it is to be stored at room temperature and used as soon as possible but within 6 hours after thawing; (3) to state that it is to be used within 4 hours after the container is entered; (4) to state that it is for intravenous administration; (5) that a filter is to be used in the administration equipment.

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Dried Human Antihaemophilic Fraction

Dried Factor VIII Fraction; Freeze-dried Human Coagulation Factor VIII

Dried Human Antihaemophilic Fraction is a preparation of a plasma protein fraction that contains the glycoprotein coagulation factor VIII together with varying amounts of von Willebrand factor, depending on the method of preparation. It is prepared from human plasma that complies with the monograph on Plasma for Fractionation.

The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU of factor VIII: C per milliliter.

Production

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection. If substances are used for the inactivation of viruses, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for the patients.

The specific activity is not less than 1 IU of Factor VIII: C per mg of total protein and not more than 80 per cent of which is fibrinogen, before the addition of any protein stabilizer.

The Factor VIII fraction is dissolved in a suitable liquid. Excipients such as a stabilizer may be added.

No antibacterial preservative is added. The solution is passed through a bacteria retentive filter distributed aseptically into the final containers and immediately frozen. It is subsequently freeze dried and the containers are closed under vaccum or under an inert gas.

For products stated to have von Willebrand factor activity for products intended for treatment of von Willebrand's disease it shall be demonstrated that the manufacturing process yields a product with a consistent composition with respect to von Willebrand factor.

Products that show flakes or particles after reconstitution for use, if a few small flakes or particles remain when the preparation is reconstituted it shall be demonstrated during validation studies that the potency is not significantly affected after passage of the preparation through the filter provided.

Description. A white or pale yellow hygroscopic powder or friable solid.

Reconstitute the preparation under examination as stated on the label immediately before carrying out the identification test (except those for solubility and water) and assays.

Identification

A. Complies with the limits of the assay.

Solubility. To a container of the preparation under examination add the volume of the solvent stated on the label at the recommended temperature. The preparation dissolves completely with gentle swirling within 10 minutes, giving a clear or slightly opalescent, colourless or slightly yellow solution.

Where the label states that the product may show a few small flakes or particles after reconstitution, reconstitute the preparation as described on the label and pass it through the filter provided: the filtered solution is clear or slightly opalescent.

Tests

pH (2.4.24). 6.5 to 7.5, determined on the reconstituted solution.

Osmolality (2.4.23). Not less than 240 mosmol per kg.

Loss on drying (2.4.19). Not more than 2.0 per cent, determined by drying 0.5g over *phosphorous pentoxide* at a pressure not exceeding 3 kPa for 24 hours.

Haemagglutinins, anti-A and anti-B. Reconstitute the powder as per the manufacturer's instruction. Dilute the solution with *saline* to produce a solution containing 3 IU of Factor VIII: C per ml. Carry out the test for Haemagglutinins, anti-A and anti-B using a suitable indirect method such as that as described below.

Prepare in duplicate serial dilution of the under examination in saline solution. To each dilution of one series add an equal volume of a 5 per cent v/v suspension of group A_1 red blood cells previously washed 3 times with *saline solution*. To each dilution of the other series add an equal volume of a 5 per cent v/v suspension of group B red blood cells previously washed 3 times with *saline solution*. Incubate the suspension at 37° for 30 minutes and then wash the cells 3 times with *saline solution*. Leave the cells in contact with a polyvalent anti-human globulin reagent for 30 minutes. Without centrifuging, examine each suspension for agglutination under a microscope. The 1 in 64 dilutions do not show agglutination.

Hepatitis B surface antigen. Dissolve in *water*. Examine the solution by a suitable sensitive method such as radioimmunoassay. Hepatitis B surface antigen is not detected.

Abnormal toxicity (2.2.1). When dissolved in *water for injection*, complies with the test for abnormal toxicity (*For Antisera and Vaccines*), injecting into each mouse a volume containing 1.5 units and into each guinea pig a volume containing 15 units.

Pyrogens (2.2.8)/or **Bacterial Endotoxins (BET)** (2.2.3). Complies with the test for pyrogen or, preferably and where justified and authorized with a validated *in-vitro* test such as BET.

Pyrogens. For the pyrogen test inject per kilogram of rabbit's mass a volume equivalent to not less than 50 IU of Factor VIII: C.

BET. Not more than 0.03 IU of endotoxin per international unit of factor VIII: C.

Sterility (2.2.11). Complies with the test for sterility.

Assay

For potency. Carry out the biological assay of human antihaemophilic fraction described below. The estimated potency is not less than 80.0 per cent and not more than 120.0 per cent of the stated potency. The confidence limits (P = 0.95) are not less than 76.0 per cent and not more than 114.0 per cent of the estimated potency using any one method.

Method A. Biological assay

The potency of human antihaemophilic fraction is determined by comparing the amount necessary to reduce the clotting time of a test mixture containing substances that cause clotting of blood with the amount of the Standard preparation necessary to produce the same effect under the conditions of the following method of assay.

Standard preparation

The Standard preparation is the 4th International Standard for Blood coagulation factor VIII:C, concentrate, human, established in 1989, consisting of an intermediate purity concentrate of human blood clotting factor VIII (supplied in ampoules containing 6.3 Units of clotting factor VIII), or another suitable preparation the potency of which has been determined in relation to the International Standard.

The Unit is the specific antihaemophilic factor contained in such an amount of the Standard preparation as the Ministry of Health & Family Welfare, Govt. of India may from time to time indicate as the quantity exactly equivalent to the Unit accepted for international use.

Special reagents

Normal serum reagent. Collect normal human blood in a dry, sterile, glass bottle, shake continuously until coagulation is complete, incubate at 37° for 3 hours, maintain at 4° overnight, remove the serum, store at –20°, dry from the frozen state and keep in a vacuum desiccator over *phosphorus pentoxide*. Dissolve a quantity of the dried serum calculated to have been obtained from 1 ml of the serum in sufficient *imidazole buffer pH 7.4* to produce 10 ml and allow to stand at 4° for 16 to 24 hours.

Phospholipid. Wash a quantity of normal human or bovine brain freed from meninges and blood vessels and macerate in a suitable blender. Weigh 1,000 to 1,300 g of the macerate and measure its volume (V). Extract with three quantities, each of $4V$ ml, of *acetone*, filter by suction and dry the precipitate at 37° for 18 hours. Extract the dried precipitate with two quantities, each of $2V$ ml, of a mixture of two volumes of *light petroleum (boiling range 30° to 40°)* and 3 volumes of *light petroleum (boiling range 40° to 60°)*, filtering each extract through a filter paper previously washed with the light petroleum mixture. Combine the extracts and evaporate to dryness at 45° at a pressure not exceeding 0.7 kPa. Dissolve the residue in $0.2V$ ml of *ether* and allow to stand at 4° until a deposit forms. Centrifuge and evaporate the clear supernatant liquid under reduced pressure until the volume is about 100 ml per kg of the original macerate. Allow to stand at 4° until a precipitate forms (12 to 24 hours) and centrifuge. To the clear supernatant liquid add 5 times its volume of *acetone*, centrifuge, discard the supernatant liquid, dry the precipitate and store protected from light in a vacuum desiccator.

Phospholipid reagent. Suspend 0.125 g of *phospholipid* in 5 ml of *water*, shake and stir until a uniform suspension is obtained. Prepare a dilution with *saline solution* that will give minimum clotting time consistent with the largest clotting time differences between consecutive dilutions of the Standard preparation and the preparation under examination. The concentration usually lies between 50 and 250 µg per ml. The diluted suspension may be kept at -20° for 6 weeks.

Clotting factor V solution. Prepare from fresh oxalated bovine plasma by fractionation at 4° with a saturated solution of *ammonium sulphate* prepared at 4°. Use the fraction precipitating between 38 per cent and 50 per cent saturation (which contains clotting factor V not significantly contaminated with clotting factor VIII), dialysed to remove *ammonium sulphate* and diluted with *saline solution* to give a solution containing between 10 per cent and 20 per cent of the amount of clotting factor V present in fresh normal human plasma.

Determine the clotting factor V content of the solution as follows. Prepare two dilutions in *imidazole buffer pH 7.4* to contain 1 volume of the solution under examination in 10 volumes and 20 volumes respectively. Test each dilution as follows. Mix 0.1 ml each of *substrate plasma deficient in clotting factor V*, the dilution under test, *thrombokinase extract* and *0.025M calcium chloride*. Record as the clotting time the interval between the addition of the *calcium chloride solution* and the first indication of fibrin formation, which may be observed visually or by mechanical means.

Similarly determine the clotting times, in duplicate, for four dilutions of pooled normal human plasma in *imidazole buffer pH 7.4* containing 1 volume in 10 volumes (equivalent to 100 per cent of clotting factor V), in 50 volumes (20 per cent),

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in 100 volumes (10 per cent), and in 1,000 volumes (1 per cent), respectively.

To calculate the result, plot the mean of the clotting times for each dilution of human plasma on double cycle log/log paper against the equivalent percentage of clotting factor V and read the percentage of clotting factor V for the two dilutions of *clotting factor V solution* by interpolation from the curve. The mean of the two results is taken as the percentage of clotting factor V in the solution.

Substrate plasma. Separate the plasma from 9 volumes of human or bovine blood collected in 1 volume of a 3.8 per cent w/v solution of *sodium citrate* or from 3.5 volumes of human or bovine blood collected in 1 volume of a solution containing 2.0 per cent w/v of *sodium acid citrate* and 2.5 per cent w/v of *dextrose*. In the former case, prepare the substrate plasma on the day of collection of the blood. In the latter case, the substrate plasma may be prepared up to 2 days after collection of the blood. Store at -20°.

Substrate plasma deficient in clotting factor V. Preferably use congenitally deficient plasma or, alternatively, prepare as follows. Separate the plasma from human blood collected in one-tenth its volume of a 1.34 per cent w/v solution of *sodium oxalate* and incubate at 37° for 24 to 36 hours. This plasma should have a clotting time, when tested by the assay method given under *clotting factor V solution*, of 70 to 100 seconds; if the clotting time is less than 70 seconds, incubate the plasma for a further 12 to 24 hours.

Method

Dissolve the contents of the sealed container of the substance under examination in the volume of the liquid stated on the label and use immediately. Reconstitute the entire contents of one ampoule of the Standard preparation as stated on the label and use immediately.

To the reconstituted Standard preparation and the preparation under examination, add sufficient *imidazole buffer pH 7.4* to produce solutions containing between 0.5 and 2 Units per ml; these solutions are stable for 15 minutes at 20°. Using a mixture of 1 volume of a 3.8 per cent w/v solution of *sodium citrate* and 5 volumes of *saline solution* as the diluent, make from the solutions three successive 2-fold dilutions in the range 1 in 16 to 1 in 256 so that all the clotting times are between 17 and 35 seconds; the dilutions must be accurately made and used immediately.

Introduce into each of six glass incubation tubes (75 mm to 100 mm) 0.1 ml each of *clotting factor V solution, phospholipid reagent* and *normal serum reagent*. To the first tube add 0.1 ml of the highest dilution of the Standard preparation, place the tube in a water-bath at 37°, add 0.1 ml of *0.05M calcium chloride* and start a stop-watch. During the next minute add 0.1 ml of the second highest dilution of the standard to a second tube, place it in the water-bath, and add 0.1 ml of *0.05M calcium chloride* at exactly 1 minute by the stop-watch. Repeat the procedure with the lowest dilution of the standard and the highest to lowest dilutions of the preparation under examination so that the *calcium chloride solution* is added at 2, 3, 4 and 5 minutes by the stop-watch, respectively.

Place in a water-bath at 37°, twelve glass tubes each containing 0.2 ml of *0.025M calcium chloride* and a further tube containing about 3 ml of *substrate plasma*. At 14 minutes 40 seconds by the stop-watch, transfer 0.1 ml of the mixture from the first incubation tube to one of the tubes containing 0.2 ml of *0.025M calcium chloride solution* and mix. At 15 minutes add 0.2 ml of the warmed substrate plasma and, using a second stop-watch, record as the clotting time the interval between the addition of the substrate plasma and the first indication of fibrin formation, which may be observed visually or by mechanical means. Repeat the procedure with the other incubation tubes at 1-minute intervals and carry out a second series of determinations at 21 to 26 minutes. The period of incubation should, if necessary, be adjusted so that the clotting times recorded in the corresponding tests in the two series of determinations do not differ by more than 5.0 per cent, showing that a stable plateau of prothrombin activator formation has been reached.

Carry out a blank determination using in place of the preparation under examination, an equal quantity of a mixture of 1 volume of a 3.8 per cent w/v solution of *sodium citrate* and 5 volumes of *saline solution*. The result of the assay is not valid unless the clotting time in the blank determination is more than 40 seconds. Calculate the result of the assay by standard statistical methods.

Method B. Carry out Chromogenic assay as stated under Assay of Human Coagulation Factor VIII (2.8.6)

For total protein. If necessary dilute an accurately measured volume of the preparation under examination with a 0.9 per cent solution of sodium *chloride* to obtain a solution containing about 15 mg of protein in 2 ml. Place 2.0 ml of a 7.5 per cent solution of *sodium molybdate* and 2 ml of a mixture of 1 volume of *nitrogen free sulphuric acid* and 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid and allow the inverted tube to drain on the filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion, and calculate the amount of protein by multiplying the result by 6.25.

For some product, especially without a protein stabilizer such as albumin, this method may not be applicable and another validated method for protein determination must be therefore performed.

For fibrinogen. Dilute 1 ml of the solution to 10 ml with the *phosphate buffered saline pH 6.5* and ionic strength 0.15. Clot 5 ml of the dilution with the minimum amount of *thrombin*

collect the clot and add 3 drops of 30.0 per cent w/v solution of copper sulphate and 1 ml of *nitrogen free sulphuric acid* and boil gently for 10 minutes. Cool; add 1 g of *anhydrous sodium sulphate* and 10 mg of selenium. Boil gently for 1 hour and cool. Then transfer to an ammonia distillation apparatus. Add 6 ml of a saturated solution of *sodium hydroxide* and pass steam through the flask, distil for 07 minutes collecting the distillate in a mixture of 5 ml of a saturated solution of *boric acid*, 5 ml of water and 1 drop of saturated solution of methyl red in alcohol containing 0.1 per cent of *methylene blue* and titrate with 0.02 M of *Hydrochloric acid*.

1 ml of 0.02M *hydrochloric acid* is equivalent to 0.00175 g of fibrinogen.

For sodium ions. To 10 ml of the solution add sufficient *water* to produce 100 ml, further dilute 10 ml to 500 ml with water and determine the content of sodium ions by method B for flame photometry, measuring at about 589nm and using *sodium solution FP* suitably diluted with water as the standard solution.

The preparation contains not more than 200 millimoles of sodium ions per liter.

Method C

Sample and Reference standard preparation

Dissolve the content of sealed container of substance under examination in the volume of the liquid stated on the label and use immediately. Reconstitute the entire contents of one ampoule of the standard preparation as stated on the label and use immediately. After reconstitution of Sample and reference standard prepare minimum three dilution of standard with suitable diluent buffer (eg.Imidazole buffer pH 7.3 containing 1 per cent Human Albumin) of each in duplicate producing the final concentration to 0.2 to 2.0 IU/ml. Same step may be performed via auto-analyser if auto dilution/multi-dilution mode is available.

Preparation of equipment

Automated coagulation analysers should be prepared for testing as per operational instruction which may include daily maintenance and cleaning of probes reaction cells. Place the reagent in reagent compartment. Program the assay procedure for volume of reagent, incubation time, order of reagent addition and other details. Calibrate the equipment using suitable international or national traceable reference standards.

System Suitability

Carry out a blank determination using in place of the preparation under examination, an equal quantity of a mixture of 1 volume of a 3.8 per cent w/v solution of sodium citrate or imidazole buffer and 5 volumes of saline solution. The result of the assay is not valid unless the clotting time in the blank determination is more than 40 seconds. Calculate the result of the assay by standard statistical methods.

Assay for von Willebrand factor

Carry out the assay of human von Willebrand factor for preparations intended for the treatment of von Willebrand's disease. Its collagen binding activity can be utilized for assays.

The estimated potency is not less than 60.0 per cent and not more than 140.0 per cent of the stated potency.

The potency of human von Willebrand factor is determined by comparing, in given conditions, its activity with the same activity of a reference preparation calibrated against the International Standard, in International Units where applicable.

The International Unit is the activity of a stated amount of the International Standard, which consists of a freeze-dried human von Willebrand factor concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organization (WHO).

Ristocetin Cofactor Assay

The ristocetin cofactor activity of von Willbrand factor is determined by measuring agglutination of a suspension of platelets in the presence of ristocetin A. The assay can be carried out for quantitative determinations by using automated instruments, or for semi-quantitative determinations by visually assessing the endpoint of agglutination in a dilution series. Quantitative assays are preferred.

Reagents

Suspension of platelets. Use standardized and, for example, formaldehyde- or paraformaldehyde-fixed preparations of freshly isolated and washed human platelets in the presence of ristocetin A.

Reference preparation. The reference preparation for von Willebrand factor is the WHO International Standard for von Willebrand factor concentrate.

Method

Semi-quantitative assay. Prepare suitable dilutions of the preparation under examination and of the reference preparation, using as diluent a solution containing 0.9 per cent w/v of *sodium chloride* and 1 to 5 per cent w/v of *human albumin*. Add to each dilution an appropriate amount of the suspension of platelets and, if necessary, of ristocetin A. Mix on a glass slide by moving it gently in circles for 1 min. Allow to stand for a further 1 min and read the result against a dark background with side lighting. The last dilution which clearly shows visible agglutination indicates the ristocetin cofactor titre of the sample. Use diluent as a negative control.

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Quantitative Assay. Reconstitute the entire contents of 1 ampoule of the reference preparation and the preparation under examination by adding the appropriate quantity of the recommended diluent (for example *water*); use immediately. Add sufficient prediluent to the reconstituted preprations to produce solutions containing 0.5-2.0 IU/ml. The prediluent consists of an isotonic non-chelating buffer containing, for example, 1-5 per cent of human or bovine albumin, and tris(hydroxymethyl) aminomethane or imidazole, appropriately buffered.

The test is performed in accordance with the manufacturer's instructions with at least 2 dilution series with as many dilutions as are needed to obtain a total of at least 3 different concentrations in the linear range of the assay. Check the validity of the assay and calculate the potency of the test preparation using the usual statistical methods.

Collagen-Binding Assay

Collagen-binding is determined by an enzyme-liked immunosorbent assay on collagen-coated microtitre plates. The method is based on the specific binding of von Willebrand factor to collagen fibrils and the subsequent binding of polyclonal anti-von Willebrand factor antibody conjugated to an enzyme, which on addition of a chromogenic substrate yields a product that can be quantitated spectrophotometrically. Under appropriate conditions, there is a linear relationship between von Willebrand factor collagen-binding and absorbance.

Reagents

Collagen. Use native equine or human fibrils of collagen type I or III. For ease of handling, collagen solution may be used.

Collagen diluent. Dissolve 50 g of *glucose* in *water*, adjust to pH 2.7-2.9 with *1 M hydrochloric acid* and dilute to 1000 ml with *water*.

Phosphate-buffered saline (PBS). Dissolve 8.0 g of *sodium chloride*, 1.05 g of *disodium hydrogen phosphate dihydrate*, 0.2 g of *sodium dihydrogen phosphate* and 0.2 g of of *potassium chloride* in *water*. Adjust to pH 7.2 using 1 M *sodium hydroxide* or 1 M *hydrochloric acid* and dilute to 1000 ml with *water*.

Washing buffer. PBS containing 0.1 per cent of polysorbate 20.

Blocking reagent. PBS containing 0.1 per cent of polysorbate 20 and 1 per cent w/v of bovine serum albumin.

Dilution buffer. PBS containing 0.1 per cent of polysorbate 20 and 5.0 per cent of bovine serum albumin.

Conjugate. Rabbit anti-human von Willebrand factor serum horseradish peroxidase conjugate. Use according to the manufacturers instructions.

Substrate Solution. Immediately before use, dissolve a tablet of *o-phenylenediamine dihydrochloride* and a tablet of *urea hydrogen peroxide* in 20 ml of *water* or use a suitable volume of hydrogen peroxide. Protect from light.

Microtitre plates. Flat-bottomed polystyrene plates with surface properties optimized for enzyme immunoassay and high protein-binding capacity.

Method

Test Solutions. Reconstitute the preparation under examination as stated on the label. Dilute with dilution buffer to produce a solution containing approximately 1 IU of von Willebrand factor. Prepare 2 series of at least 3 further dilutions using dilution buffer.

Reference solutions. Reconstitute the reference preparation as directed. Dilute with dilution buffer to produce a solution containing approximately 1 IU of von Willebrand factor. Prepare 2 series of at least 3 further dilutions using dilution buffer.

Allow the solution of collagen to warm to room temperature. Dilute with collagen dilutent to obtain a solution containing 30-75 µg/ml of collagen, mix gently to produce a uniform suspension of collagen fibrils. Pipette 100 µl into each well of the microtitre plate. Cover the plate with plastic film and incubate at 37° overnight. Empty the wells of the collagen-coated plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times. Add 250 µl of blocking reagent to each well, cover the plate with plastic film and incubate at 37° for 1 hour. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Add 100 µl each of the test solutions or reference solutions to the wells. Add 100 µl of dilution buffer to a series of wells to serve as negative control. Cover the plate with plastic film and incubate at 37° for 2 h. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Prepare a suitable dilution of the conjugate (for example, a dilution factor of 1 to 4000) with *PBS* containing 0.5 per cent w/v of *bovine serum albumin* and add 100 µl to each well. Cover the plate with plastic film and incubate at 37° for 2 hours. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a towel. Repeat this operation 3 times.

Add 100 µl of substrate solution to each of the wells and incubate at room temperature for 20 minutes in the dark. Add 100 µl of 1 M *hydrochloric acid* to each of the wells.

Measure the absorbance at 492 nm. Use the absorbance values to estimate the potency of the preparation to be examined using the usual statistical method (5.7).

The assay is invalid if the absorbencies measured for the negative controls are more than 0.05.

Storage. In an airtight container, protected from light.

Labelling. The label states (1) the number of International Units of factor VIII C and, where applicable (2) the amount of protein in the container (3) the name and quantity of any added substance (4) the name and volume of the liquid to be used for reconstitution (5) where applicable, that the preparation may show the presence of a few small flakes or particles after reconstitution (6) that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

Fibrin Sealant Kit

Fibrin sealent kit is essentially composed of two components, namely fibrinogen concentrate (component 1), a protein fraction containing human fibrinogen and a preparation containing human thrombin (component 2). A fibrin clot is rapidly formed when the two thawed or reconstituted components are mixed. Other ingredients (for example, human coagulation factor XIII, a fibrinolysis inhibitor or calcium ions) and stabilizer (for example human albumin solution may be added). No antimicrobial preservative is added.

Human constituents are obtained from plasma that complies with the requirements of the monograph on Human Plasma for Fractionation. No antibiotic is added to the plasma used.

When thawed or reconstituted as stated on the label, component 1 contains not less than 4.0 per cent of clottable protein; the thrombin activity of component 2 varies over a wide range (approximately 4- 1000 IU/ml).

Production

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and any residue are such as not to compromise the safety of the preparation for patients.

Constituents or mixtures of constituents are passed through a bacteria retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze dried and the containers are closed under vacuum or filled with oxygen free nitrogen or other suitable inert gas before being closed. In either case, they are closed so as to exclude micro-organisms.

If the human coagulation factor XIII content in component 1 is greater than 10 units per ml, the assay of coagulation factor XIII is carried out.

Description. Freeze dried constituents are hygroscopic white or pale yellow, powder or friable solid. Frozen constituents are colorless or pale yellow or opaque solids. Liquid constituents are colorless or pale yellow.

For the freeze dried or frozen constituents, reconstitute or thaw as stated on the label, immediately before carrying out the Identification and Tests, except those for solubility and water.

Component 1 (Fibrinogen Concentrate)

Identification

A. Complies with the limits of the assay of fibrinogen.

B. Complies with the limits of the assay of factor XIII (where applicable).

Tests

pH (2.4.24). 6.5 to 8.0

Solubility. Freeze dried concentrates dissolve within 20 minutes in the reconstituted solution at the temperature stated on the label, forming an almost colourless, clear or slightly turbid solution.

Stability of the solution. No gel formation appears at room temperature during 120 minutes following thawing or reconstitution.

Water. Determine by suitable method such as semi-micro determinations (2.3.43), loss on drying (2.4.19) or near infrared spectrophotometry (2.4.6), the water content is within the limits approved by the competent authority.

Sterility (2.2.11). Complies with the test for sterility.

Assay

Fibrinogen (clottable protein)

The estimated content in mg of clottable protein is not less than 70.0 per cent and not more than 130.0 per cent of the content stated on the label. The estimated content in mg of clottable protein should be within the limits as stated on the label, if fibrinogen content (Clottable protein) is stated on the label as range (within a specified lower limit and Upper limit).

Mix 0.2 ml of the reconstituted preparation with 2 ml of a suitable *buffer solution* pH 6.6 to 7.4 containing sufficient

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human thrombin (approximately 3 IU per ml) and calcium (0.05 mol per l). Maintain at 37° for 20 minutes, separate the precipitate by centrifugation (5000 rpm, 20 minutes), wash thoroughly with a 0.9 per cent of *sodium chloride* and determine the protein as nitrogen by sulphuric acid digestion (2.3.30). Calculate the protein content by multiplying the result by 6. If for a particular preparation this method cannot be applied, use another validated method for determination of fibrinogen.

Factor XIII

When the label indicates that the human coagulation factor XIII activity is greater than 10 units per ml, the estimated activity is not less than 80.0 per cent and not more than 120.0 per cent of the activity stated on the label.

Make at least 3 suitable dilutions of thawed or reconstituted component I and of human normal plasma (reference preparation) using as diluent coagulation factor XIII deficient plasma or another suitable diluent. Add to each dilution suitable amount of the following reagents.

(a) activator reagent, containing bovine or human thrombin, a suitable buffer, calcium chloride and a suitable inhibitor such as Gly-Pro-Arg-Pro-Ala-NH_2 which inhibits clotting of the sample but does not prevent coagulation factor XIII activation by thrombin.

(b) detection reagent, containing a suitable factor XIII_a specific peptide substrate, such as Leu-Gly-Pro-Gly-Glu-Ser-Lys-Val-Ile-Gly- NH_2 and *glycine ethyl ester* as 2nd substrate in a suitable *buffer solution.*

(c) NADH reagent, containing *glutamate dehydrogenase, α- ketoglutarate* and NADH in a suitable *buffer solution.*

After mixing, the absorbance changes (ΔA per min) measured at a wavelength of 340 nm, after the linear phase of the reaction is reached.

1 unit of factor XIII is equal to the activity of 1 ml of human normal plasma.

Calculate the activity of the test preparation by the usual statistical methods. The confidence limits (P=0.95) are not less than 80.0 per cent and not more than 125.0 per cent of the estimated activity.

Component 2 (Thrombin Preparation)

Identification

Complies with the limits of the assay of thrombin.

Tests

pH (2.4.24). 5.0 to 8.0.

Solubility. Freeze dried concentrates dissolve within 5 minutes in the reconstituted solution at the temperature stated on the label, forming an almost colourless, clear or slightly turbid solution.

Water. Determine by a suitable method such as semi micro determinations (2.3.43), loss on drying (2.4.19) or near infrared spectrophotometry (2.4.6), the water content is within the limits approved by the competent authority.

Sterility. (2.2.11). Complies with test for sterility.

Assay

Thrombin

The estimated activity is not less than 80.0 per cent and not more than 125.0 per cent of the activity stated on the label.

If necessary, dilute the reconstituted preparation under examination to approximately 2 -20 IU of thrombin per ml using as diluent a suitable buffer pH 7.3 to 7.5, such as *imidiazole buffer solution pH 7.3 for Thrombin Assay* containing 1 per cent of *human albumin* or bovine albumin. To a suitable volume of the dilution, add a suitable volume of fibrinogen solution (0.1 per cent of clottable protein) warmed to 37° and start measurement of the clotting time immediately. Repeat the procedure with each of at least 3 dilutions, in the range stated above of a reference preparation of thrombin, calibrated in IU. Calculate the activity of the test preparation by the usual statistical methods.

The confidence limits (P=0.95) are not less than 80.0 per cent and not more than 125.0 per cent of the estimated activity.

Storage. Store protected from light, and moisture.

Labelling. The label states (1) the amount of fibrinogen (mg of clottable protein) thrombin (IU) per container, and coagulation factor XIII, if this is greater than 10 Units per ml; (2) Where applicable, the name and the volume of solvent to be used to reconstitute the components.

Hepatitis B Immunoglobulin

Human Hepatitis B Immunoglobulin

Category. Immunological product.

Hepatitis B Immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma from selected and/or immunized donors having antibodies against hepatitis B surface antigen. Human normal immunoglobulin may be added.

It complies with the monograph on Human Normal Immunoglobulin, except for the minimum number of donors and the minimum total protein content.

Tests

Potency. The potency is determined by comparing the antibody titre of the immunoglobulin under examination with that of a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (2.2.14).

The International Unit is the activity contained in a stated amount of the International Reference Preparation of hepatitis B immunoglobulin. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

The stated potency is not less than 100 Units per ml. The estimated potency is not less than the stated potency. The confidence limits (P = 0.95) of the estimated potency are not less than 80.0 per cent and not more than 125.0 per cent.

Storage. Store protected from light, the liquid preparation in sealed, colourless glass containers and the freeze-dried preparation in an airtight colourless glass container.

Labelling. The label states (1) for liquid preparations, the volume of the preparation in the container and the protein content expressed in g per litre; (2) for freeze-dried preparations, the quantity of protein in the container; (3) the route of administration; (4) for freeze-dried preparations, the name or composition and the volume of the reconstituting liquid to be added; (5) where applicable, that the preparation is suitable for use in the prophylaxis of hepatitis A infection; (6) where applicable, the anti-hepatitis A virus activity in International Units per ml; (7) where applicable, the name and amount of antimicrobial preservative in the preparation; (8) the number of International Units per container.

Human Albumin

Human Normal Albumin; Human Albumin Solution

Human albumin is an aqueous solution of albumin component obtained from human plasma that complies with the requirement of the monograph on Plasma for Fractionation.

Production

Separation of albumin is carried out under control conditions particularly on pH, ionic strength and temperature so that in the final product not less than 95.0 per cent of total protein is albumin. Human albumin is prepared as a concentrated solution containing 15.0 to 25.0 per cent of total protein or as an isotonic solution containing 3.5 to 5.0 per cent of total protein. No antimicrobial agent is added at any stage during preparation and all processing steps are conducted in a manner to minimize the risk of contamination from either microorganisms or other deleterious matter. The solution is sterilized by filtration and distributed aseptically into sterile containers which are closed so as to prevent contamination. Then solution is then heated and maintained at 60° ± 0.5° for 10 hours. Finally the containers are incubated at 30 -32° for not less than 14 days or at 20 -25° for not less than 4 weeks and examined visually for evidence of microbial contamination. Those showing abnormalities such as abnormal colour, turbidity, microbial contamination or presence of a typical particle must be discarded.

Description. A clear, slightly viscous liquid, it is almost colourless to greenish yellow or amber to green, depending on protein concentration and the method of fractionation used.

Identification

A. Double Immunodiffusion. Precipitation test with a suitable range of species specific antisera which gives positive results for the presence of protein of human origin and negative results with antisera specific to plasma protein for other species.

B. Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and preparation under examination, both diluted to contain 1 per cent of protein. The main component of the preparation under examination corresponds to the main component of the normal human serum. The preparation may show the presence of small quantities of other plasma proteins.

Tests

pH (2.4.24). Dilute the preparation under examination with a 0.9 per cent solution of *sodium chloride R* to produce a solution containing 1.0 per cent of protein; pH of the resulting solution 6.7 to 7.3.

Haem content. Dilute the preparation under examination using a 0.9 per cent solution of *sodium chloride* to obtain a solution containing 1.0 per cent of protein. The absorbance of the solution measured at 403 nm using 0.9 percent solution of Sodium chloride as the compensation liquid is not more than 0.15 (2.4.7).

Molecular Size Distribution. Determined by liquid chromatography (2.4.14).

Test solution; dilute the preparation under examination with a 0.9 per cent solution of *sodium chloride* to a suitable concentration. Concentration in the range of 0.4 -1.2 per cent and injection of 50 - 600 µg of protein are usually suitable.

Chromatographic System

- a stainless steel column 0.6 m x 7.5 mm or 0.3 m x 7.8 mm with *Hydrophilic silica gel for chromatography*, of a grade suitable for fractionation of globular proteins with relative molecular masses in the range 10,000 – 500,000,
- mobile phase: a mixture of 4.873 g of *disodium hydrogen phosphate dihydrate*, 1.741 g of *sodium dihydrogen*

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phosphate monohydrate, 11.688 g of *sodium chloride* and 50 mg of *sodium azide* in 1000 ml *water*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 280 nm.

The peak due to polymers and aggregates is located in the part of the chromatogram representing the void volume. Disregard the peak due to the stabilizer, the area of the peak due to polymers and aggregate is not more than 10.0 per cent of the total area of the chromatogram.

Protein Composition. Determined by zone electrophoresis (2.4.12). Not less than 95.0 per cent w/v of the total protein as albumin.

Use strips of suitable cellulose acetate gel or agarose gel as the supporting medium and *barbital buffer solution pH 8.6* as the electrolyte solution.

If cellulose acetate is the supporting material, the method described below can be used. If agarose gels are used, and because they are normally part of an automated system, the manufacturer's instructions are followed instead.

Test solution. Dilute the preparation under examination with a 0.9 per cent solution of *sodium chloride* to a protein concentration of 2.0 per cent w/v.

Reference solution. Dilute human albumin for electrophoresis RS with a 0.9 per cent solution of *sodium chloride* to a protein concentration of 2.0 per cent.

To a strip apply 2.5 µl of the test solution as a 10 mm band or apply 2.5 µl per ml if a narrower strip is used.

To another strip, apply in the same manner the same volume of the reference solution. Apply suitable electric field such that the most rapid band migrates at least 30 mm. Treat the strips with *amido black 10 B solution* for 5 minutes. Decolourise with the mixture of 10 volumes of *glacial acetic acid* and 90 volumes of *methanol* until the background is just free of colour. Develop the transparency of the strips with the mixture of 19 volumes of *glacial acetic acid* and 81 volume of *methanol* Measure the absorbance of the band at 600 nm in an instrument having a linear response over the range of measurement. Calculate the result as the mean of 3 measurements of each strips.

System suitability. The test is not valid if the production of the protein in the principal band is not within the limits stated in the leaflet supplied with *human albumin for electrophoresis*.

In the electropherogram obtained with the test solution on *cellulose acetate* or on *agarose gel*, not more than 5.0 per cent of the protein has a mobility different from that of the principal band.

Prekallikrein activator. Not more than 35 IU per mL, determined as described in the Test for Prekallikrein activator in the monograph for Human Normal Immunoglobulin for Intravenous use.

Pyrogens (2.2.8)/ or **Bacterial Endotoxin (BET)** (2.2.3). Complies with the test for pyrogen or, preferably and where justified and authorized with a validated *in-vitro* test such as the BET.

Pyrogens. Complies with the test for pyrogen using 3 ml per kg of the rabbit weight irrespective of the protein content.

BET. "Not more than 0.5 IU of endotoxin per ml for solutions with a protein content not greater than 5.0 per cent, less than 1.3 IU of endotoxin per ml for solution with a protein content greater than 5.0 per cent but not greater than 20.0 per cent and less than 1.7 IU of endotoxin per ml for solution with a protein content greater than 20.0 per cent but not greater than 25.0 per cent."

Sterility (2.2.11). Complies with the tests for sterility.

Abnormal toxicity (2.2.1). Complies with the test for abnormal toxicity (*For Antisera and Vaccine)*, and 0.5 ml of the solution for each mouse and 5 ml for each guinea pig irrespective of the protein content.

Assay

For protein. The preparation under examination contains not less than 95.0 per cent and not more than 105.0 per cent of the protein stated on the label. Determine by either Method I or II.

Method I

Dilute to about 0.75 per cent w/v of total protein with *saline solution*. Take 2 ml of the solution in a round bottom centrifuge tube. Add 2 ml of 7.5 per cent w/v solution of *sodium molybdate* and 2 mL of mixture of 30 volumes of *water* and 1 volume of *nitrogen free sulphuric acid*. Shake, centrifuge for 5 minutes. Decant the supernatant liquid and let the inverted tube stand on a filter paper to drain the fluid. Carry out method F for Determination of nitrogen (2.3.30) on the residue thus obtained and calculate the protein content.

Method II

Determine by method G for determination of nitrogen (2.3.30) and multiply the result by 6.25 to obtain the content of protein.

For Sodium. Dilute to 0.01 per cent w/v of protein with water and determine by Method A for atomic absorption spectrophotometery (2.4.2), or by Method B for flame photometry (2.4.4), measuring at about 589 nm and using *sodium solution FP* suitably diluted with *water* as the standard solution.

It contains not less than 95.0 per cent and not more than 105.0 percent of Na stated on the label and in any case not more than 160 mmol/L.

For potassium. Dilute To 0.25 per cent w/v of protein with *water* and determine by Method A (2.4.2) for atomic absorption spectrophotometry, or by Method B for flame photometry (2.4.4), measuring at about 767 nm and using *potassium solution FP* suitably diluted with *water* as the standard solution.

The preparation under examination contains not more than 2 millimoles of K per litre.

Human albumin intended for administration to patients undergoing dialysis or to premature infants complies with the following additional test.

Aluminium (2.3.8). Not more than 200 µg of Al per liter. Determine by atomic absorption spectrophotometry (2.4.2), with a furnace as a atomic generator and measuring at 309.3nm and using as standard solutions a suitable range of dilutions in water of *aluminium standard solution* (10ppm Al) further diluted, as necessary with a solution containing 0.17 per cent w/v of *magnesium nitrate* and 0.05 per cent w/v of *octoxinol 10* in a solution of *nitiric acid* containing 1 per cent w/v of *nitric Acid*. Prepare suitable dilutions of the preparations under examination and *human albumin for Aluminium validation RS* with water. Dilute the solutions, as necessary, with the *magnesium nitrate – Octoxinol 10-nitirc acid solution* used for dilution of the standard solution. The test is not valid unless the Aluminium content determined for *Human albumin for Aluminium validation RS* is within 20 per cent of the stated value.

NOTE— Wash all equipments with a solution containing 20.0 per cent w/v of nitric acid before use and use plastic containers only to prepare solutions.

Storage. Protected from light at a temperature between 2° and 25°. Human albumin stored at 2° to 8° may be expected to continue to meet the requirements of the monograph for 5 years from the date on which it was heated at 60° for 10 hours. Human albumin stored at a temperature not exceeding 25° may be expected to continue to meet the requirements of the monograph for 3 years from the date on which it was heated at 60° for 10 hours.

Labelling. The label states (1) the name of the preparation; (2) the volume of the preparation; (3) the content of protein expressed in grams per litre; (4) the content of sodium expressed in millimoles per litre; (5) that the product is not to be used if it is cloudy or if a deposit has formed; (6) the name and concentration of any added substances (for example stabilizer); (7) that the contents must not be used more than 4 hours after the container has been penetrated and any remnant portion must be discarded; (8) Storage condition; (9) the date after which the solution is not intended to be used; (10) either that the preparation is suitable for administration to patients undergoing dialysis or to premature infants or that it is not intended for such purpose.

Human Coagulation Factor VII

Human Coagulation Factor VII is a plasma protein fraction that contains the single-chain glycoprotein factor VII and may also contain small amounts of the activated form, the two-chain derivative factor VIIa. It may also contain coagulation factors II, IX and X and protein C and protein seconds. It is obtained from human plasma that complies with the monograph on Human Plasma for Fractionation.

The potency of the preparation, reconstituted as stated on the label, is not less than 15 IU of factor VII per ml.

Production

The method of preparation is designed to minimise activation of any coagulation factor (to minimise potential thrombogenicity) and includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

The specific activity is not less than 2 IU of factor VII per mg of total protein, before the addition of any protein stabiliser.

The factor VII fraction is dissolved in a suitable liquid. Heparin, antithrombin and other auxiliary substances such as a stabiliser may be added. No antimicrobial preservative is added. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas.

Consistency of the method

The consistency of the method of production with respect to the activities of factors II, IX and X of the preparation, expressed in International Units relative to the activity of factor VII, shall be demonstrated.

The consistency of the method of production with respect to the activity of factor VIIa of the preparation shall be demonstrated. The activity of factor VIIa may be determined, for example, using a recombinant soluble tissue factor that does not activate factor VII but possesses a cofactor function specific for factor VIIa; after incubation of a mixture of the recombinant soluble tissue factor with phospholipids reagent and the dilution of the test sample in factor VII-deficient plasma, *calcium chloride* is added and the clotting time determined; the clotting time is inversely related to the factor VIIa activity of the test sample.

Description. A hygroscopic powder or friable solid that may be white, pale yellow, green or blue.

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Reconstitute the preparation under examination as stated on the label immediately before carrying out the Identification, Tests (except those for solubility and water) and Assay.

Identification

It complies with the limits of the Assay.

Tests

pH (2.4.24). 6.5 to 7.5.

Osmolality (2.4.23). Minimum 240 mosmol per kg.

Total protein. If necessary, dilute an accurately measured volume of the reconstituted preparation with a 0.9 per cent solution w/v of *sodium chloride* to obtain a solution expected to contain about 15 mg of protein in 2 ml. To 2.0 ml of the solution in a round-bottomed centrifuge tube add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid* and 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.3.30) and calculate the amount of protein by multiplying the result by 6.25.

Activated coagulation factors (2.8.3). For each of the dilutions, the coagulation time is not less than 150 seconds.

Heparin. If heparin has been added during preparation, determine the amount present by the assay of heparin in coagulation factor concentrates (2.8.9). The preparation under examination contains not more than the amount of heparin stated on the label and in any case not more than 0.5 IU of heparin per International Unit of factor VII.

Thrombin. If the preparation under examination contains heparin, determine the amount present as described in the test for heparin and neutralise the heparin by addition of *protamine sulphate* (10 µg of protamine sulphate neutralises 1 IU of heparin). In each of 2 test-tubes, mix equal volumes of the reconstituted preparation and a 0.3 per cent solution of *fibrinogen*. Keep one of the tubes at 37° for 6 hours and the other at room temperature for 24 hours. In a third tube, mix a volume of the *fibrinogen solution* with an equal volume of a solution of *human thrombin* (1 IU per ml) and place the tube in a water-bath at 37°. No coagulation occurs in the tubes containing the preparation under examination. Coagulation occurs within 30 seconds in the tube containing thrombin.

Factor II

Determine the assay of human coagulation factor II (2.8.4).

The estimated content is not more than 125.0 per cent of the stated content. The confidence limits (P = 0.95) are not less than 90.0 per cent and not more than 111.0 per cent of the estimated potency.

Factor IX

Determine the assay of human coagulation factor IX (2.8.7).

The estimated content is not more than 125.0 per cent of the stated content. The confidence limits (P = 0.95) are not less than 80.0 per cent and not more than 125.0 per cent of the estimated potency.

Factor X

Determine the assay of human coagulation factor X (2.8.8).

The estimated content is not more than 125.0 per cent of the stated content. The confidence limits (P = 0.95) are not less than 90.0 per cent and not more than 111.0 per cent of the estimated potency.

Water. Determine by semi-micro determination of water (2.3.43), loss on drying (2.4.19) or near-infrared spectrophotometry (2.4.6), the water content is within the limits approved by the competent authority.

Pyrogens (2.2.8) or **Bacterial endotoxins** (2.2.3). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

Pyrogens. Complies with the test for pyrogens. Inject per kg of the rabbit's mass a volume equivalent to not less than 30 IU of factor VII.

BET. Where the bacterial endotoxin test is used, the preparation under examination contains not more than 0.1 Endotoxin Unit per IU of factor VII.

Sterility (2.2.11). Complies with the test for sterility.

Assay

Determine the assay of human coagulation factor VII (2.8.5).

The estimated potency is not less than 80.0 per cent and not more than 125.0 per cent of the stated potency. The confidence limits (P = 0.95) are not less than 80.0 per cent and not more than 125.0 per cent of the estimated potency.

Storage. Store protected from light.

Labelling. The label states (1) the number of International Units of factor VII per container; (2) the maximum content of International Units of factor II, factor IX and factor X per container; (3) the amount of protein per container; (4) the name and quantity of any added substances, including where applicable, heparin; (5) the name and volume of the liquid to be used for reconstitution; (6) that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

Human Coagulation Factor VIII (rDNA)

Human Coagulation Factor VIII (rDNA) is a freeze-dried preparation of glycoproteins having the same activity as coagulation factor VIII in human plasma. It acts as a cofactor of the activation of factor X in the presence of factor IXa, phospholipids and calcium ions. It circulates in plasma mainly as a two-chain glycosylated protein with 1 heavy (relative molecular mass of about 2,00,000) and 1 light (relative molecular mass 80,000) chain held together by divalent metal ions. Human coagulation factor VIII (rDNA) is prepared as full-length factor VIII (octocog alfa), or as a shortened two-chain structure (relative molecular mass 90,000 and 80,000), in which the B-domain has been deleted from the heavy chain (moroctocog alfa).

Full-length human rDNA coagulation factor VIII contains 25 potential *N*-glycosylation sites, 19 in the B domain of the heavy chain, 3 in the remaining part of the heavy chain (relative molecular mass 90,000) and 3 in the light chain (relative molecular mass 80,000). The different products are characterised by their molecular size and post-translational modification and/or other modifications.

Production

Human coagulation factor VIII (rDNA) is produced by recombinant DNA technology in mammalian cell culture. It is produced under conditions designed to minimise microbial contamination.

Purified bulk factor VIII (rDNA) may contain added human albumin and/or other stabilising agents, as well as other auxiliary substances to provide, for example, correct pH and osmolality.

The specific activity is not less than 2,000 IU of factor VIII:C per mg of total protein before the addition of any protein stabiliser, and varies depending on purity and the type of modification of molecular structure of factor VIII.

The quality of the bulk preparation is controlled using reference preparations.

Reference preparations

During development, reference preparations are established for subsequent verification of batch consistency during production, and for control of bulk and final preparation. They are derived from representative batches of purified bulk factor VIII (rDNA) that are extensively characterised by tests including those described below and whose procoagulant and other relevant functional properties have been ascertained and compared, wherever possible, with the International Standard for factor VIII concentrate. The reference preparations are suitably characterised for their intended purpose and are stored in suitably sized aliquots under conditions ensuring their stability.

Purified bulk factor VIII (rDNA)

The purified bulk complies with a suitable combination of the following tests for characterisation of integrity of the factor VIII (rDNA). Where any substance added during preparation of the purified bulk interferes with a test, the test is carried out before addition of that substance. Where applicable, the characterisation tests may alternatively be carried out on the finished product.

Specific biological activity or ratio of factor VIII activity to factor VIII antigen

Determine the assay of human coagulation factor VIII (2.8.6). The protein content, or where a protein stabiliser is present, the factor VIII antigen content, is determined by a suitable method and the specific biological activity or the ratio of factor VIII activity to factor VIII antigen is calculated.

Protein composition

The protein composition is determined by a selection of appropriate characterisation techniques which may include peptide mapping, Western blots, HPLC, gel electrophoresis, capillary electrophoresis, mass spectrometry or other techniques to monitor integrity and purity. The protein composition is comparable to that of the reference preparation.

Molecular size. Using size-exclusion chromatography (2.4.16), the molecular size distribution is comparable to that of the reference preparation.

Peptide mapping (2.3.47). There is no significant difference between the test protein and the reference preparation.

Carbohydrates/sialic acid. To monitor batch-to-batch consistency, the monosaccharide content and the degree of sialylation or the oligosaccharide profile are monitored and correspond to those of the reference preparation.

Final lot

It complies with the tests under Identification, Tests and Assay.

Excipients. 80.0 per cent to 120.0 per cent of the stated content, determined by a suitable method, where applicable.

Description. A white or slightly yellow powder or friable mass.

Identification

A. It complies with the limits of the Assay.

B. The distribution of characteristic peptide bands corresponds with that of the reference preparation (SDS-PAGE or Western blot).

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Tests

Reconstitute the preparation as stated on the label immediately before carrying out the Tests (except those for solubility and water) and Assay.

pH (2.4.24). 6.5 to 7.5.

Osmolality (2.4.23). Minimum 240 mosmol per kg.

Water. Determine by semi-micro determination of water (2.3.43), loss on drying (2.4.19) or near infrared spectrophotometry (2.4.6), the water content is within the limits approved by the competent authority.

Sterility (2.2.11). Complies with the test for sterility.

Bacterial endotoxins (2.2.3). Less than 3 IU in the volume that contains 100 IU of factor VIII activity.

Assay

Determine the assay of human coagulation factor VIII (2.8.6).

The estimated potency is not less than 80.0 per cent and not more than 125.0 per cent of the stated potency. The confidence limits (P = 0.95) are not less than 80.0 per cent and not more than 120.0 per cent of the estimated potency.

Storage. Store protected from light.

Labelling. The label states (1) the factor VIII content in International Units; (2) the name and amount of any excipient; (3) the composition and volume of the liquid to be used for reconstitution.

Human Coagulation Factor IX

Human Coagulation Factor IX is a plasma protein fraction containing coagulation factor IX, prepared by a method that effectively separates factor IX from other prothrombin complex factors (factors II, VII and X). It is obtained from human plasma that complies with the monograph on human plasma for fractionation. The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU of factor IX per ml.

Production

The method of preparation is designed to maintain functional integrity of factor IX, to minimize activation of any coagulation factor (to minimize potential thrombogenicity) and includes a step or steps that have been shown to remove or to inactivate known agents of infections; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to comprise the safety of the preparations for patients.

The specific activity is not less than 50 IU of factor IX per mg of total protein, before the addition of any pro stabilizer.

The factor IX fraction is dissolved in a suitable liquid. Hep anti-thrombin and other auxiliary substances such a stabilizer may be included. No anti-microbial preservativ added. The solution is passed through a bacteria reten filter, distributed aseptically into the final containers immediately frozen. It is subsequently freeze-dried and containers are closed under vaccum or under an inert ga

Consistency of the method

The consistency of the method of production is evaluated suitable analytical procedure that are determine during proc development and which normally include (1) assay of fac IX (2) determination of activated coagulation factor (3) determination of activities of factor II, VII and X whi shall be shown to be not more than 5 per cent of the activity factor IX.

Description. A white or pale yellow, hygroscopic powder friable solid.

Reconstitute the preparation under examination as state on the label, immediately before carrying out the Identification, Tests (except those for solubility and water and assay.

Solubility. To a container of the preparation to be examine add the volume of the liquid stated on the label at the recommended temperature. The preparation dissolve completely with gentle swirling within 10 minute, giving clear or slightly opalescent, colorless or slightly yello solution

Identification

It complies with the limits of the assay.

Tests

pH (2.4.24). 6.5 to 7.5.

Osmolality (2.4.23). Minimum 240 mosmol per kg.

Total protein. If necessary, dilute an accurately measure volume of the preparation under examination with a 0.9 pe cent w/v solution of *sodium chloride*, to obtain a solutio which may be expected to contain about 15 mg of protein 2 ml. To 2 ml of that solution in a round bottomed centrifug tube, add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a mixture of 1 volume of nitrogen fre sulphuric acid and 30 volumes of *water*. Shake, centrifuge fo 5 minutes, decant the supernatant liquid and allow the inverte tube to drain on filter paper. Determine the nitrogen in th residue by the method of sulphuric acid digestion (2.3.30) and calculate the amount of protein by multiplying the result by 6.25.

For some products, especially those without a protein stabilizer such as albumin, this method may not be applicable. Another validated method for protein determination must therefore be performed.

Activated coagulation factor (2.8.3). If necessary, dilute the preparation under examination to contain 20 IU of factor IX per ml. For each of the dilution the coagulation time is not less than 150 seconds.

Heparin (2.8.9). If heparin has been added during preparation, determine the amount by the assay of heparin in coagulation factor concentrates. The preparation under examination contains not more than the amount of heparin stated on the label and in any case not more than 0.5 IU of heparin per IU of factor IX.

Water. Determine by suitable method such as semi micro determination of water (2.3.43), loss on drying (2.4.19) or near infrared spectrophotometry(2.4.6), the water content is within the limits approved by the competent authority.

Sterility (2.2.11). Complies with the test for sterility.

Pyrogens (2.2.8) or **Bacterial Endotoxin (BET)** (2.2.3). Complies with the test for pyrogen or, preferably and where justified and authorized with a validated *in-vitro* test such as the Bacterial Endotoxin test.

Pyrogens. Complies with the test for pyrogens. Inject per kg of the rabbit's mass a volume equivalent to not less than 50 IU of factor IX.

BET. Not more than 0.03 IU of endotoxin per International Unit of Factor IX.

Assay. Determine the assay of human blood coagulation factor IX (2.8.7).

The estimated potency is not less than 80.0 per cent and not more than 125.0 per cent of the stated potency. The confidence limits (P=0.95) are not less than 80.0 per cent and not more than 125.0 per cent of the estimated potency.

Storage. Store in an air tight container protected from light.

Labelling. The label states (1) the number of IU of factor IX per container; (2) the amount of protein per container; (3) the name and the quantity of any added substance including, where applicable, heparin; (4) the name and the volume of the liquid to be used for reconstitution; (5) that the transmission of infectious agents can not be totally excluded when medicinal products prepared from human blood or plasma administered; (6) Recommended storage temperature.

Human Coagulation Factor IX Complex

Category. Coagulation factor.

It is a sterile, freeze dried powder consisting of partially purified Factor IX fraction, as well as concentrated factors II, VII and X fractions, of venous plasma obtained from healthy human donors. It contains no preservative.

It complies with the requirements of the test for potency in having not less than 80.0 per cent and not more than 120.0 per cent of the potency stated on the label in factor IX Units by comparison with the factor IX standard or with a working reference that has been calibrated with it.

Expiration date. The expiration date is not later than 2 years from the date of manufacture.

Storage. Store in hermetic containers in a refrigerator.

Labelling. The label states (1) a warning that it is to be used within 4 hours after constitution; (2) that it is for intravenous administration; (3) that a filter is to be used in the administration equipment.

Human Normal Immunoglobulin

Normal Immunoglobulin; Immune Human Serum Globulin; Human Gamma Globulin

Human Normal Immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly IgG. Other proteins may be present. Human normal immunoglobulin contains the IgG antibodies of normal subjects. It is intended for intramuscular or subcutaneous administration.

Human normal immunoglobulin is obtained from plasma that complies with the requirements of the monograph on *Plasma for Fractionation*. No antibiotic is added to the plasma used.

Production

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses, it shall have been shown that any residues present in the final product have no adverse effects on the patients treated with immunoglobulin.

The product shall have been shown, by suitable tests in animals and evaluation during clinical trials, to be well tolerated when administered intramuscularly or subcutaneously.

Human Normal Immunoglobulin is prepared from pooled material from not fewer than 1,000 donors by a method that has been shown to yield a product that (a) does not transmit

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infection; (b) at a protein concentration of 16 per cent, contains antibodies for at least 2 of which (one viral and one bacterial) an International Standard or Reference Preparation is available, the concentration of such antibodies being at least 10 times that in the initial pooled material.

It is prepared as a stabilized solution, for example in a 0.9 per cent w/v solution of sodium chloride, a 2.25 per cent solution of glycine or, if the preparation is to be freeze-dried, a 6 per cent w/v solution of *glycine*. Multidose preparation contains an antimicrobial preservative.

Single dose preparation does not contain an antimicrobial preservative. Any antimicrobial preservative or stabilizing agent used shall have been shown to have no deleterious effect on the final product in the amount present. The solution is passed through a bacteria-retentive filter. The preparation may subsequently be freeze-dried and the containers closed under vaccum or under an inert gas.

The stability of the preparation is demonstrated by suitable test carried out during development studies.

If the human normal immunoglobulin is intended for subcutaneous administration, the production method shall have been shown to consistently yield products that comply with the test for Fc function of immunoglobulin as stated in the monograph for Human Normal Immunoglobulin for Intravenous Use.

Description. The liquid preparation is clear and colourless or pale yellow or light brown; during storage it may show formation of slight turbidity or a small amount of particulate matter. The freeze-dried preparation is a hygroscopic, white or slightly yellow powder or solid, friable mass.

For the freeze-dried preparation, reconstitute as stated on the label immediately before carrying out the Identification and the Tests, except those for solubility and water.

Identification

A. Double Immunodiffusion: Precipitation test with a suitable range of species specific antisera which gives positive results for the presence of the protein of human origin and negative results with antisera specific to plasma protein for other species.

B. Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum compare normal human serum and the preparation under examination both diluted to contain 1 per cent w/v of protein. The main component of the preparation under examination corresponds to the IgG component of normal serum. The preparation under examination may show the presence of small quantities of other plasma proteins. If human albumin has been added as a stabilizer it may be seen as a major component.

Tests

Solubility. For the freeze-dried preparation, add the volume of the liquid stated on the label. The preparation dissolves completely within 20 minutes at 20-25°.

pH (2.4.24). 5.0 to 7.2

Dilute the preparation under examination with a 9 per cent solution of *sodium chloride* to obtain a solution containing 1 per cent of protein.

Assay for total protein. Human normal immunoglobulin contains not less than 90.0 per cent and not more than 110.0 per cent of the quantity of protein stated on the label and in any case not less than 10.0 per cent w/v and not more than 18.0 per cent w/v of protein. Determine by either Method I or II.

Method I

Dilute a suitable volume with water to produce a solution containing 1 per cent w/v of protein. Take 1.5 ml of the dilution in a round bottom centrifuge tube. Add 5 ml of *water*, mix add 0.2 ml of 7.5 per cent w/v solution of *sodium molybdate* and 1 ml of mixture consisting of 1 volume of *nitrogen-free sulphuric acid* and 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid and allow the inverted tube to drain on filter paper. To the residue in the tube add 3 drops of 30 per cent w/v solution of *copper sulphate* and 1 ml of *nitrogen-free sulphuric acid* and boil gently for 10 minutes, cool, add 1 g of *anhydrous sodium sulphate* and 10 mg of *selenium*, boil gently for 1 hour and cool. Transfer to an ammonia distillation apparatus, add 6 ml of a saturated solution of *sodium hydroxide* and pass steam through the flask; distil for seven minutes, collecting the distillate in a mixture of 5 ml of a saturated solution of *boric acid*, 5 ml of water, and 1 drop saturated solution of *methyl red* in alcohol containing 0.1 per cent of methylene blue and titrate with 0.02 M *hydrochloric acid*.

Method II

Determine by method G for Determination of nitrogen (2.3.3) and multiply the result by 6.25 to obtain the content of protein.

Protein composition. Determine by zone electrophoresis (2.4.12)

Use strips of suitable cellulose acetate gel as the supporting medium and *barbital buffer pH 8.6* as the electrolyte solution.

Test solution. Dilute the preparation under examination with 0.9 per cent solution of *sodium chloride* to a protein concentration of 5 per cent .

Reference solution. Reconstitute *human immunoglobulin for electrophoresis RS and* dilute with a 0.9 per cent solution of *sodium chloride* to a protein concentration of 5 per cent.

To a strip apply 2.5 µl of the test solution as a 10 mm band or apply 0.25 µl per mm if a narrower strip is used, to another strip apply in the same manner the same volume of the reference solution. Apply a suitable electric field such that the albumin band of normal human serum applied on a control strip migrates at least 30mm. Stain the strips with *amido black 10B solution* for 5 minutes. Decolourize with a mixture of 10 volumes of *glacial acetic acid* and 90 volumes of *methanol* so that the background is just free of colour. Develop the transparency of the strips with a mixture of 19 volumes of *glacial acetic acid* and 81 volumes of *methanol*. Measure the absorbance of the bands at 600 nm in an instrument having a linear response over the range of measurement. Calculate the result as the mean of 3 measurements of each strip.

System suitability. In the electropherogram obtained with the reference solution on cellulose acetate or on agarose gels, the proportion of protein in the principal band is within the limits stated in the leaflet accompanying the reference preparation.

Interpretation. In electropherogram obtained with the tests solution on cellulose acetate or on agarose gels, not more than 10 per cent of protein has mobility different from that of the principal band.

Molecular size. Determine by liquid chromatography (2.4.16).

Test solution. Dilute the preparation under examination with a 0.9 per cent solution of *sodium chloride* to a suitable concentration. A concentration in the range of 0.4-12 per cent and injection of 50-600 µg of protein are usually suitable.

Reference solution. Dilute *human immunoglobulin (molecular size) RS* with a 0.9 per cent solution of *sodium chloride* to the same protein concentration as the test solution.

Chromatographic system

- a stainless steel column 60 cm x 7.5 mm or 30 cm x 7.8 mm packed with hydrophilic silica gel for chromatography, of a grade suitable for fractionation of globular proteins with relative molecular masses in the range 10,000 to 500,000,
- mobile phase: A mixture of 4.873 g of *disodium hydrogen phosphate dihydrate*, 1.741g of *sodium dihydrogen phosphate monohydrate*, 11.688 g of *sodium chloride* and 50 mg of *sodium azide* in 1000 ml of *water*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 280 nm.

In the chromatogram obtained with the reference solution, the principal peak corresponds to IgG monomer and there is a peak corresponding to dimer with a relative retention to the principal peak of about 0.85. Identify the peaks in the chromatogram obtained with the test solution by comparison with a chromatogram obtained with the reference solution; any peak with a retention time shorter than that a dimer corresponds to polymers and aggregates.

Interpretation

In the chromatogram obtained with the test solution:

(a) *relative retention*. for monomer and dimer, the relative retention to the corresponding peak to the chromatogram obtained with the reference solution is 1±0.02;

(b) *peak area*. the sum of the peak areas of monomer and dimer represent not less than 85.0 per cent of the total area of the chromatogram and the sum of the peak area of polymers and aggregates represents not more than 10 per cent of the total area of the chromatogram.

Loss on drying (2.4.19). Not more than 2.0 per cent, determined on 0.5 g, by drying over phosphorous pentoxide at a pressure not exceeding 3 pa for 24 hours.

Sterility (2.2.11). Complies with the test for sterility.

Pyrogens (2.2.8)/or **Bacterial Endotoxin (BET)** (2.2.3). Complies with the test for pyrogens or, preferably and where justified and authorized with a validated *in-vitro* test such as the BET.

Pyrogens. Complies with the test of pyrogens, using 1 ml of the preparation under examination per kilogram of the rabbit's weight.

BET. Not more than 5 IU of endotoxin per ml.

Abnormal Toxicity (2.2.1). Complies with the test for abnormal toxicity, general test, injecting 0.5 ml into each mouse and 5 ml into each guinea-pig.

Antibody to hepatitis B surface antigen. Minimum 0.5 IU per g of immunoglobulin, determined by a suitable immunochemical method.

Antibody to hepatitis A virus. If intended for use in the prophylaxis of hepatitis A, it complies with the following additional requirement. Determine the antibody content by comparison with a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity.

The International Unit is the activity contained in a stated amount of the International Standard for anti hepatitis-A immunoglobulin. The equivalence in International Units of the International Standard is stated by WHO.

Human Hepatitis A Immunoglobulin RS is calibrated in International Units by comparison with the International Standards.

The stated potency is not less than 100 IU per ml. the estimated potency is not less than the stated potency. The confidence limits (P=0.95) of the estimated potency are not less than 80.0 per cent and not more than 125.0 per cent.

Additional tests for human normal immunoglobulin for subcutaneous administration:

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Anti-A and Anti-B haemagglutinin. If Human Normal Immunoglobulin is intended for subcutaneous administration, it complies with the test for Anti-A and Anti-B haemagglutinin as stated in the monograph for Dried Human Haemophilic Fraction.

Anti-D antibodies. If Human normal Immunoglobulin is intended for subcutaneous administration, it complies with the test for Anti-D antibodies as stated in the monograph for human normal immunoglobulin intravenous.

Storage. For the liquid preparation, store in a colourless glass container, protected from light, at the temperature stated on the label. For the freeze-dried preparation, store in an airtight colourless glass container, protected from light.

Labelling. The label states (1) for liquid preparation, the volume of the preparation in the container and the protein content expressed in per cent; (2) for freeze-dried preparation, the quantity of protein in the container; (3) The amount of immunoglobulin in the container; (4) The route of administration; (5) For freeze-dried preparation, the name or composition and the volume of the reconstituting liquid to be added; (6) The distribution of subclasses of IgG present in the preparation; (7) Where applicable, the amount of albumin added as a stabilizer; (8) The maximum content of IgA; (9) Recommended storage temperature.

Human Normal Immunoglobulin for Intravenous use

Human Normal Immunoglobulin for Intravenous Administration

Human Normal Immunoglobulin for Intravenous Administration is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G (IgG). Other proteins may be present. Human normal immunoglobulin for Intravenous Administration contains the IgG antibodies of normal subjects. This monograph does not apply to products intentionally prepared to contain fragments or chemically modified IgG.

Human Normal Immunoglobulin for Intravenous Administration is obtained from plasma that complies with the requirements of the monograph on *Plasma for Fractionation*. No antibiotic is added to the plasma used.

Production

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses, it shall have been shown that any residues present in the final product have no adverse effects on the patients treated with immunoglobulin.

The product shall have been shown, by suitable tests in animals and evaluation during clinical trials, to be well tolerated when administered intravenously.

Human Normal Immunoglobulin for Intravenous Administration is prepared from pooled material from not fewer than 1,000 donors by a method that has been shown to yield a product that (a) does not transmit infection; (b) at an immunoglobulin concentration of 50g per litre, contains antibodies for at least 2 of which (one viral and one bacterial) an International Standard or Reference Preparation is available, the concentration of such antibodies being at least 3 times that in the initial pooled material; (c) has a defined distribution of immunoglobulin G subclasses; (d) complies with the test for Fc function of immunoglobulin.

Test for Fc function of immunoglobulin. The Fc function for the preparation under the examination, its value is not less than that stated in the leaflet accompanying the reference preparation.

Stabilized human blood. Collect group O human red blood into ACD anticoagulant solution. Store the stabilised blood at 4° for not more than 3 weeks.

Phosphate buffered saline pH 7.2. Dissolve 1.022 g of *anhydrous disodium hydrogen phosphate*, 0.336 g of *anhydrous sodium dihydrogen phosphate* and 8.766 g of *sodium chloride* in 800 ml of *water* and dilute to 1,000 ml with the same solvent.

Magnesium and calcium stock solution. Dissolve 1.103 g of *calcium chloride* and 5.083 g of *magnesium chloride* in *water* and dilute to 25 ml with the same solvent.

Barbital buffer stock solution. Dissolve 207.5 g of *sodium chloride* and 25.48 g of *barbital sodium* in 4,000 ml of *water* and adjust to pH 7.3 using *1 M hydrochloric acid*. Add 12.5 ml of *magnesium and calcium stock solution* and dilute to 5,000 ml with *water*. Filter through a membrane filter (pore size 0.22 µm). Store at 4° in glass containers.

Albumin barbital buffer solution. Dissolve 0.150 g of *bovine albumin* in 20 ml of *barbital buffer stock solution* and dilute to 100 ml with *water*.

Tannic acid solution. Dissolve 10 mg of *tannic acid* in 100 ml of *phosphate-buffered saline pH 7.2*. Prepare immediately before use.

Guinea-pig complement. Prepare a pool of serum from the blood of not fewer than 10 guinea-pigs. Separate the serum from the clotted blood by centrifugation at about 4°. Store the serum in small amounts below -70°. Immediately before starting complement-initiated haemolysis, dilute to 125 to 200 CH_{50} per ml with albumin barbital buffer solution and store in an ice-bath during the test.

Rubella antigen. Suitable rubella antigen for haemagglutination-inhibition titre (HIT). Titre > 256 HA units.

Preparation of tanned human red blood cells. Separate human red blood cells by centrifuging an appropriate volume of stabilised human blood and wash the cells at least 3 times with *phosphate-buffered saline pH 7.2* and suspend at 2 per cent v/v in *phosphate-buffered saline pH 7.2*. Dilute 0.1 ml of *tannic acid solution* to 7.5 ml with *phosphate-buffered saline pH 7.2* (final concentration 1.3 mg per litre). Mix 1 volume of the freshly prepared dilution with 1 volume of human red blood cell suspension and incubate at 37° for 10 minutes. Collect the cells by centrifugation (400 to 800 g for 10 minutes), discard the supernatant and wash the cells once with *phosphate-buffered saline pH 7.2*. Resuspend the tanned cells at 1 per cent v/v in *phosphate-buffered saline pH 7.2*.

Antigen coating of tanned human red blood cells. Take a suitable volume (*Vs*) of tanned cells, add 0.2 ml of *rubella antigen* per 1.0 ml of tanned cells and incubate at 37° for 30 minutes Collect the cells by centrifugation (400 to 800 g for 10 minutes) and discard the supernatant, leaving a volume of 200 µl. Add a volume of *albumin barbital buffer solution* equivalent to the discarded supernatant, resuspend and collect the cells as described and repeat the washing procedure. Make up the remaining 200 µl to three-quarters of *Vs*, thereby obtaining the initial volume (*Vi*). Mix 900 µl of *albumin barbital buffer solution* with 100 µl of *Vi*, which is thereby reduced to the residual volume (*Vr*), and determine the initial absorbance at 541 nm (*A*). Dilute *Vr* by a factor equal to *A* using *albuminbarbital buffer solution*, thereby obtaining the final adjusted volume $Vf = Vr \times A$ of sensitised human red blood cells and adjusting *A* to 1.0 ± 0.1 for a tenfold dilution.

Antibody binding of antigen-coated tanned human red blood cells. Prepare the following solutions in succession and in duplicate, using for each solution a separate half-micro cuvette (for example, disposable type) or test-tube.

Test solutions. If necessary, adjust the immunoglobulin under examination to pH 7, for example by addition of *1 M sodium hydroxide*. Dilute volumes of the preparation under examination containing 30 mg and 40 mg of immunoglobulin with *albumin barbital buffer solution* and adjust the volume to 900 µl.

Reference solutions. Prepare as for the test solutions using *human immunoglobulin RS*.

Complement control. 900 µl of *albumin barbital buffer solution*.

Add to each cuvette/test-tube 100 µl of sensitised *human red blood cells* and mix well.

Incubate at room temperature for 15 minutes, add 1000 µl of *albumin barbital buffer solution*, collect the cells by centrifugation (1000 *g* for 10 minutes) of the cuvette/test-tube and remove 1900 µl of the supernatant. Replace the 1900 µl with *albumin barbital buffer solution* and repeat the whole of the washing procedure, finally leaving a volume of 200 µl. Test samples may be stored in sealed cuvette/test-tubes at 4° for 24 hours.

Complement-initiated haemolysis. To measure haemolysis, add 600 µl of *albumin barbital buffer solution* warmed to 37° to the test sample, re-suspend the cells carefully by repeated pipetting (not fewer than 5 times) and place the cuvette in the thermostated cuvette holder of a spectrophotometer. After 2 minutes, add 200 µl of *diluted guinea-pig complement* (125 to 200 CH_{50}/ml), mix thoroughly by pipetting twice and start immediately after the second pipetting the time-dependent recording of absorbance at 541 nm, using *albumin barbital buffer solution* as the compensation liquid. Stop the measurement if absorbance as a function of time has clearly passed the inflexion point.

Evaluation. Determine the slope (*S*) of the haemolysis curve at the approximate inflexion point by segmenting the steepest section in suitable time intervals Δt (for example, $\Delta t = 1$ minute) and calculate *S* between adjacent intersection points, expressed as ΔA per minute. The largest value for *S* serves as (S_{exp}). In addition, determine the absorbance at the start of measurement (A_s) by extrapolating the curve, which is almost linear and parallel to the time axis within the first few minutes. Correct (S_{exp}) using the expression

$$S' = \frac{S_{exp}}{A_s}$$

Calculate the arithmetic mean of the values of S' for each preparation. Calculate the index of Fc function (I_{Fc}) from the expression.

$$I_{FC} = \frac{100 \times (\overline{S'} - \overline{S'_C})}{\overline{S'_S} - \overline{S'_C}}$$

Where,

$\overline{S'}$ = arithmetic mean of the corrected slope for the preparation under examination,

$\overline{S'_S}$ = arithmetic mean of the corrected slope for the reference preparation,

$\overline{S'_C}$ = arithmetic mean of the corrected slope for the complement control.

Calculate the index of Fc function for the preparation under examination; the value is not less than that stated in the leaflet accompanying the reference preparation.

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Human Normal Immunoglobulin for Intravenous Administration is prepared as a stabilised solution or as a freeze- dried preparation. A stabilizer may be added. In both cases the preparation is passed through a bacteria-retentive filter. The preparation may subsequently be freeze-dried and the containers closed under vaccum or under an inert gas. No antimicrobial preservative is added either during fractionation or at the stage of the final bulk solution.

The stability of the preparation is demonstrated by suitable tests carried out during development studies.

Description. The liquid preparation is clear or slightly opalescent and colorless or pale yellow. The freeze-dried preparation is a hygroscopic, white or slightly yellow powder or solid friable mass.

For the freeze-dried preparation, reconstitute as stated on the label immediately before carrying out the identification and the tests, except those for solubility and water.

Identification

A. Double Immunodiffusion: Precipitation test with a suitable range of species specific antisera which gives positive results for the presence of protein of human origin and negative results with antisera specific to plasma protein for other species.

B. Examine by a suitable Immunoelectrophoresis technique. Using antiserum to normal human serum compare normal human serum and the preparation under examination both diluted to contain 1 per cent w/v of protein. The main component of the preparation under examination corresponds to the IgG component of normal human serum. The preparation under examination may show the presence of small quantities of other plasma proteins. If human albumin has been added as a stabilizer it may be seen as a major component.

Tests

pH (2.4.24). 4.0 to 7.4

Dilute the preparation under examination with a 0.9 per cent w/v solution of *sodium chloride* to obtain a solution containing 1 per cent of protein.

Osmolality (2.4.23). Minimum 240 mosmol per kg.

Total protein. Minimum 3 per cent w/v and between 90.0 to 110.0 per cent of the quantity of protein stated on the label.

Determine by either Method I or II.

Method I

Dilute the preparation under the examination with a 0.9 percent solution of *sodium chloride* to obtain a solution containing about 15 mg of protein in 2 ml. Take 2 ml of the solution in a round bottom centrifuge tube. Add 2 ml of 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of mixture of 30 volumes of water and 1 volume of *nitrogen free sulphuric acid*. Shake, centrifuge for 5 minutes. Decant the supernatant liquid and invert the inverted tube stand on a filter paper to drain the fluid. Determine the nitrogen in the centrifugation residue by method of sulphuric acid digestion (2.3.30) and calculate the protein content.

Method II

Determine by method G for Determination of nitrogen (2.3.) and multiply the result by 6.25 to obtain the content of protein.

Protein composition. Determine by zone electrophoresis (2.4.12).

Use strips of suitable cellulose acetate gel as the support medium and *barbital buffer pH8.6* as the electrolyte solution.

Test solution. Dilute the preparation under examination with 0.9 per cent w/v solution of *sodium chloride* to a immunoglobulin concentration of 3.0 per cent w/v.

Reference solution. Reconstitute *human immunoglobulin for electrophoresis reference preparation* and dilute with 0.9 per cent w/v solution of *sodium chloride* to a protein concentration of 3.0 per cent w/v.

To a strip apply 4 µl of the test solution as a 10mm band apply 0.4 µl per mm if a narrower strip is used, to another strip apply in the same manner the same volume of the reference solution. Apply a suitable electric field such that the albumin band of normal human serum applied on a control strip migrates at least 30mm. stain the strips with *amido black 10B solution* for 5 minutes. Decolourize with a mixture of 10 volumes of *glacial acetic acid* and 90 volumes of *methanol* so that the background is just free of colour. Develop the transparency of the strips with a mixture of 19 volumes of *glacial acetic acid* and 81 volumes of *methanol*. Measure the absorbance of the bands at 600 nm in an instrument having a linear response over the range of measurement. Calculate the result as the mean of 3 measurements of each strip.

System suitability. In the electropherogram obtained with the reference preparation, the proportion of protein in the principal band is within the limits stated in the leaflet accompanying the reference preparation.

Results. In electropherogram obtained with the tests solution not more than 5.0 per cent of protein has mobility different from that of the principal band. This limit is not applicable if albumin has been added to the preparation as a stabilizer; for such preparations, a test for proteins compositions is carried out during manufacture before addition of the stabilizer.

Molecular size. Determine by liquid chromatography (2.4.14).

Test solution: Dilute the preparation under examination with 0.9 per cent w/v solution of *sodium chloride* to obtain a concentration in the range of 0.4 to 1.2 per cent w/v an injection of 50-600 µg of protein are usually suitable.

Reference solution: Dilute *human immunoglobulin RS* with a 0.9 per cent w/v solution of *sodium chloride* to the same protein concentration as the test solution.

Chromatographic system

- a stainless steel column 60 cm x 7.5mm or 30 cm x 7.8mm
- packed with hydrophilic silica,
- mobile phase: a mixture of 4.873 g of *disodium hydrogen phosphate dihydrate*, 1.741g of *sodium dihydrogen phosphate monohydrate*, 11.688 g of *sodium chloride* and 50 mg of *sodium azide in* 1000 ml of *water*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 280 nm.

In the chromatogram obtained with the reference solution, the principal peak corresponds to IgG monomer and there is a peak corresponding to dimer with a relative retention to the principal peak of about 0.85. Identify the peaks in the chromatogram obtained with the test solution by comparison with a chromatogram obtained with the reference solution; any peak with a retention time shorter than that a dimer corresponds to polymers and aggregates.

Results. In the chromatogram obtained with the test solution:

(a) *relative retention:* for monomer and dimer, the relative retention to the corresponding peak to the chromatogram obtained with the reference solution is 1±0.02;

(b) *peak area:* the sum of the peak areas of monomer and dimer represent not less than 90.0 per cent of the total area of the chromatogram and the sum of the peak area of polymers and aggregates represents not more than 3.0 per cent of the total area of the chromatogram. This requirement does not apply to products where albumin has been added as a stabilizer; for products stabilized with albumin, a test for distribution of molecular size is carried out during manufacture before addition of the stabilizer.

This requirement does not apply to products containing IgM and IgA other than IgG.

Anticomplementary activity. The consumption of complement is not greater than 50.0 per cent (1 CH_{50} per mg of immunoglobulin).

Test for anticomplementary activity of immunoglobulin

For the measurement of anticomplementary activity (ACA) of immunoglobulin, a defined amount of test material (10 mg of immunoglobulin) is incubated with a defined amount of guinea-pig complement (20 CH_{50}) and the remaining complement is titrated; the anticomplementary activity is expressed as the percentage consumption of complement relative to the complement control considered as 100.0 per cent.

The haemolytic unit of complement activity (CH_{50}) is the amount of complement that, in the given reaction conditions, will produce the lysis of 2.5×10^8 out of a total of 5×10^8 optimally sensitised red blood cells.

Magnesium and calcium stock solution. Prepare as stated earlier.

Barbital buffer stock solution. Prepare as stated earlier.

Gelatin solution. Dissolve 12.5 g of *gelatin* in about 800 ml of *water* and heat to boiling in a water-bath. Cool to 20° and dilute to 10 litres with *water*. Filter through a membrane filter (pore size: 0.22 µm). Store at 4°. Use clear solutions only.

Citrate solution. Dissolve 8.0 g of *sodium citrate*, 4.2 g of *sodium chloride* and 20.5 g of *glucose* in 750 ml of *water*. Adjust to pH 6.1 using a 10.0 per cent w/v solution of *citric acid* and dilute to 1,000 ml with *water*.

Gelatin barbital buffer solution. Add 4 volumes of *gelatin solution* to 1 volume of *barbital buffer stock solution* and mix. Adjust to pH 7.3, if necessary, using *1 M sodium hydroxide* or *1 M hydrochloric acid*. Maintain at 4°. Prepare fresh solutions daily.

Stabilised sheep blood. Collect one volume of sheep blood into one volume of citrate solution and mix. Store at 4° for not less than 7 days and not more than 28 days. (Stabilised sheep blood and sheep red blood cells are available from a number of commercial sources.)

Haemolysin. Antiserum against sheep red blood cells prepared in rabbits.

Guinea-pig complement. Prepare a pool of serum from the blood of not fewer than ten guinea-pigs. Separate the serum from the clotted blood by centrifugation at about 4°. Store the serum in small amounts below -70°.

Method

Preparation of standardised 5 per cent sheep red blood cell suspension. Separate sheep red blood cells by centrifuging an appropriate volume of stabilised sheep blood and wash the cells at least three times with *gelatin barbital buffer solution* and prepare as a 5 per cent v/v suspension in the same solution. Measure the cell density of the suspension as follows: add 0.2 to 2.8 ml of *water* and centrifuge the lysed solution for 5 minutes at 1,000 g; the cell density is suitable if the absorbance (2.4.7) of the supernatant liquid at 541 nm is 0.62 ± 0.01. Correct the cell density by adding *gelatin barbital buffer solution* according to the formula:

$$V_f = \frac{V_i \times A}{0.62}$$

Where, V_f = final adjusted volume,

V_i = initial volume,

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A = absorbance of the original suspension at 541 nm.

The adjusted suspension contains about 1×10^9 cells per ml.

Haemolysin titration

Prepare haemolysin dilutions as shown in Table 1.

Add 1.0 ml of 5 per cent *sheep red blood cell suspension* to each tube of the haemolysin dilution series, starting at the 1:75 dilution, and mix. Incubate at 37° for 30 minutes.

Transfer 0.2 ml of each of these incubated mixtures to new tubes and add 1.10 ml of *gelatin barbital buffer solution* and 0.2 ml of diluted *guinea-pig complement* (for example, 1:150). Perform this in duplicate.

As the unhaemolysed cell control, prepare three tubes with 1.4 ml of *gelatin barbital buffer solution* and 0.1 ml of *5 per cent sheep red blood cell suspension*.

As the fully haemolysed control, prepare three tubes with 1.4 ml of *water* and 0.1 ml of *5 per cent sheep red cell suspension*.

Incubate all tubes at 37° for 60 minutes and centrifuge at 1,000 g for 5 minutes. Measure the absorbance (2.4.7) of the supernatants at 541 nm and calculate the percentage degree of haemolysis in each tube using the expression:

$$\frac{A_a - A_1}{A_b - A_1} \times 100$$

Where, A_a = absorbance of tubes with haemolysin dilution,

A_b = mean absorbance of the three tubes with full haemolysis,

A_1 = mean absorbance of the three tubes with no haemolysis.

Plot the percentage degree of haemolysis as the ordinate against the corresponding reciprocal value of the haemolysin dilution as the abscissa on linear graph paper. Determine the optimal dilution of the haemolysin from the graph by inspection. Select a dilution such that further increase in the amount of haemolysin does not cause appreciable change in the degree of haemolysis. This dilution is defined as one minimal haemolytic unit (1 MHU) in 1.0 ml. The optimal haemolytic haemolysin dilution for preparation of sensitised sheep red blood cells contains 2 MHU per ml.

The haemolysin titration is not valid unless the maximum degree of haemolysis is 50.0 per cent to 70.0 per cent. If the maximum degree of haemolysis is not in this range, repeat the titration with more or less diluted complement solution.

Table - 1

Required dilution of haemolysin	Prepared using		
	Gelatin barbital buffer solution Volume ml	Dilution (1 : ...)	Haemolysin Volume ml
7.5	0.65	undiluted	0.1
10	0.90	undiluted	0.1
75	1.80	7.5	0.2
100	1.80	10	0.2
150	1.00	75	1.0
200	1.00	100	1.0
300	1.00	150	1.0
400	1.00	200	1.0
600	1.00	300	1.0
800	1.00	400	1.0
1200	1.00	600	1.0
1600	1.00	800	1.0
2400	1.00	1200	1.0
3200*	1.00	1600	1.0
4800*	1.00	2400	1.0

*discard 1.0 ml of the mixture.

Preparation of optimised sensitised sheep red blood cells (haemolytic system)

Prepare an appropriate volume of diluted haemolysin containing 2 MHU/ml and an equal volume of *standardised 5 per cent sheep red blood cell suspension*. Add the haemolysin dilution to the standardised cell suspension and mix. Incubate at 37° for 15 minutes, store at 2° to 8° and use within 6 hours.

Titration of complement. Prepare an appropriate dilution of complement (for example, 1:250) with *gelatin barbital buffer solution* and perform the titration in duplicate as shown in Table 2.

Add 0.2 ml of sensitised sheep red blood cells to each tube, mix well and incubate at 37° for 60 minutes. Cool the tubes in an ice-bath and centrifuge at 1,000 g for 5 minutes. Measure the absorbance of the supernatant liquid at 541 nm and calculate the degree of haemolysis (Y) using the expression:

$$\frac{A_c - A_1}{A_b - A_1}$$

Where, A_c = absorbance of tubes 1 to 12,

A_b = mean absorbance of tubes with 100 per cent haemolysis,

A_1 = mean absorbance of cell controls with 0 per cent haemolysis.

Plot $Y/(1 - Y)$ as the abscissa against the amount of diluted complement in ml as the ordinate on log–log graph paper. Fit the best line to the points and determine the ordinate for the 50.0 per cent haemolytic complement dose where $Y/(1 - Y) = 1.0$. Calculate the activity in haemolytic units (CH_{50}/ml) from the expression:

$$\frac{C_d}{C_a \times 5}$$

where, C_d = reciprocal value of the complement dilution,

C_a = volume of diluted complement in ml resulting in 50.0 per cent haemolysis,

5 = scaling factor to take account of the number of red blood cells.

The test is not valid unless the plot is a straight line between 15.0 per cent and 85.0 per cent haemolysis and the slope is 0.15 to 0.40, and preferably 0.18 to 0.30.

Table - 2

Tube Number	Volume of diluted complement in ml (for example 1:250)	Volume of gelatin barbital buffer solution in ml
1	0.1	1.2
2	0.2	1.1
3	0.3	1.0
4	0.4	0.9
5	0.5	0.8
6	0.6	0.7
7	0.7	0.6
8	0.8	0.5
9	0.9	0.4
10	1.0	0.3
11	1.1	0.2
12	1.2	0.1
Three tube as cell control at 0 per cent haemolysis	-	1.3
Three tube at 100 per cent haemolysis	-	1.3 ml of water

Test for anticomplementary activity

Prepare a complement dilution having 100 CH_{50} per ml by diluting titrated *guinea-pig complement* with *gelatin barbital buffer solution*. If necessary, adjust the immunoglobulin under examination to pH 7. Prepare incubation mixtures as follows for an immunoglobulin containing 50 mg per ml:

Table - 3

Immunoglobulin under examination	Complement control (in duplicate)	
Immunoglobulin (50 mg per ml)	0.2 ml	—
Gelatin barbital buffer	0.6 ml	0.8 ml
Complement	0.2 ml	0.2 ml

Carry out the test on the immunoglobulin under examination and prepare ACA negative and positive controls using *human immunoglobulin RS*, as indicated in the leaflet accompanying the reference preparation. Higher or lower volumes of sample and of *gelatin barbital buffer solution* are added if the immunoglobulin concentration varies from 50 mg per ml; for example, 0.47 ml of *gelatin barbital buffer solution* is added to 0.33 ml of immunoglobulin containing 30 mg per ml to give 0.8 ml. Close the tubes and incubate at 37° for 60 minutes. Add 0.2 ml of each incubation mixture to 9.8 ml of *gelatin barbital buffer solution* to dilute the complement. Perform complement titrations as described above on each tube to determine the remaining complement activity (Table 2). Calculate the anticomplementary activity of the preparation under examination relative to the complement control considered as 100.0 per cent, from the expression:

$$\frac{a - b}{a} \times 100$$

where, a = mean complement activity (CH_{50}/ml) of complement control,

b = complement activity (CH_{50}/ml) of tested sample.

The test is not valid unless:

a. the anticomplementary activities found for ACA negative control and ACA positive control are within the limits stated in the leaflet accompanying the reference preparation,

b. the complement activity of the complement control (a) is in the range 80 to 120 CH_{50} per ml.

Prekallikrein activator. Maximum 35 IU per ml calculated with the reference to a dilution of the preparation to be examined containing 3.0 per cent of immunoglobulin.

Test for prekallikrein activator

Prekallikrein activator (PKA) activates prekallikrein to kallikrein and may be assayed by its ability to cleave a chromophore from a synthetic peptide substrate so that the rate of cleavage can be measured spectrophotometrically and the concentration of PKA calculated by comparison with a reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of the International Standard which consists of freeze-dried

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prekallikrein activator. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Reagents. *Prekallikrein activator in albumin RS* is calibrated in International Units by comparison with the International Standard.

Buffer A. Dissolve 6.055 g of *tris(hydroxymethyl)-aminomethane*, 1.17 g of *sodium chloride*, 50 mg of *hexadimethrine bromide* and 0.100 g of *sodium azide* in *water*. Adjust to pH 8.0 with *2 M hydrochloric acid* and dilute to 1000 ml with *water*.

Buffer B. Dissolve 6.055 g of *tris(hydroxymethyl)-aminomethane* and 8.77 g of *sodium chloride* in *water*. Adjust to pH 8.0 with *2 M hydrochloric acid* and dilute to 1,000 ml with *water*.

Preparation of prekallikrein substrate

To avoid coagulation activation, blood or plasma used for the preparation of prekallikrein must come into contact only with plastics or silicone-treated glass surfaces.

Draw 9 volumes of human blood into 1 volume of anticoagulant solution (ACD, CPD or 3.8 per cent w/v solution of *sodium citrate*) to which 1 mg per ml of *hexadimethrine bromide* has been added. Centrifuge the mixture at 3,600 g for 5 minutes. Separate the plasma and centrifuge again at 6,000 g for 20 minutes to sediment platelets. Separate the platelet-poor plasma and dialyse against 10 volumes of buffer A for 20 hours. Apply the dialysed plasma to a chromatography column containing agarose-DEAE for ion exchange chromatography which has been equilibrated in buffer A and is equal to twice the volume of the plasma. Elute from the column with buffer A at 20 ml per cm 2 per hours. Collect the eluate in fractions and record the absorbance (2.4.7) at 280 nm. Pool the fractions containing the first protein peak so that the volume of the pool is about 1.2 times the volume of the platelet-poor plasma.

Test the substrate pool for absence of kallikrein activity by mixing 1 part with 20 parts of the pre-warmed *chromogenic substrate solution* to be used in the assay and incubate at 37° for 2 minutes. The substrate is suitable if the increase in absorbance is less than 0.001 per minute. Add to the pooled solution 0.7 per cent w/v of *sodium chloride* and filter using a membrane filter (porosity 0.45 µm). Freeze the filtrate in portions and store at -25°; the substrate may be freeze-dried before storage.

Carry out all procedures from the beginning of the chromatography to freezing in portions during a single working day.

Method. The assay may be carried out using an automated enzyme analyser or a suitable microtitre plate system allowing kinetic measurements, with appropriate software for calculation of results. Standards, samples and prekallikrein substrate may be diluted as necessary using buffer B.

Incubate diluted standards or samples with prekallikrein substrate for 10 minutes such that the volume of the undiluted sample does not exceed 1/10 of the total volume of the incubation mixture to avoid errors caused by variation in ionic strength and pH in the incubation mixture. Incubate the mixture or a part thereof with at least an equal volume of a solution of a suitable synthetic chromogenic substrate, known to be specific for kallikrein (for example, *N-benzoyl*-L-*prolyl*-L-*phenylalanyl*-L-*arginine 4-nitroanilide acetate* or D-*prolyl*-L-*phenylalanyl*-L-*arginine-4-nitroanilide-dihydrochloride*), dissolved in buffer B. Record the rate of change in absorbance per minute for 2 to 10 minutes at the wavelength specific for the substrate used. Prepare a blank for each mixture of sample or standard using buffer B instead of prekallikrein substrate.

Depending on the method used ΔA per minutes has to be corrected by subtracting the value obtained for the corresponding blank without the prekallikrein substrate. The results may be calculated using a standard curve, a parallelline or a slope ratio assay or any other suitable statistical method. Plot a calibration curve using the values thus obtained for the reference preparation and the respective concentrations; use the curve to determine the PKA activity of the preparation under examination.

Anti-A and anti-B haemagglutinins. Carry out the tests for anti-A and anti-B haemagglutinins as stated under monograph for Dried human haemophilic fraction. If the preparation to be examined contains more than 3.0 per cent of immunoglobulin dilute to this concentration before preparing the dilution to be used in the test. The 1 to 64 dilutions do not show agglutination.

Anti-D antibodies. It complies with the test for anti-D antibodies in human immunoglobulin for intravenous administration.

Test for anti-D antibodies in human immunoglobulin for intravenous administration

Materials

Phosphate-buffered saline (PBS). Dissolve 8.0 g of *sodium chloride*, 0.76 g of *anhydrous disodium hydrogen phosphate*, 0.2 g of *potassium chloride*, 0.2 g of *potassium dihydrogen phosphate* and 0.2 g of *sodium azide* in *water* and dilute to 1,000 ml with the same solvent.

Papain solution. Use serological grade papain from a commercial source, the activity of which has been validated.

Red blood cells. Use pooled red blood cells from not fewer than 3 donors of group OR_2R_2 and 3 donors of group Orr

respectively. Wash the cells 4 times with PBS or until the supernatant is clear. Centrifuge the cells at 1, 800 g for 5 minutes to pack. Treat the packed red cells with papain solution according to the manufacturer 's instructions. Store in Alsever's solution for not more than 1 week.

Microtitre plates. Use V-bottomed rigid micro-titre plates.

Reference standards. *Immunoglobulin (anti-D antibodies test) reference preparation* and *Immunoglobulin (anti-D antibodies test negative control) reference preparation* are suitable for use as the reference preparation and negative control, respectively.

Method

Reference preparation and negative control solutions. Reconstitute the reference preparation and the negative control according to instructions. Dilute the reconstituted preparations with an equal volume of *PBS* containing 0.2 per cent w/v of *bovine albumin* and then prepare a further 7 serial two-fold dilutions using *PBS* containing 0.2 per cent w/v of *bovine albumin* to give a total dilution range from 1/2 to 1/256. Make 2 independent sets of dilutions for each preparation. Add 20 µl of each dilution to the microtitre plate.

Test solutions. Initially dilute the test samples to give a starting immunoglobulin G (IgG) concentration of 2.5 per cent w/v using *PBS* containing 0.2 per cent w/v of *bovine albumin* and then prepare a further 7 serial two-fold dilutions using *PBS* containing 0.2 per cent w/v of *bovine albumin* to give a total dilution range from 1/2 to 1/256. Make 2 independent sets of dilutions for each test sample. Add 20 µl of each dilution to the microtitre plate.

Prepare 3 per cent v/v suspensions of papain-treated D-positive (OR_2R_2) and D-negative (Orr) red cells in *PBS* containing 0.2 per cent w/v of *bovine albumin*. Add 20 µl of D-positive cells to one dilution series of each of the test sample, the reference preparation and the negative control, and 20 µl of *D-negative cells* to the other dilution series of each of the test samples, the reference preparation and the negative control. Mix by shaking the plate on a shaker for 10 seconds.

Centrifuge the plate at 80 g for 1 minutes to pellet the cells.

Place the plate at an angle of approximately 70°. Read after 4 to 5 minutes (or until the cells have streamed in the wells containing the negative control and the wells where the D-negative cells have been added). A cell button at the bottom of the well indicates a positive result. A stream of cells represents a negative result.

Record the endpoint titre as the reciprocal of the highest dilution that gives rise to a positive result.

The titre of the preparation under examination is not greater than the titre of the reference preparation.

Antibody to hepatitis B surface antigen. Minimum 0.5 IU per g of immunoglobulin, determine by a suitable immunochemical method.

Immunoglobulin A. As determined by a suitable immunochemical method, the content of the immunoglobulin A is not more than the maximum content stated on the label.

Water. Determine by a suitable method, such as the semi-micro determination of water(2.3.43), loss on drying (2.4.19) or near infrared spectrophotometry (2.4.6), the water content is within the limits approved by the competent authority.

Sterility (2.2.11). Complies with the test for sterility.

Pyrogens (2.2.8) / or **Bacterial Endotoxin (BET)** (2.2.3). Complies with the test for pyrogen or, preferably and where justified and authorized with a validated in-vitro test such as the BET.

Pyrogens. Complies with the test for pyrogen. Inject per kg of the rabbit's mass a volume equivalent to 0.5 g of immunoglobulin but not more than 10 ml per kg of body mass

BET. Where the bacterial endotoxin test is used, the product contains less than 0.5 IU of endotoxin per ml for solution with a protein content not more than 5.0 per cent, and less than 1 IU of endotoxin per milliliter for solutions with the protein content greater than 5.0 per cent but not greater than 10.0 per cent.

Storage. For the liquid preparation, store in a colourless glass container, protected from light, at the temperature stated on the label. For the freeze-dried preparation, store in an airtight colourless glass container, protected from light, at a temperature not exceeding 25°.

Labelling. The label states (1) for liquid preparation, the volume of the preparation in the container and the protein content expressed in grams per litre; (2) for freeze-dried preparation, the quantity of protein in the container; (3) The amount of immunoglobulin in the container; (4) The route of administration; (5) For freeze-dried preparation, the name or composition and the volume of the reconstituting liquid to be added; (6) The distribution of subclasses of IgG present in the preparation; (7) Where applicable, the amount of albumin added as a stabilizer; (8) The maximum content of IgA.

Human Plasma Protein Fraction

Plasma Protein Solution; Human Albumin Fraction (Saline); Plasma Protein Fraction; PPF

Human plasma protein fraction is a sterile isotonic aqueous solution of proteins containing albumin and globulins obtained from human Plasma that complies with the monograph of Plasma for fractionation. It is prepared as an

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isotonic solution containing 4.0 to 5.0 per cent w/v of total protein. It contains no fibrinogen or antibodies.

Production

The separation of protein may be done by precipitation with suitable organic solvents under controlled conditions, particularly of pH, ionic strength and temperature, so that in the final product not less than 85.0 per cent of the total protein is albumin. Residual solvents, if present, may be removed by freeze drying or other suitable treatment. Alternative methods of preparation which shall not affect the integrity of the product and shall have been shown to yield consistently a product which is safe for intravenous injection may be adopted.

The product is dissolved in *water* and sufficient quantities of suitable stabilizer against the effect of heat, like *sodium caprylate* in a suitable concentration and sufficient *sodium chloride,* to adjust the sodium ions to between 130 and 160 millimoles per litre, may be added but no antibiotic or antimicrobial preservative is added at any stage during preparation. The solution is sterilized by filtration through a bacteria retentive filter and distributed aseptically into sterile container which are then closed so as to prevent microbial contamination. The solution in its final container is heated at 60°±0.5° and maintained at this temperature for 10 hours. The containers are then incubated at 30°-32° for not less than 14 days or at 20°-25° for not less than 4 weeks and examined visually for evidence of microbial contamination. Those showing abnormalities such as abnormal color, turbidity, presence of atypical particles or microbial contamination are discarded.

Human plasma protein fraction contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of total protein.

Description. A clear, almost colorless or pale yellow liquid; almost odourless. On storage a dust-like precipitate may develop but it disappears on shaking.

Identification

A. Double Immunodiffusion. Precipitation test with a suitable range of species specific antisera which gives positive results for the presence of protein of human origin and negative results with antisera specific to plasma protein for other species.

B. Immunoelectrophoresis technique. Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and preparation under examination, both diluted to contain 1 per cent w/v of protein. The main component of the preparation under examination corresponds to the Albumin component of normal human serum. The solution may show the presence of small quantities of other plasma proteins.

Tests

pH (2.2.24). Dilute with sufficient *saline solution* to prod solution containing 1 per cent w/v of protein; pH of resu solution 6.7 to 7.3.

Molecular Size Distribution. Determine by L Chromatography (2.4.14).

Test solution; Dilute the preparation under examination wi 0.9 per cent solution of sodium chloride to a suit concentration. Concentration in the range of 0.4 to 12 cent and injection of 50 to 600 µg of protein are usually suit

Chromatographic System
- a stainless steel column 0.6 m x 7.5 mm or 0.3 m x 7.8 with *Hydrophilic silica gel*, of a grade suitable fractionation of globular proteins with relative molec masses in the range 10,000 – 500,000,
- mobile phase: A mixture of 4.873 g of *disodium hydro phosphate dihydrate,* 1.741 g of *sodium dihydro phosphate monohydrate,* 11.688 g of *sodium chlor* and 50 mg of *sodium azide,* in 1000 ml of *water,*
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 280 nm.

The peak due to polymers and aggregates is located in part of the chromatogram representing the void volum Disregard the peak due to the stabilizer, the area of the pe due to polymers and aggregate is not more than 10.0 per c of the total area of the chromatogram.

Protein Composition. Determined by zone electrophore (2.4.12).

Use strips of suitable cellulose acetate gel or agarose gel the supporting medium and barbital buffer solution pH 8.6 the electrolyte solution.

If cellulose acetate is the supporting material, the meth described below can be used. If agarose gels are used, s because they are normally part of an automated system, manufacturer's instructions are followed instead.

Test solution. Dilute the preparation under examination wi 0.9 per cent solution of sodium chloride to a prot concentration of 2.0 percent.

Reference solution. Dilute *human albumin for electrophores RS* with a 0.9 per cent solution of sodium chloride to a prot concentration of 2.0 per cent.

To a strip apply 2.5 µl of the test solution as a 10 mm band apply 2.5 µl per ml if a narrower strip is used.

To another strip, apply in the same manner the same volu of the reference solution. Apply suitable electric field s that the most rapid band migrates at least 30 mm. Treat strips with *amido black 10 B solution* for 5 minu Decolourise with the mixture of 10 volumes of *glacial ac*

acid and 90 volumes of *methanol* until the background is just free of colour. Develop the transparency of the strips with the mixture of 19 volumes of *glacial acetic acid* and 81 volume of *methanol.* Measure the absorbance of the band at 600 nm in an instrument having a linear response over the range of measurement. Calculate the result as the mean of 3 measurements of each strips.

In the electropherogram obtained with the reference solution on cellulose acetate or on agarose gel, the proportion of protein in the principal band is within the limits stated in the leaflet accompanying the reference preparation.

Results: In the electropherogram obtained with the test solution on *cellulose acetate* or on *agarose gel,* not more than 15 per cent of the protein is contained in the bands other than the principal band.

Haem content. Dilute the preparation under examination using a 0.9 per cent solution of sodium chloride to obtain a solution containing 1 per cent of protein. The absorbance of resulting solution shall be not more than 0.15 when measured by spectrophotometery (2.4.7) at 403 nm.

Sterility (2.2.11). Complies with the tests for sterility.

Abnormal toxicity (2.2.1). Complies with the tests for abnormal toxicity. *(For Antisera and Vaccines)* and 0.5 ml of the solution for each mouse and 5 ml for each guinea pig irrespective of the protein content.

Pyrogens (2.2.8)/or **Bacterial Endotoxins (BET)** (2.2.3). Complies with the test for pyrogen or, preferably and where justified and authorized with a validated *in-vitro* test such as the Bacterial Endotoxin test.

Pyrogens. Complies with the test for pyrogen using 3 ml/kg of the rabbit weigh or

BET. Where the bacterial endotoxin test is used, the preparation under examination contains less than 0.5 IU of endotoxin per milliliter for solution with a protein content not greater than 5.0 per cent, less than 1.3 IU of endotoxin per ml for solution with a protein content greater than 5.0 per cent but not greater than 20.0 per cent, and less than 1.7 IU of endotoxin per ml for solution with a protein content greater than 20.0 per cent but not greater than 25.0 per cent.

Assay

For total protein. Determine by either Method I or II.

Method I

Dilute to about 0.75 per cent w/v of total protein with *saline solution*. Take 2 ml of the solution in a round bottom centrifuge tube. Add 2 ml of 7.5 per cent w/v solution of *sodium molybdate* and 2 mL of mixture of 30 volumes of *water* and 1 volume of *nitrogen free sulphuric acid*. Shake, centrifuge for 5 minutes. Decant the supernatant liquid and let the inverted tube stand on a filter paper to drain the fluid. Carry out method F for Determination of nitrogen (2.3.30) on the residue thus obtained and multiply the result by 6.25 to obtain the protein content.

Method II

Determine by method G for determination of nitrogen (2.3.30) and multiply the result by 6.25 to obtain the content of protein.

For Sodium. Not less than 95.0 per cent and not more than 105.0 per cent of the stated amount and, in any case not more than 160 millimoles of sodium per litre, determine by Method A for atomic absorption spectrophotometry (2.4.2), measuring at 589 nm and using *sodium solution AAS* suitably diluted with water to prepare the standard solutions.

For Potassium. Not more than 50 µmol of K per g of protein, determined by atomic absorption spectrophotometry (2.4.2), measuring at 766 nm and using *sodium solution AAS* suitably diluted with water to prepare the standard solutions.

Storage. Store protected from light at a temperature between 2° and 25°.

Labelling. The label states (1) the volume in the container; (2) the total amount of protein in the container expressed in g per litre or as percentage; (3) the concentration of sodium and potassium ions expressed in millimoles per litre; (4) the names and concentrations of any stabilizing agents and any other additives in the final solution; (5) the type of source material used to manufacture the product; (6) the words "do not use if turbid"; (7) that the contents must not be used more than 4 hours after the container has been penetrated and any remnant portion must be discarded; (8) the storage conditions.

Human Prothrombin Complex

Sterile plasma protein fraction containing human coagulation factor IX together with variable amounts of human coagulation factors II, VII and X; the presence and proportion of these additional factors depends on the method of fractionation. It is obtained from human plasma that complies with the monograph on Human plasma for fractionation. The preparation may contain excipients such as stabilisers, heparin and antithrombin. The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU of human coagulation factor IX per millilitre. If the content of any of the factors is stated as a single value, the estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency; if the content of any of the factors is stated as a range, the estimated potency is not less than the lower limit and not greater than the upper limit of the stated range.

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Production

The method of preparation is designed to minimise activation of any coagulation factor (to minimise potential thrombogenicity) and includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

The specific activity is not less than 0.6 IU of factor IX per mg of total protein, before the addition of any protein stabiliser.

The prothrombin complex fraction is dissolved in a suitable liquid. Heparin, antithrombin and other auxiliary substances such as a stabiliser may be added. No antimicrobial preservative is added. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas.

Description. A white or slightly coloured powder or friable solid, very hygroscopic.

Reconstitute the preparation under examination as stated on the label immediately before carrying out the Identification, Tests (except those for solubility and water) and Assay.

Identification

It complies with the limits of the assay for coagulation factor IX activity and, where applicable, those for factors II, VII and X.

Tests

pH (2.4.24). 6.5 to 7.5.

Osmolality (2.4.23). Minimum 240 mosmol per kg.

Total protein. If necessary, dilute an accurately measured volume of the reconstituted preparation with a 0.9 per cent w/v solution of *sodium chloride* to obtain a solution expected to contain about 15 mg of protein in 2 ml. To 2.0 ml of the solution in a round-bottomed centrifuge tube add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid* and 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.3.30) and calculate the amount of protein by multiplying the result by 6.25.

Activated coagulation factors (2.8.3). If necessary, dilute the preparation under examination to contain 20 IU of factor IX per ml. For each of the dilutions, the coagulation time is not less than 150 seconds.

Heparin. If heparin has been added during preparation, determine the amount present by the assay of heparin in coagulation factor concentrates (2.8.9). The preparation under examination contains not more than the amount of heparin stated on the label and in any case not more than 0.5 IU of heparin per International Unit of factor IX.

Thrombin. If the preparation under examination contains heparin, determine the amount present as described in the test for heparin and neutralise it by addition of *protamine sulphate* (10 µg of protamine sulphate neutralises 1 IU of heparin). In each of 2 test-tubes, mix equal volumes of the reconstituted preparation and a 0.3 per cent w/v solution of *fibrinogen*. Keep one of the tubes at 37° for 6 hours and the other at room temperature for 24 hours. In a third tube, mix a volume of the fibrinogen solution with an equal volume of a solution of *human thrombin* (1 IU per ml) and place the tube in a water-bath at 37°. No coagulation occurs in the tubes containing the preparation under examination. Coagulation occurs within 30 seconds in the tube containing thrombin.

Water. Determine by semi-micro determination of water (2.3.43), loss on drying (2.4.19) or near-infrared spectrometry (2.4.6), the water content is within the limits approved by the competent authority.

Pyrogens (2.2.8) or **Bacterial endotoxins (BET)** (2.2.3). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

Pyrogens. Complies with the test for pyrogens. Inject per kg of the rabbit's mass a volume of the reconstituted preparation equivalent to not less than 30 IU of factor IX.

BET. Where the bacterial endotoxin test is used, the preparation to be examined contains not more than 0.05 Endotoxin Unit per IU of factor IX.

Sterility (2.2.11). Complies with the test for sterility.

Assay

Factor IX

Determine the assay of human coagulation factor IX (2.8.7).

The estimated potency is not less than 80.0 per cent and not more than 125.0 per cent of the stated potency. The confidence interval (P = 0.95) of the estimated potency is not greater than 80.0 per cent to 125.0 per cent.

Factor II

Determine the assay of human coagulation factor II (2.8.4).

The estimated potency is not less than 80.0 per cent and not more than 125.0 per cent of the stated potency. The confidence interval (P = 0.95) of the estimated potency is not greater than 90.0 per cent to 111.0 per cent.

Factor VII

If the label states that the preparation contains factor VII, Determine the assay of human coagulation factor VII (2.8.5).

The estimated potency is not less than 80.0 per cent and not more than 125.0 per cent of the stated potency. The confidence interval (P = 0.95) of the estimated potency is not greater than 80.0 per cent to 125.0 per cent.

Factor X

Determine the assay of human coagulation factor X (2.8.8).

The estimated potency is not less than 80.0 per cent and not more than 125.0 per cent of the stated potency. The confidence interval (P = 0.95) of the estimated potency is not greater than 90.0 per cent to 111.0 per cent.

Storage. Store protected from light.

Labelling. The label states (1) The number of International Units of human coagulation factor IX, and the number or range of International Units of human coagulation factor II per container; (2) Where applicable, the number or range of International Units of human coagulation factor VII and human coagulation factor X. per container; (3) where applicable, that the preparation contains protein C and/or protein S; (4) the amount of protein per container; (5) the name and quantity of any added substances, including where applicable, heparin; (6) the name and quantity of the liquid to be used for reconstitution; (7) that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

Plasma for Fractionation

Human Plasma for Fractionation

Human Plasma for Fractionation is the liquid part of human blood remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant, or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure; it is intended for the manufacture of plasma-derived products.

Production

Donors

Only a carefully selected, healthy donor who, as far as can be ascertained after medical examination, laboratory blood tests and a study of the donor's medical history, is free from detectable agents of infection transmissible by plasma-derived products may be used.

Immunisation of donors

Immunisation of donors to obtain immunoglobulins with specific activities may be carried out when sufficient supplies of material of suitable quality can not be obtained from naturally immunised donors. Recommendations for such immunisations are formulated by the World Health Organisation (*Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives,* WHO Technical Report Series, No. 840, 1994 or subsequent revision).

Records

Records of donors and donations made are kept in such a way that, while maintaining the required degree of confidentiality concerning the donor's identity, the origin of each donation in a plasma pool and the results of the corresponding acceptance procedures and laboratory tests can be traced.

Laboratory tests

Laboratory tests are carried out for each donation to detect the following viral markers:

1. Antibodies against human immunodeficiency virus 1 (anti-HIV-1),
2. Antibodies against human immunodeficiency virus 2 (anti-HIV-2),
3. Antibodies against hepatitis C virus (anti-HCV),
4. Hepatitis B surface antigen (HBsAg).

Pending complete harmonisation of the laboratory tests to be carried out, the competent authority may require that a test for alanine aminotransferase (ALT) also be carried out.

The test methods used are of suitable sensitivity and specificity and comply with the regulations in force. If a repeat-reactive result is found in any of these tests, the donation is not accepted.

Individual plasma units

The plasma is prepared by a method that removes cells and cell debris as completely as possible. Whether prepared from whole blood or by plasmapheresis, the plasma is separated from the cells by a method designed to prevent the introduction of micro-organisms. No antibacterial or antifungal agent is added to the plasma. The containers comply with the requirements for plastic containers for blood and blood components (6.2). The containers are closed so as to prevent any possibility of contamination.

If 2 or more units are pooled prior to freezing, the operations are carried out using sterile connecting devices or under

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aseptic conditions and using containers that have not previously been used.

When obtained by plasmapheresis, plasma intended for the recovery of proteins that are labile in plasma is frozen by cooling rapidly in a chamber at -30° or below as soon as possible and at the latest within 24 hours of collection.

When obtained from whole blood, plasma intended for the recovery of proteins that are labile in plasma is separated from cellular elements and is frozen by cooling rapidly in a chamber at -30° or below as soon as possible and at the latest within 24 hours of collection.

When obtained from whole blood, plasma intended solely for the recovery of proteins that are not labile in plasma is separated from cellular elements and frozen in a chamber at -20° or below as soon as possible and at the latest within 72 hours of collection.

It is not intended that the determination of total protein and factor VIII shown below be carried out on each unit of plasma. They are rather given as guidelines for good manufacturing practice, the test for factor VIII being relevant for plasma intended for use in the preparation of concentrates of labile proteins.

The total protein content of a unit of plasma depends on the serum protein content of the donor and the degree of dilution inherent in the donation procedure. When plasma is obtained from a suitable donor and using the intended proportion of anticoagulant solution, a total protein content complying with the limit of 5 per cent is obtained. If a volume of blood or plasma smaller than intended is collected into the anticoagulant solution, the resulting plasma is not necessarily unsuitable for pooling for fractionation. The aim of good manufacturing practice must be to achieve the prescribed limit for all normal donations.

Preservation of factor VIII in the donation depends on the collection procedure and the subsequent handling of the blood and plasma. With good practice, 0.7 IU/ml can usually be achieved, but units of plasma with a lower activity may still be suitable for use in the production of coagulation factor concentrates. The aim of good manufacturing practice is to conserve labile proteins as much as possible.

Total protein. Carry out the test using a pool of not less than 10 units. Dilute the pool with a 0.9 per cent solution of *sodium chloride* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of this solution in a round-bottomed centrifuge tube add 2 ml of a 7.5 per cent solution of *sodium molybdate* and 2 ml of a mixture of 1 volume of *nitrogen-free-sulphuric acid* and 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.3.30) and calculate the protein content by multiplying the quantity of nitrogen by 6.25. The total protein content is not less than 5.0 per cent.

Factor VIII

Carry out the test using a pool of not less than 10 units. Dilute the samples under examination, if necessary, at 37°. Determine the assay of factor VIII (2.8.6), using a reference plasma calibrated against the International Standard for human coagulation factor VIII in plasma. The activity is not less than 0.7 IU per ml.

Pooled plasma

During the manufacture of plasma products, the final homogeneous pool of plasma (for example, after removal of cryoprecipitate) is tested for HBsAg, HCV antibodies and for HIV antibodies using test methods of suitable sensitivity and specificity; the pool must give negative results in these tests.

The plasma pool is also tested for hepatitis C virus RNA using a validated nucleic acid amplification technique (2.8.1). A positive control with 100 IU per ml of hepatitis C virus RNA and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors. The plasma pool complies with the test if it is found non-reactive for hepatitis C virus RNA.

Description. Before freezing, a clear to slightly turbid liquid without visible signs of haemolysis; it may vary in color from light yellow to green.

Storage. Frozen plasma is stored in conditions designed to maintain the temperature at or below -20°; for accidental reasons, the storage temperature may rise above -20° on one or more occasions during storage but the plasma is nevertheless considered suitable for fractionation if all the following conditions are fulfilled (1) the total period of time during which the temperature exceeds -20° does not exceed 72 hours; (2) the temperature does not exceed -15° on more than one occasion; (3) the temperature at no time exceeds -5°.

Labelling. The label enables each individual unit to be traced to a specific donor.

Plasma (Pooled and Treated for Virus Inactivation)

Human Plasma (Pooled and Treated for Virus Inactivation)

Category. Blood product.

Plasma (pooled and treated for virus inactivation) is a frozen or freeze-dried, sterile, non-pyrogenic preparation obtained from human plasma derived from donors belonging to the same ABO blood group. The preparation is thawed or reconstituted before use to give a solution for infusion.

The human plasma used complies with the monograph on Plasma for Fractionation.

Production

The units of plasma to be used are cooled to -30° or lower within 6 hours of separation of cells and always within 24 hours of collection.

The pool is prepared by mixing units of plasma belonging to the same ABO blood group.

The pool of plasma is tested for hepatitis B surface antigen (HBsAg) and for HIV antibodies using test methods of suitable sensitivity and specificity; the pool must give negative results in these tests.

Hepatitis A virus RNA

The plasma pool is tested using a validated nucleic acid amplification technique (2.8.1). A positive control with 100 IU of hepatitis A virus RNA per ml and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors. The pool complies with the test if it is found non-reactive for hepatitis A virus RNA.

Hepatitis C virus RNA

The plasma pool is tested using a validated nucleic acid amplification technique (2.8.1). A positive control with 100 IU of hepatitis C virus RNA per ml and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors. The pool complies with the test if it is found non-reactive for hepatitis C virus RNA.

Hepatitis C virus RNA for NAT testing reference preparation is suitable for use as a positive control.

To limit the potential burden of B19 virus in plasma pools, the plasma pool is also tested for B19 virus using a validated nucleic acid amplification technique (2.8.1).

B19 virus DNA

The plasma pool contains not more than 10.0 IU per µl.

A positive control with 10 IU of B19 virus DNA per µl and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors.

B19 virus DNA for NAT testing reference preparation is suitable for use as a positive control.

The method of preparation is designed to minimise activation of any coagulation factor (to minimise potential thrombogenicity) and includes a step or steps that have been shown to inactivate known agents of infection; if substances are used for the inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

Inactivation process

The solvent-detergent process, which is one of the methods used to inactivate enveloped viruses, uses treatment with a combination of *tributyl phosphate* and *octoxinol 10*; these reagents are subsequently removed by oil extraction or by solid phase extraction so that the amount in the final product is less than 2 µg per ml for *tributyl phosphate* and less than 5 µg per ml for *octoxinol 10*. No antimicrobial preservative is added.

The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen; it may subsequently be freeze-dried.

Plastic containers comply with the requirements for sterile plastic containers for human blood and blood components (6.4.1).

Glass containers comply with the requirements for glass containers for pharmaceutical use (6.2.1).

Description. The frozen preparation, after thawing, is a clear or slightly opalescent liquid free from solid and gelatinous particles. The freeze-dried preparation is an almost white or slightly yellow powder or friable solid.

Thaw or reconstitute the preparation under examination as stated on the label immediately before carrying out the Identification, Tests and Assay.

Identification

A. Examine by electrophoresis (2.4.12) comparing with normal human plasma. The electropherograms show the same bands.

B. It complies with the test for anti-A and anti-B haemagglutinins (see Tests).

Tests

pH (2.4.24). 6.5 to 7.6.

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Osmolality (2.4.23). Minimum 240 mosmol per kg.

Total protein. Minimum 4.5 per cent w/v.

Dilute with a 0.9 per cent w/v solution of *sodium chloride* to obtain a solution containing about 15 mg of protein in 2 ml. Place 2.0 ml of this solution in a round-bottomed centrifuge tube and add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid* and 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.3.30) and calculate the quantity of protein by multiplying the result by 6.25.

Activated coagulation factors (2.8.3). Complies with the test for activated coagulation factors. Carry out the test with 0.1 ml of the preparation under examination instead of 10-fold and 100-fold dilutions. The coagulation time for the preparation under examination is not less than 150 seconds.

Anti-A and anti-B haemagglutinins (2.8.13). The presence of haemagglutinins (anti-A or anti-B) corresponds to the blood group stated on the label.

Hepatitis A virus antibodies

Minimum 1 IU per ml, determined by a suitable immunochemical method (2.2.14).

Human hepatitis A immunoglobulin reference preparation is suitable for use as a reference preparation.

Irregular erythrocyte antibodies

The preparation under examination does not show the presence of irregular erythrocyte antibodies when examined without dilution by an indirect antiglobulin test.

Citrate. Maximum 25 mmol per litre.

Determine by liquid chromatography (2.4.14).

Test solution. Dilute the preparation under examination with an equal volume of a 0.9 per cent w/v solution of *sodium chloride*. Filter the solution using a filter with 0.45 µm pores.

Reference solution. Dissolve 0.3 g of *sodium citrate* in *water* and dilute to 100.0 ml with the same solvent.

Chromatographic system
- a stainless steel column 30 cm x 7.8 mm, packed with cation exchange resin (9 µm),
- mobile phase: 0.051 per cent w/v solution of *sulphuric acid*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 10 µl.

Equilibrate the column for about 15 minutes.

Inject the reference solution and the test solution. The retention time of citrate is about 10 minutes.

Calcium. Maximum 5.0 mmol per litre, determined by Meth A for atomic absorption spectrometry (2.4.2), measuring 622 nm and using calcium hollow-cathode lamp and transmission band preferably of 0.5 nm and the atomisa device as air-acetylene or acetylene-propane flame.

Potassium. Maximum 5.0 mmol per litre, determined by Meth A for atomic emission spectrometry (2.4.3), measuring 766.5 nm.

Sodium. Maximum 2.00 × 10^2 mmol per litre, determined Method A for atomic emission spectrometry (2.4.3), measur at 589 nm.

Water. Determine by semi-micro determination of wa (2.3.43), loss on drying (2.4.19) or near-infra spectrophotometry (2.4.6), the water content is within the lim approved by the competent authority (freeze-dried produc

Pyrogens (2.2.8) or **Bacterial endotoxins (BET)** (2.2.3). complies with the test for pyrogens or, preferably and wher justified and authorised, with a validated *in vitro* test such a the bacterial endotoxin test.

Pyrogens. Complies with the test for pyrogens. Inject 3 ml pe kg of the rabbit's mass.

BET. Where the bacterial endotoxin test is used, th preparation to be examined contains not more than 0. Endotoxin Unit per ml.

Sterility (2.2.11). Complies with the tests for sterility.

Assay

Factor VIII

Carry out the assay of human coagulation factor VIII (2.8.6 using a reference plasma calibrated against the Internationa Standard for blood coagulation factor VIII in plasma.

The estimated potency is not less than 0.5 IU per ml. Th confidence limits (P = 0.95) are not less than 80.0 per cent an not more than 120.0 per cent of the estimated potency.

Factor V

Carry out the assay of human coagulation factor V describe below using a reference plasma calibrated against th International Standard for blood coagulation factor V in plasma

Using *imidazole buffer solution pH 7.3*, prepare at leas 3 two-fold dilutions of the preparation under examination preferably in duplicate, from 1 in 10 to 1 in 40. Test each dilution as follows: mix 1 volume of *plasma substrate deficient in factor V*, 1 volume of the dilution under examination, 1 volume of *thromboplastin* and 1 volume of a 0.35 per cent w/v solution of *calcium chloride*; measure the coagulation times, i.e. the interval between the moment at which the calcium chloride solution is added and the 1st indication of the formation of fibrin, which may be observed visually or by means of a suitable apparatus.

In the same manner, determine the coagulation time of 4 two-fold dilutions (1 in 10 to 1 in 80) of human normal plasma in *imidazole buffer solution pH 7.3*.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods for a parallel-line assay.

The estimated potency is not less than 0.5 IU per ml. The confidence limits (P = 0.95) are not less than 80.0 per cent and not more than 120.0 per cent of the estimated potency.

Factor XI

Carry out the assay of human coagulation factor XI (2.8.14) using a reference plasma calibrated against the International Standard for blood coagulation factor XI in plasma.

The estimated potency is not less than 0.5 IU per ml. The confidence limits (P = 0.95) are not less than 80.0 per cent and not more than 125.0 per cent of the estimated potency.

Protein C. Carry out the assay of human protein C (2.8.15) using a reference plasma calibrated against the International Standard for human protein C in plasma.

The estimated potency is not less than 0.7 IU per ml. The confidence limits (P = 0.95) are not less than 80.0 per cent and not more than 120.0 per cent of the estimated potency.

Protein S. Carry out the assay of human protein S (2.8.16) using a reference plasma calibrated against the International Standard for human protein S in plasma.

The estimated potency is within the limits approved for the particular product. The confidence limits (P = 0.95) are not less than 80.0 per cent and not more than 120.0 per cent of the estimated potency.

Plasmin inhibitor (α_2 -antiplasmin)

Carry out the assay of human plasmin inhibitor (2.8.17) using a reference plasma calibrated against human normal plasma. 1 unit of factor V is equal to the activity of 1 ml of human normal plasma. Human normal plasma is prepared by pooling plasma units from not less than 30 donors and stored at -30° or lower.

The estimated potency is not less than 0.2 units per ml. The confidence limits (P = 0.95) are not less than 80.0 per cent and not more than 120.0 per cent of the estimated potency.

Labelling. The label states (1) the ABO blood group; (2) the method used for virus inactivation.

Platelet Concentrate

Platelets separated from whole blood within 4 to 6 hours of collection and suspended in 40 to 50 ml of plasma are designated as platelet concentrate.

Production

Platelet Concentrate is prepared from units of whole blood that have not been allowed to cool below 20°. Platelet rich plasma (PRP) is separated within 4-6 hours after completion of the phlebotomy. The final component should contain resuspended platelets in an amount of plasma adequate to maintain an acceptable pH generally 40 to 70 ml is used.

Tests

Four PC per month should be assayed for pH and for platelet, RBC and leucocyte counts.

PRP		
	Counts	3.0 - 4.5 x 10^5 per µl (approximately)
	Volume	170 - 250 ml (approximately)
	Total Count	9 x 10^{10} per bag (approximately)
PRC		
	Counts	8 - 10 x 10^5 per µl 350 ml bag (approximately)
		10 - 12 x 10^5 per µl 450 ml bag (approximately)
	Volume	40 - 50 ml per bag
Platelet counts		> 5.5 x 10^{10} per bag in 75 per cent of bags (if prepared from 450 ml bags)
		> 4.2 x 10^{10} per bag in 75 per cent of bags (if prepared from 350 ml bags)
Leucocyte count		< 0.12 x 10^9 per bag.
RBC counts		< 1.2 x 10^9 per bag.
pH		< 6.3 at the end of 5 days storage.

Expiration time. The expiration time is not more than 72 hours from the time of collection of the source material.

Storage. Store at 20° to 22° in polyvinylchloride plastic bags. Preserve at the temperature relevant to the volume of resuspension plasma, either between 20° and 22° or between 1° and 6°, the latter except during shipment, when the temperature may be between 1° and 10°.

Labelling. In addition to the labelling requirements of Whole Blood applicable to this product, label it to state the volume of original plasma present, the kind and volume of anticoagulant solution present in the original plasma, the blood group designation of the source blood, and the hour of expiration on the stated expiration date. Where labelled for storage at 20° to 22°, label it also to state that a continuous gentle agitation shall be maintained, or where labelled for storage at 1° to 6°, to state that such agitation is optional. Label it also with the type and result of a serologic test for syphilis, or to indicate that it

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was nonreactive in such test; with the type and result of a test for hepatitis B surface antigen, or to indicate that it was nonreactive in such test; with a warning that it is to be used as soon as possible but not more than 6 hours after entering the container; to state that a filter is to be used in the administration equipment; and to state that the instruction circular provided is to be consulted for directions for use.

Rabies Immunoglobulin

Human Rabies Immunoglobulin

Category. Immunological product.

Rabies Immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma from donors immunised against rabies. It contains specific antibodies neutralising the rabies virus. Human normal immunoglobulin may be added.

It complies with the monograph on Human Normal Immunoglobulin, except for the minimum number of donors and the minimum total protein content.

Tests

Potency. The potency is determined by comparing the dose of immunoglobulin required to neutralise the infectivity of a rabies virus suspension with the dose of a reference preparation, calibrated in International Units, required to produce the same degree of neutralisation (2.2.14). The test is performed in sensitive cell cultures and the presence of unneutralised virus is revealed by immunofluorescence.

The International Unit is the specific neutralising activity for rabies virus in a stated amount of the International Standard for anti-rabies immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Human rabies immunoglobulin reference preparation is calibrated in International Units by comparison with the International Standard.

Carry out the test in suitable sensitive cells. It is usual to use the BHK 21 cell line, grown in the medium described below, between the 18th and 30th passage levels counted from the ATCC seed lot. Harvest the cells after 2 to 4 days of growth, treat with *trypsin* and prepare a suspension containing 500,000 cells per ml (cell suspension). Ten minutes before using this suspension, add 10 µg of *diethylamino-ethyldextran* per ml if necessary, to increase the sensitivity of the cells.

Use a fixed virus strain grown in sensitive cells, such as the CVS strain of rabies virus adapted to growth in the BHK 21 cell line (seed virus suspension). Estimate the titre of the seed virus suspension as follows.

Prepare a series of dilutions of the viral suspension. In chambers of cell-culture slides (8 chambers per slide), pla 0.1 ml of each dilution and 0.1 ml of medium and add 0.2 ml the cell suspension. Incubate in an atmosphere of carb dioxide at 37° for 24 hours. Carry out fixati immunofluorescence staining and evaluation as descri below. Determine the end-point titre of the seed vi suspension and prepare the working virus dilut corresponding to 100 $CCID_{50}$ per 0.1 ml.

For each assay, check the amount of virus used by perform a control titration: from the dilution corresponding 100 $CCID_{50}$ per 0.1 ml, make three ten-fold dilutions. Add 0.1 of each dilution to four chambers containing 0.1 ml of medi and add 0.2 ml of the cell suspension. The test is not vali unless the titre lies between 30 $CCID_{50}$ and 300 $CCID_{50}$.

Dilute the reference preparation to a concentration of 2 IU per ml using non-supplemented culture medium (stock reference dilution, stored below -80°). Prepare two suitable predilutions (1:8 and 1:10) of the stock reference dilution so that the dilution of the reference preparation that reduces the number of fluorescent fields by 50 per cent lies within the four dilutions of the cell-culture slide. Add 0.1 ml of the medium to each chamber, except the first in each of two rows, to which ad respectively 0.2 ml of the two predilutions of the stoc reference dilution transferring successively 0.1 ml to the oth chambers.

Dilute the preparation under examination 1 in 100 using no supplemented medium (stock immunoglobulin dilution) to reduce to a minimum errors due to viscosity of the undiluted preparation and make three suitable predilutions so that the dilution of the preparation under examination that reduces the number of fluorescent fields by 50 per cent lies within the four dilutions of the cell-culture slide. Add 0.1 ml of the medium to all the chambers except the first in each of three rows, to which add respectively 0.2 ml of the three predilutions of the stock immunoglobulin dilution. Prepare a series of two-fold dilutions transferring successively 0.1 ml to the other chambers.

To all the chambers containing the dilutions of the reference preparation and the dilutions of the preparation under examination, add 0.1 ml of the virus suspension corresponding to 100 $CCID_{50}$ per 0.1 ml (working virus dilution), shake manually, allow to stand in an atmosphere of carbon dioxide at 37° for 90 minutes, add 0.2 ml of the cell suspension, shake manually and allow to stand in an atmosphere of carbon dioxide at 37° for 24 hours.

After 24 hours, discard the medium and remove the plastic lids. Wash the cell monolayer with *phosphate buffered saline pH 7.4* and then with a mixture of 20 volumes of *water* and 80 volumes of *acetone* and fix in a mixture of 20 volumes of *water* and 80 volumes of *acetone* at -20° for 3 minutes. Spread on the slides *fluorescein-conjugated rabies antiserum* ready for use. Allow to stand in an atmosphere with a high level of moisture at 37° for 30 minutes. Wash with *phosphate buffered saline pH 7.4* and dry. Examine twenty fields in each chamber at a magnification of 250, using a microscope equipped for fluorescence readings. Note the number of fields with at least one fluorescent cell. Check the test dose used in the virus titration slide and determine the dilution of the reference preparation and the dilution of the preparation under examination that reduce the number of fluorescent fields by 50 per cent, calculating the two or three dilutions together using probit analysis. The test is not valid unless the statistical analysis shows a significant slope of the dose-response curve and no evidence of deviation from linearity or parallelism.

The stated potency is not less than 150 Units per ml. The estimated potency is not less than the stated potency and is not greater than twice the stated potency. The confidence limits (P = 0.95) of the estimated potency are not less than 80.0 per cent and not more than 125.0 per cent.

Culture Medium for Growth of BHK 21 Cells

Commercially available media that have a slightly different composition from that shown below may also be used.

Sodium chloride	6.4 g
Potassium chloride	0.40 g
Calcium chloride, anhydrous	0.20 g
Magnesium sulphate, heptahydrate	0.20 g
Sodium dihydrogen phosphate, monohydrate	0.124 g
Glucose monohydrate	4.5 g
Ferric nitrate, nonahydrate	0.10 mg
L-Arginine hydrochloride	42.0 mg
L-Cystine	24.0 mg
L-Histidine	16.0 mg
L-Isoleucine	52.0 mg
L-Leucine	52.0 mg
L-Lysine hydrochloride	74.0 mg
L-Phenylalanine	33.0 mg
L-Threonine	48.0 mg
L-Tryptophan	8.0 mg
L-Tyrosine	36.0 mg
L-Valine	47.0 mg
L-Methionine	15.0 mg
L-Glutamine	0.292 g
i-Inositol	3.60 mg
Choline chloride	2.0 mg
Folic acid	2.0 mg
Nicotinamide	2.0 mg
Calcium pantothenate	2.0 mg
Pyridoxal hydrochloride	2.0 mg
Thiamine hydrochloride	2.0 mg
Riboflavin	0.2 mg
Phenol red	15.0 mg
Sodium hydrogen carbonate	2.75 g
Water to	1000 ml

The medium is supplemented with:

Foetal calf serum (heated at 56° for 30 minutes)	10 per cent
Tryptose phosphate broth	10 per cent
Benzylpenicillin sodium	60 mg/l
Streptomycin	0.1 g/l

Storage. Store protected from light, the liquid preparations in sealed, colourless glass containers and the freeze-dried preparation in an airtight colourless glass container.

Labelling. The label states (1) for liquid preparations, the volume of the preparation in the container and the protein content expressed in g per litre; (2) for freeze-dried preparations, the quantity of protein in the container; (3) the route of administration; (4) for freeze-dried preparations, the name or composition and the volume of the reconstituting liquid to be added; (5) where applicable, that the preparation is suitable for use in the prophylaxis of hepatitis A infection; (6) where applicable, the anti-hepatitis A virus activity in International Units per ml; (7) where applicable, the name and amount of antimicrobial preservative in the preparation; (8) the number of International Units per container.

Tetanus Immunoglobulin

Human Tetanus Immunoglobulin

Category. Immunological product.

Tetanus Immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma containing specific antibodies against the toxin of *Clostridium tetani*. Human normal immunoglobulin may be added.

It complies with the monograph on Human Normal Immunoglobulin, except for the minimum number of donors and the minimum total protein content.

Production

During development, a satisfactory relationship shall be established between the potency determined by immunoassay

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as described under Potency and that determined by means of the following test for toxin-neutralising capacity in mice.

Toxin-neutralising capacity in mice. The potency is determined by comparing the quantity necessary to protect mice against the paralytic effects of a fixed quantity of tetanus toxin with the quantity of a reference preparation of human tetanus immunoglobulin, calibrated in International Units, necessary to give the same protection.

The International Unit of antitoxin is the specific neutralising activity for tetanus toxin contained in a stated amount of the International Standard, which consists of freeze-dried human immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Human tetanus immunoglobulin reference preparation is calibrated in International Units by comparison with the International Standard.

Selection of animals. Use mice weighing 16g to 20g.

Preparation of the test toxin. Prepare the test toxin by a suitable method from the sterile filtrate of a culture in liquid medium of *C. tetani*. The two methods shown below are given as examples and any other suitable method may be used.

1. To the filtrate of an approximately 9-day culture, add 1 to 2 volumes of *glycerol* and store the mixture in the liquid state at a temperature slightly below 0°.
2. Precipitate the toxin by addition to the filtrate of *ammonium sulphate*, dry the precipitate *in vacuo* over *diphosphorus pentoxide*, reduce to a powder and store dry, either in sealed ampoules or *in vacuo* over *diphosphorus pentoxide*.

Determination of test dose of toxin (Lp/10 dose). Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.5 IU of antitoxin per ml. If the test toxin is stored dry, reconstitute it using a suitable liquid. Prepare mixtures of the solution of the reference preparation and the test toxin such that each contains 2.0 ml of the solution of the reference preparation, one of a graded series of volumes of the test toxin and sufficient of a suitable liquid to bring the volume to 5.0 ml. Allow the mixtures to stand, protected from light, for 60 minutes. Using six mice for each mixture, inject a dose of 0.5 ml subcutaneously into each mouse. Observe the mice for 96 hours. Mice that become paralysed may be euthanised. The test dose of toxin is the quantity in 0.5 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, paralysis in all six mice injected with the mixture, within the observation period.

Determination of potency of the immunoglobulin. Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.5 IU of antitoxin per ml. Prepare a solution of the test toxin in a suitable liquid such that it contains five lethal doses per ml. Prepare mixtures of the solution of the test toxin and the immunoglobulin under examination such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the immunoglobulin under examination and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 ml) that contains 1 IU and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand, protected from light, for 60 minutes. Using six mice for each mixture, inject subcutaneously a dose of 0.5 ml into each mouse. Observe the mice for 96 hours. Mice that become paralysed may be euthanised. The mixture that contains the largest volume of immunoglobulin that fails to protect the mice from paralysis contains 1 IU. This quantity is used to calculate the potency of the immunoglobulin in IU per ml.

The test is not valid unless all the mice injected with mixtures containing 2.0 ml or less of the solution of the reference preparation show paralysis and all those injected with mixtures containing more do not.

Tests

Potency. The potency is determined by comparing the antibody titre of the immunoglobulin under examination with that of a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (2.2.14).

The International Unit is the activity contained in a stated amount of the International Standard for anti-tetanus immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Human tetanus immunoglobulin reference preparation is calibrated in International Units by comparison with the International Standard.

The stated potency is not less than 100 Units of tetanus antitoxin per ml. The estimated potency is not less than the stated potency. The confidence limits (P = 0.95) of the estimated potency are not less than 80.0 per cent and not more than 125.0 per cent.

Storage. Store protected from light, the liquid preparations in sealed, colourless glass containers and the freeze-dried preparation in an airtight colourless glass container.

Labelling. The label states (1) for liquid preparations, the volume of the preparation in the container and the protein content expressed in g per litre; (2) for freeze-dried preparations, the quantity of protein in the container; (3) the route of administration; (4) for freeze-dried preparations, the name or composition and the volume of the reconstituting liquid to be added; (5) where applicable, that the preparation is suitable for use in the prophylaxis of hepatitis A infection; (6) where applicable, the anti-hepatitis A virus activity in International Units per ml; (7) where applicable, the name and amount of antimicrobial preservative in the preparation; (8) the number of International Units per container.

Whole Human Blood

Whole Blood (Human)

Whole Human Blood is blood drawn aseptically from selected human donors and mixed with a suitable anticoagulant.

Whole Human Blood is the final mixture of blood and anticoagulant solution contains not less than 9.7 per cent w/v of haemoglobin, calculated from the haemoglobin content of the donor's blood and the dilution due to the anticoagulant solution. It is obtained from healthy donors who must:

(a) be in the age range of 18 to 60 years and be in good health as indicated in part by normal temperature and blood pressure within normal limits;

(b) not be pregnant, if females;

(c) not have undergone major surgery within 6 months of donation;

(d) as far as can be ascertained after clinical and laboratory examination and the study of medical history of the donor be free from disease transmissible by blood transfusion;

(e) have blood containing not less than 12.5 per cent w/v of haemoglobin;

(f) be free from acute respiratory diseases;

(g) be free from any infectious skin disease at the site of phlebotomy;

(h) have no history of malarial fever within 12 months of donation;

(i) have no history of viral hepatitis or of close contact with an individual having viral hepatitis within 12 months of donation and have blood that has given negative results in tests for the presence of hepatitis-B antigen;

(j) have blood that has been tested with negative results for evidence of syphilitic infection, HCV antibodies, HIV antibodies and malarial parasites.

The examinations and tests to be carried out are decided by the National Regulatory Authority.

The frequency of donations of whole blood shall not exceed once every 3 months with a maximum volume of 1.5 litres in any consecutive 12 month period.

The blood is drawn aseptically through a closed system into a suitable sterile container containing a specific amount of Anticoagulant Citrate Dextrose Solution (ACD Solution) or Anticoagulant Citrate Phosphate Dextrose Solution (CPD Solution) which is placed before the container is sterilised. The quantity of anticoagulant solution should not exceed 22.0 per cent v/v of the final volume of the mixture. No antimicrobial preservative is added.

During the withdrawal of blood the container is gently agitated to ensure thorough mixing. When withdrawal is complete the container is immediately sealed and cooled to 2° to 8°. It is not opened until immediately before transfusion. With every container of blood, a separate sample mixed with the appropriate quantity of anticoagulant solution, is collected for compatibility and other tests; this small container is firmly attached to the main container.

Whole Human Blood in containers from which samples have been removed for tests should not be used for transfusion. Consequently, it is not intended that the Tests and the Assay should be carried out on the contents of the container. The Blood Bank or the service collecting the blood is responsible for ensuring that the conditions in which the blood is collected and stored are such that, if and when tested, the blood will comply with the requirements of the monograph.

Blood group. Determine the blood group (in the sample accompanying each donation) under the ABO system (2.8.10), by examination of both corpuscles and serum, and under the Rh system (2.8.10), by examination of the corpuscles.

Description. Deep red fluid which, on standing separates into a lower layer of sedimented-red cells and a yellowish, almost clear upper layer of plasma, free from visible signs of haemolysis, with a greyish layer between the two consisting of leucocytes and thrombocytes. A layer containing emulsified fat may form on the surface.

Tests

Sterility (2.2.11). Complies with the tests for sterility, determined by Method B.

Assay. Determine the haemoglobin content by photometric haemoglobinometry (2.8.11).

Storage. Store in colourless, transparent and sterile containers into which it was originally drawn. The containers should be provided with a hermetic, contamination-proof closure. Store at a temperature between 2° to 8°.

Labelling. The label states (1) the distinctive code number by reference to which the details of the donor are available;

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(2) the ABO group with the approved colour scheme for different groups as specified by the National Regulatory Authority; (3) the Rh group; (4) the total volume of fluid, the proportion of blood, and the nature and volume of anticoagulant solution; (5) the date on which the blood was withdrawn; (6) the expiry date which should not exceed 21 days from the date of withdrawal of blood; (7) the storage conditions; (8) that the blood must not be used for transfusion if there is any visible evidence of haemolysis or deterioration; (9) for blood of group O whether haemol are present or not and if they are, that the blood mu administered only to recipients of blood group O; (10) th blood has given negative results in the tests for the pre of malarial parasites, hepatitis-B antigen, syphilis and antibodies and any other tests prescribed by the Nat Regulatory Authority.

BIOTECHNOLOGY DERIVED THERAPEUTIC PRODUCTS

General Monograph

Biotechnology Derived Therapeutic Products 4567

Therapeutic Monoclonal Antibodies for Human Use 4576

Monographs

Calcitonin (Salmon) 4584

Calcitonin (Salmon) Injection 4586

Erythropoietin Concentrated Solution 4588

Erythropoietin for Injection 4594

Erythropoietin Injection 4597

Filgrastim Concentrated Solution 4601

Filgrastim Injection 4605

Follicle Stimulating Hormone 4607

Follicle Stimulating Hormone Concentrated Solution 4615

Follicle Stimulating Hormone Injection 4623

Human Insulin 4626

Insulin Injection 4628

Insulin Aspart 4628

Insulin Aspart Injection 4630

Insulin Lispro 4632

Insulin Lispro Injection 4634

Biphasic Insulin Aspart Injection 4636

Biphasic Insulin Lispro Injection 4638

Biphasic Isophane Insulin Injection 4640

Isophane Insulin Injection 4642

Insulin Glargine 4642

Insulin Glargine Injection 4644

Interferon Alfa-2 Concentrated Solution 4646

Interferon Alfa-2a Injection 4649

Interferon Alfa-2b Injection 4651

Interferon Beta-1a Concentrated Solution 4653

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Interferon Beta-1a Injection 4658
Pegfilgrastim 4662
Recombinant Streptokinase Bulk Solution 4667
Recombinant Streptokinase for Injection 4668
Rituximab 4669
Rituximab Injection 4676
Somatropin 4682
Somatropin Concentrated Solution 4684
Somatropin for Injection 4687
Teriparatide 4690
Teriparatide Concentrated Solution 4696
Teriparatide Injection 4699

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Biotechnology Derived Therapeutic Products

Biotechnology has brought about the discovery and development of a new class of human therapeutics. Advances in genetics, cellular and molecular biology have allowed scientists to identify and develop a number of new products. These products provide significant clinical benefits, and in many cases, provide therapies where no effective treatment existed.

Proteins are the workhorse molecules of the cell. Due to their biological significance, proteins can make excellent drug candidates. In the living cell, genes, in the form of DNA, provide the basic instructions in creating biologically functional proteins. Biotechnology is the process that produces protein molecules in sufficient quantities for therapeutic use, which have very specific physiological roles that already exist in the human body *albeit* in limited quantities. Techniques of genetic engineering using bacteria, yeast and eukaryotic cells in culture or cultured animal cells are employed to incorporate in these cells the information needed to produce a human protein with therapeutic potential. Once engineered, these cells can be grown in large quantities by fermentation or large-scale cultivation of animal or human cells. The addition of genetic material to cells gives the engineered proteins their name, recombinant proteins.

Gene Cloning and Protein Expression

The basic principle involved in gene cloning includes the insertion of a select fragment of DNA representing the gene for a peptide/protein into an episomal circular DNA, called the vector, which transports the gene into a host cell. The vehicle containing the inserted gene then replicates along with the host cell. The cellular machinery of the host cell transcribes and translates the genes including the inserted gene into proteins. The vector with an inserted foreign gene is called a *recombinant DNA molecule* and the protein produced as a consequence of the inserted gene by the host cell is called *recombinant Protein*. The commonly used vectors for bacterial transformations are *Plasmids*, which are small circular DNAs found in bacteria and other microorganisms. Other vectors include viruses such as bacteriophages (for bacterial hosts) or animal viruses for eukaryotic host cells (e.g., Epstein-Barr virus, Simian Virus etc). Major advances have been made in the past decade for efficient and stable expression of foreign proteins in mammalian cells in culture.

In addition, transgenic animals and plants have been produced which express foreign genes inserted into their genome for tissue/organ specific expression. Although large-scale production of therapeutic proteins has been accomplished, the current edition of the Indian Pharmacopoeia does not contain products produced by this process and hence no further discussion on this aspect is included in this chapter.

Monoclonal Antibodies

Antibodies are proteins produced by differentiated B-lymphocytes. Kohler and Milstein first fused a normally short-lived antibody producing B-cell obtained from the spleen of mice immunized with an antigen to a rapidly dividing cancer cell (mouse myeloma) to produce a cell line (hybridoma), which was both immortal and a producer of antibodies that recognized a single epitope on an antigen, hence, the name monoclonal antibodies (mAbs). Monoclonal antibodies can be produced in cell culture or in live animals. When the hybridoma cells are injected into the peritoneal cavity of mice, they produce tumors secreting an antibody-rich fluid called ascites fluid. Production in cell culture is usually preferred since cell culture in fermentation chambers can be used to produce antibodies on a larger scale.

Humanized monoclonal antibodies

The standard procedure of producing monoclonal antibodies yields mouse antibodies. For therapeutic applications in humans these proteins are unsuitable since the human immune system recognizes mouse antibodies as foreign, causing systemic inflammatory effects and are removed rapidly from circulation. Application of recombinant DNA technology has overcome this problem. The DNA that encodes the binding portion of monoclonal mouse antibodies is ligated to the human antibody producing DNA. Such a construct is then cloned suitably in a vector and introduced into a mammalian expression system to produce these half-mouse and half-human antibodies. Depending on how big a part of the mouse antibody is used, such proteins are either referred to as Chimeric or humanized monoclonal antibodies. Monoclonal antibodies have been generated and approved to treat cancer, cardio-vascular disease, inflamatory diseases, muscular degeneration, transplant rejection, viral infection and others.

Therapeutic recombinant monoclonal antibodies

Recombinant monoclonal antibodies for therapeutic applications belong to the IgG class of immunoglobulins. Immunoglobulins are made up of four polypeptide chains, two light (either derived from λ and k light chain antibody class) and two heavy chains (derived from one of the α, β, γ, ε, μ heavy chain antibody class, and the α class is further classified into subclass 1 and 2, and the γ is subclassed as 1, 2, 3 and 4). The type of heavy chain determines the immunoglobulin isotype (IgA, IgD, IgG, IgE and IgM respectively). The four-polypeptide chains are cross-linked by disulfide bridges (-S-S- bonds). Light chains comprise approximately 220 amino acid residues whereas heavy chains are made of 440-550 amino acids. Each chain has two distinct regions namely, "constant" and "variable", the former being

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proximal to the C-terminal region (111-220 or 440-550 residues for light chain and heavy chain respectively) whereas the latter is towards the N- terminal sequence of the polypeptide (1-110) for light chain as well as heavy chain. The amino acids of the constant region of immunoglobulin are uniform from one antibody to another within the same isotype (e.g., IgG), while the variable region, found in both the light and heavy chains, consist of different amino acids. These regions are referred to as hypervariable region or "Complementary Determining Regions (CDRs)" representing antibody diversity, each specific for the recognition of the antigen. An antibody molecule is generally represented as a "Y" shaped structure. The arms of the Y structure are connected to the stem region by a flexible hinge. IgG can be proteolytically cleaved by partial digestion with papain, which results in three distinct ~50 KD fragments: two identical fragments designated Fab fragments and one Fc fragment. The Fab fragments are the arms of the antibody containing the entire light (L) chain and the N-terminal half of the Heavy (H) chain. These fragments contain the IgG's antigen binding domains. The Fc fragment derives from the stem portion of the Y structure and contains the c-terminal halves of the two H chains. The Fc region is responsible for the activation of phagocytosis, complement dependent cellular toxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC).

The IgG-Fc region is glycosylated at Asn 297, which is essential for the conformation of the antibody molecule that allows interaction with effector ligands. The IgG-Fc glycoform pattern of monoclonal chimeric and "humanized" recombinant therapeutic antibodies produced in CHO or NSO cells show wide variation from clone to clone, which is dependent on the process and cell culture conditions. Under certain conditions a number of abnormally glycosylated products that lack potency and potentially immunogenic products are produced (e.g., galactose-α (1,3)-galactose and N-glycolylneuraminic acid) which are not therapeutically acceptable. However, successive rounds of selection of cell lines overcome these problems. Another important variable in efficacy due to glycosylation is the ability of the IgG molecule to mediate ADCC. This property is particularly important in antibody therapeutics for oncology indications. The ability to elicit ADCC greatly varies with different glycoforms. Therefore, the cell lines have been engineered by the introduction of a glycosyl transferase (GnTIII) and/or inactivation/deletion of fucosyl transferase in the CHO cells, which has resulted in products with 20 to 100-fold increase in ADCC.

Processes for the production of recombinant therapeutic proteins including mAbs

Prokaryotes

The qualitative and quantitative demand for recombinant proteins is steadily increasing. Molecular biologists are constantly challenged by the need to improve and optimize the existing expression systems to meet the steadily increasing qualitative and quantitative demand for recombinant proteins. The continuous evolution of novel expression systems is paralleled by growing concerns about the safety of these novel pharmaceuticals, which has resulted in the regulatory authorities setting high standards for certification. One of the strategies used by researchers in this field involves sourcing new genetic elements for incorporation into expression systems by systematically analyzing the rich natural diversity of microorganisms. There are, in addition, numerous tools for modifying microorganisms and for re-engineering existing biological pathways or processes to meet the needs of the pharmaceutical industry. Although many prokaryotic hosts are described in literature for the production of recombinant proteins, *E.coli* is the most preferred host because its genetics are well understood. The popular cloning host has been *E.coli* BL21 and its derivative BL 21λDE3. However there are some disadvantages such as the recombinant protein produced may not have a proper secondary structure due to the non-formation of intra-molecular disulfide bonds. Many recombinant proteins tend to aggregate when over expressed in *E.coli*. *E.coli* proteins begin their sequence with an N-formyl methionine, which may not be removed by its enzyme systems. Proteolytic enzymes of *E.coli* may partially degrade the recombinant protein.

The principal element in recombinant protein expression technology is the recombinant plasmid, which contains the gene that codes for the protein of interest. The gene expression regulatory elements such as the promoter sequence in the plasmid play a key role in the production of mRNA of the recombinant gene. The other important regulatory process employed in gene expression is the application of gene induction. Induction is the phenomenon wherein the transcription of the gene is turned on by the addition of a chemical to the growth medium. The most popular system used in academic research is the *lac* promoter, which is induced by Isopropyl thiogalactopyranoside (IPTG). However, in the industrial production of recombinant therapeutic proteins, addition of chemical inducers is not desired, hence auto-inducible promoters which are up-regulated upon changes in the culture medium or the growth phase of the bacteria or the regulation of cell cycle, are employed for improvement in production efficiency. Overproduction of proteins most often results in misfolded structures, which are insoluble and accumulate in the cytoplasm as "inclusion bodies". Cloning and expressing a group of proteins collectively called "Chaperones" overcome this problem. Some of the chaperones whose mode of action is partially understood include, Gro EL/ Gro ES, DnaK, DnaJ and GrpE. Fusion of the gene of interest with a partner gene results in a recombinant protein, which produces both gene products linked in tandem. Such fusions

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also increase expression levels as well as provide a means for a convenient process of purification by affinity chromatography. A number of fusion partner genes are reported in literature but the most common partner proteins are maltose binding protein, glutathione-S-transferase and thioredoxin. For ease of recombinant protein purification, short sequence tags at N- or C-terminal are genetically engineered which result in recombinant protein products with additional amino acid sequences. These additional sequences have to be cleaved from the final therapeutic product. Several methods for precise excision of tag sequences have been described which include enzymatic and chemical processes. Another strategy employed for enhancing protein expression is the use of "bicistronic" vectors. In this strategy, a short sequence of a gene efficiently expressed is linked as a non-translatable transcriptional fusion to the gene of interest. Secretion of recombinant proteins or their translocation to periplasmic space of bacteria is a procedure employed in the expression of foreign proteins in prokaryotes. This procedure requires the addition of signal sequences from secretory pathways or leader sequences for transport to periplamic space. *OmpA* and *PelB* signal peptides have been shown to efficiently transport recombinant proteins to bacterial periplasm. A good example is the production of correctly folded full-length antibodies within the periplasm of *E. coli*.

Eukaryotes

Yeasts

Similar to prokaryotes in growth kinetics achieving high density, yeasts secrete large amounts of recombinant proteins. *Saccharomyces sp, Pichia pastoris, Hansenula polymorpha,* and *Kluyveromyces lactis* are some of the yeasts, which are currently in use for the production of therapeutic recombinant proteins. Yeasts are the preferred eukaryotes for the production of recombinant therapeutic proteins that cannot be produced from prokaryotes because of incorrect folding or the requirement for glycosylation. *Sacharomyces cerevisiae* is genetically well characterized, its growth physiology well understood and hence, it is the prevalent yeast species in pharmaceutical production process. However, *Pichia pastoris* is also gaining ground as a preferred host for heterologous protein expression because of its superior secretion efficiency and high yields. *Pichia pastoris* and *Hansenula polymorpha* are methanol metabolizing yeasts (methylotrophic yeasts), which contain strong promoters for the genes of methanol assimilating enzymes such as methanol oxidase and alcohol oxidase. As the enzymes of these pathways account for more than 30 per cent of the total protein content of the organism, the metabolic efficiency with respect to secreted protein is significantly higher. Although attempts are being made to humanize N-linked glycosylation in the yeast *Pichia pastoris,* the employability of yeasts for the expression of human therapeutic proteins might reach a limit if the product has a highly specific post-translational modification for its pharmacological activity/properties. In such cases, mammalian cell expression systems are employed for the production of therapeutic proteins.

Mammalian Cell Lines

Animal Cell expression systems produce proteins, which have high degree of similarity to human proteins with respect to the pattern and capacity of post translational modifications. The most commonly used cell lines for the expression of therapeutic proteins are as follows:

Chinese Hamster Ovary (CHO) Cell Line

Baby Hamster Kidney (BHK) Cell Line

HEK Human Embryo Kidney (HEK) Cell Line

Human Retinal Embryonic Cell Line (PER C.6)

Mouse Myeloma Cell Line (NSO)

Mammalian cell lines produce therapeutic proteins with proper folding, assembly and post-translational modifications, which results in pharmacologically active products in terms of quality and reproducibility. However, there remains a possibility of transmission of viral or pyrogenic substances carried over in the final product. There is a trend now to use cell lines, which are of human origin since they possess all mechanisms to produce products most similar if not identical to naturally occurring proteins in the human body.

Immortalized cell lines transfected with foreign therapeutic protein genes are used for the production of the respective proteins. There are basically two processes of immortalizing mammalian cells; (1) *Viral transformation*: Induction of immortalization by inactivating the tumor suppressor genes of mammalian cells. Viral genes including Epstein - Barr virus (EBV), Simian Virus 40 (SV 40) T antigen, adenovirus and human papillomavirus (HPV) can induce immortalization. Although the method is quite reliable, there are reports of aneuploidy and loss of some properties of the primary cells. (2) *Modification of telomere of somatic cells*. Telomerase reverse transcriptase protein (TERT), which is inactive in most somatic cells, when exogenously expressed, the cells are able to maintain telomere lengths sufficient to avoid replicative senescence thus, rendering the cells immortal. The latter method is preferred for therapeutic protein expression. Analysis of several telomere-immortalized cell lines has verified that the cells maintain a stable genotype and retain critical phenotype markers.

Development of a cell line stably expressing a desired protein gene starts with the construction of expression vectors. Different expression vectors both viral and non-viral have been designed to transfer foreign genes into mammalian cells. The choice of the vector is dependent on the host cell, the

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level of the desired product formed and safety. Typically an expression vector consists of (i) a constitutive or inducible promoter (ii) a transcription terminator and (iii) a cassette with an origin of replication and a selection marker for vector production in bacteria. Strong constitutive promoters are of viral genomes (e.g., human cytomegalovirus (HCMV), simian virus 40 and Rous sarcoma virus). Amongst the non-viral promoters the promoter of human elongation factor-1-α (EF-1α) is similar in strength to the HCMV promoter. The desired therapeutic protein gene is isolated as a cDNA, with one intron sequence, which is preferably located between the promoter and the coding sequence of the gene of interest and inserted into the vector at a suitable site. The vector containing the gene insert is then propogated in the bacterial host. The vector is then recovered from the bacteria, linearized and introduced into the mammalian cell by transfection. There are many reagents/methods to effectively mediate gene transfer. Calcium phosphate facilitated transfection, electroporation, lipofection, micro injection, biolistic and polymer mediated transfer are routinely used. For selection of recombinant cell, a second gene is transferred that confers to the recipient cells a selective advantage. The most common selector genes are dihydrofolate reductase (DHFR) and glutamine synthetase (GS). In both cases, selection occurs in the absence of appropriate metabolite (hypoxanthine and thymidine in the case of DHFR and glutamine in the case of GS), thus preventing the growth of non-transformed cells. When the transfected foreign gene is integrated into a "good site" on the chromosome of the host by recombination, the resultant cell line will stably express the desired gene. Selection pressure results in several fold amplification of genes including the integrated genes when high concentrations of respective inhibitors are used (for. e.g., Methotrexate inhibition of DHFR and Methionine sulphoximine inhibition of GS).

There are two main ways of culturing animal cells for the production of recombinant proteins, viz., (a) Adherent cell culture and (b) Suspension culture. In adherent cell culture technology the mammalian cells (e.g., CHO cells) are seeded into roller bottles that are filled to about 25 per cent capacity with the medium and slowly rotated allowing cells to adhere. The rotation provides the continuous contact of the cells with the medium and oxygen is supplied by the "head space" in the bottle. Following attainment of confluency, the product is harvested from the decanted supernatant. The suspension culture method is more widely used for the mass production of recombinant proteins, where the capacity of the mammalian cells for single cell suspension growth is exploited for scalability to very large volumes. The transition of adherent cells to suspension cultivation requires a selection of media formulations. The seeding inoculum begins with a small volume of cell suspension, which is gradually expanded so that sufficient cell numbers are generated for the final production phase. In the suspension culture process the changing composition of the cell medium during the production phase can affect the quality of the product due to degradation enzymes released by the cells as well as the molecular composition of the product due to limitations of nutrients.

General Techniques used in down-stream processing of recombinant proteins

The design of the purification process of a recombinant protein and its scale-up capabilities assumes significant importance since the regulatory authorities would approve the "product by process". Thus, the approved process must be followed even if new developments in separation technologies provide significant advantages.

Liquid chromatography is the core of preparative protein purification and all supplementary procedures like extraction, centrifugation, ultrafiltration, and dialysis serve to prepare the protein solution for chromatography. A series of chromatographic steps usually termed as capture, intermediate purification and polishing, make use of different intrinsic features of proteins, is usually required to achieve sufficient separation of the target from contaminants. Common modes of chromatography include ion exchange chromatography, affinity chromatography, hydrophobic interaction chromatography, gel filtration and reverse phase chromatography. The selection of chromatographic procedures and their arrangements in a suitable order are based on the properties of the recombinant protein particularly its tolerance to organic solvents, its susceptibility to denaturation due to changes in pH, temperature, degradation and oxidation.

In the case of secreted recombinant proteins, downstream processing starts with the collection of the fermentation supernatant after separating the cell mass while if the recombinant protein is intracellular, the process begins with the extraction of soluble proteins from the cell mass. In the former case the target protein may be highly dilute with large amounts of inorganic salts and media proteins such as fetal calf serum, yeast extract and other growth promoting substances, which may limit the choice of the capture step. In the case of the target protein expressed as an intracellular protein, the process of extraction from the cell mass is employed, which leads to a highly complex mixture of recombinant and host biomolecules including host proteins, nucleic acids, lipids, carbohydrates and other cell components. In addition to commonly employed technologies such as continuous centrifugation, tangential flow filtration and fast flow chromatography two novel techniques viz.; affinity tags and expanded bed adsorption techniques are gaining importance in the purification protocols for recombinant proteins. Affinity tags are short peptide sequences genetically fused to a target protein. A peptide of six consecutive histidine residues (His-6-tag) is the most widely used tag peptide, which is fused either at the N- or C-terminal of the protein of interest. This tag facilitates the purification of the protein by immobilized metal ion (generally Nickel) affinity column (IMAC). For this technique, a metal chelating ligand, e.g., iminodiacetic acid or nitrilotriacetic acid is immobilized on a chromatographic matrix and charged with transition metal ions leaving one or more coordinating sites free for interaction with the analyte. Proteins with the His-6-tag will bind to the ligand tightly to allow quantitative capture of the target molecule from a complex feed stream. The bound target protein is recovered by step-gradient elution using imidazole or by acidification. A potential problem is the presence of leached metal ions in the eluate, which must be removed in a subsequent step of purification. His tag facilitated purification process has yielded therapeutic recombinant proteins without affecting the folding, bioactivity and biodistribution profiles.

Expanded bed adsorption (EBA) is another novel technique, which is employed for preparative protein purification when large volumes of particulate raw materials are involved in the initial purification step. EBA combines the advantages of batch- or fluidized bed methods with superior adsorption characteristics of packed bed columns by a novel design of matrix particles. With the exception of gel filtration, all modes of biochromatography are compatible for EBA. However, ion-exchange media are most extensively used.

Post-Translational Modifications of Proteins

The primary structure of proteins, which is the linear sequence of amino acids, is encoded by the gene sequence of the cell. However, proteins undergo a variety of modifications subsequent to their synthesis. Enzymes within the cell mediate these modifications and such modifications are attributed to certain properties, such as solubility, transportability, resistance to proteolysis, folding, receptor binding, signal transduction etc., in the native milieu of the cell. However, with the advent of production of proteins for therapeutic applications in heterologous expression systems, post-translational modifications (PTMs) have assumed special significance from the point of view of setting specifications and quality assurance. Proteins undergo a broad range of PTMs during production in the host systems and undergo further changes in bio-physiochemical and structural properties, during its processing, handling and storage; the most common of them are listed below.

i) Acylation
ii) Phosphorylation
iii) Sulphonation
iv) Proteolysis (terminal or domain deletion)
v) Glycosylation (N-linked, O-linked)
vi) Aggregation
vii) High order structural change (conformational change or denaturation)
viii) Amidation or deamidation
ix) Carbamylation
x) Carboxylation
xi) Formylation
xii) Formation of gamma carboxyglutamic acid
xiii) Methylation
xiv) Succinimide forms
xv) Aspartate isomerization
xvi) Disulfide linkage
xvii) Oxidation

Glycosylation represents the most important PTM since a great diversity exists among different expression systems. For example, prokaryotic expression systems result in aglycosylated proteins often resulting in insoluble inclusion bodies. Yeasts on the other hand, add carbohydrate side chains of high mannose content, CHO and murine cells can also add sugars not normally found in human proteins some of them are known to be immunogenic.

Posttranslational modifications of recombinant therapeutic proteins are significant in terms of potency, stability and toxicity including immunogenecity of the biopharmaceutical product/s. This emphasizes the need for inclusion in the pharmacopoeial monographs, meticulous description of specifications and rigorous analytical data to characterize each biopharmaceutical product.

The mere demonstration that variations in the post translational modifications are found in recombinant glycoproteins produced in different expression systems, does not automatically negate the biological efficacy or the safety of the product. In many instances it has been clearly demonstrated through clinical trials that the products are safe and efficacious (e.g. recombinant factor VIII produced by BHK cells and CHO cells have different glycosylation profiles but both are safe and efficacious). Therefore, comparability of the same protein produced by different processes has to be governed by a pragmatic approach. Recently, the secretory pathway of *Pichia pastoris* has been genetically re-engineered to perform sequential glycosylation reactions that mimic early processing of N-glycans in humans.

Glycosylation of Proteins

More than one third of the biopharmaceticals currently in clinical use have an absolute requirement for glycosylation and hence they are produced in eukaryote expression systems. Oligosaccharides are attached co-translationally through specific aspargine (N-linked) or serine or threonine residues (O-linked). While the consensus sequence for N-glycosylation has been shown to be Asn-x-ser/Thr as an essential but not sufficient sequence, there is no such consensus sequence

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known for O-glycosylation sites. In some cases of recombinant proteins, glycosylation may not be essential for therapeutic application, but as pharmaceutical products, glycosylated preparations have been found to have lowered protein aggregation and better thermal stability which are desired properties.

Immunogenicity is yet another parameter, which is a matter of concern with regard to recombinant therapeutic proteins, produced in different expression systems.

γ-Carboxylation and β-hydroxylation of Proteins

These are particularly found in proteins involved in the process of Blood Coagulation. An enzyme, carboxylase, converts target glutamic acid residues in the protein to γ-carboxyglutamic acid residues, while hydroxylase enzyme catalyses the conversion of either the target aspartic acid or the target aspargine residues to their corresponding β-hydroxy derivatives (β-hydroxyaspartate and β-hydroxyaspargine respectively

O-sulfonation

Tyrosine O-sulfonation is a post-translational modification limited to a few proteins. Tyrosine O-sulfonation is catalyzed by two enzymes, viz., tyrosylprotein sulfotransferases (TPST-1 and TPST-2) that transfer the sulfuryl ($-SO_3^-$) group from phosphoadenosylphosphosulphate to certain tyrosyl residues in the protein. Tyrosine sulfation occurs in all mammalian cell lines but not in yeasts and prokaryotes. The functional importance of tyrosine sulfation is presumed to be in protein-protein interactions.

Amidation

Modification of the carboxy terminal of proteins and peptides with an amide group is widely found in vertebrate and invertebrate proteins and peptides but not in yeasts and prokaryotes. Bioactive peptides such as vasopressin, oxytocin, gastrin, calcitonin, substance P and Neuropeptide Y are amidated at their C-terminal. Although amidation is of widespread occurrence in mammalian bioactive peptides and polypeptides, the biological significance of amidation is less understood. It is presumed that this PTM may contribute to stability and in increasing the hydrophobicity of the peptide to facilitate binding to its receptor. Most bioactive peptides are chemically synthesized and subsequently amidated enzymatically using a-amidating enzyme.

Covalent modification of Therapeutic Proteins

Many therapeutic recombinant proteins are characterized by low protease stability, quick renal excretion and high immunogenicity. Covalent modification of proteins is a process, which to some extent, if not all overcomes problems. Covalent conjugation of polyethylene glycol (PEG) to therapeutic proteins is one of the most important techniques (Molecule Altering Structural Chemistry (MASC)), employed for the development of second-generation biopharmaceuticals. PEgylated proteins have been shown to be less susceptible to proteolytic degradation, less immunogenic due to masking of epitopes and have reduced renal clearance rates. Early procedures of PEgylation resulted in mixtures of PEG substituted positional isomers. But later developments in the chemistry of ligation which includes different coupling techniques using different activated poly ethylene glycol and introduction of linkers between PEG and the protein targets, have resulted in site-specific PEgylation. Several PEGylated biopharmaceuticals have received regulatory approvals, which include PEG-IFN, PEG-human growth hormone and PEG-granulocyte macrophage colony stimulating factor. In all cases PEGylation increased product plasma half-life which resulted in reduced frequency of administration.

Proteins by nature are heterogeneous but in the context of recombinant DNA derived protein products produced in various systems add to more complexities. In the chain of processes starting from cloning of the gene to the production of therapeutic protein, there could be many products formed such as truncated polypeptides, differently folded proteins, scrambled disulfide linkages, varying extents and different pattern of glycosylation. In addition, there could be process related contaminants resulting in impurities in the final product. Proteins have pleomorphic physiological effects. Thus, although the recombinant therapeutic protein is optimized for one biological activity, it may have a deleterious effect on another activity, which may result in adverse effects. Therefore, pharmacopoeial monographs should include precise specifications for each formulation of therapeutic proteins, and analytical characterization.

Specifications

They comprise ICH recommendations of characterization parameters that include appearance, identity, quantity, and purity including impurity profile, stability, and biological potency. These parameters that are numerical limits are established by a list of tests, analytical procedures, and other criteria, to which a drug substance or drug product should conform. Conformity of each batch of production to "specification test accept-criteria" is an important part of the batch quality control release (certificate of analysis, COA). The specifications are developed in the early part of process and product development, scale up, non-GMP manufacture, and cGMP manufacture for clinical trials wherein the active drug is fully characterized for its physiochemical properties, biological activity, immunochemical properties, purity, and quantity. The acceptance criteria are related to the analysis method chosen and established during the development process.

Identity

An important part of the overall characterization program for a recombinant protein is the identity tests confirming molecular weight, isoelectric point, primary structure, higher order structures (secondary, tertiary and quaternary) and possible posttranslational modifications. Protein structure and function are closely related. Even minor deviations in the three-dimensional conformation or in the posttranslational modifications (e.g., glycosylation, phosphorylation, or acylation pattern) may result in altered biological activity or in an adverse imunogenic/allergic response. During the drug development phase extensive characterizations are carried out in order to confirm the chemical and physical properties of the recombinant product with its natural counterpart. Only a minority of the identity tests will be used for batch releases that are specified in the relevant monographs.

The standard methods for determination of molecular weight (MW) are Electrospray Mass Spectra (EMS), Matrix Assisted Laser Desorption/Ionization (MALDI)-Time of Flight (TOF), High Performance Size Exclusion Chromatography (HP-SEC), and Analytical Ultracentrifugation (AUC). Various sedimentation velocity measurements (sedimentation velocity, difference sedimentation, and sedimentation equilibrium) are used to gain information on shape and conformation. This is a useful check of homogeneity.

The isoelectric point (pI) is the pH level where the protein has no net charge. The standard methods for determination of pI are Isoelectric focusing in polyacrylamide gels (IEF) and Capillary Electrophoresis (CE)-IEF. The separation principle is based on charge heterogeneity caused by differences in amino acid residue charges. The pI may also be determined by means of 2D-electrophoresis, where molecules are separated according to pI and MW.

The primary structure provides information on the amino acid sequence of the protein. For most proteins a primary structure analysis comprises N-terminal sequencing by Edman degradation, C-terminal analysis, and peptide mapping followed by High Performance Reversed Phase Chromatography (RP-HPLC) purification and subsequent determination of the MW of the fragments by mass spectrometry. The amino acid sequence is often supported by total amino acid analysis, comparison of the cloned gene sequence, and the molecular weight determination. Comparative fingerprints between the natural and the recombinant protein are also used to confirm primary structure identity.

The secondary structure provides information on disulfide bond arrangement, a-helix, and b-sheet content. The standard methods for determination of disulfide arrangements are peptide mapping by RP-HPLC or Sodium dodecyl sulphate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE). Structural information is obtained by means of fluorescence, far UV circular dichroism, and infrared absorption using Fourier Transformed Infra Red Spectroscopy (FTIR). The spectroscopic methods are very powerful when used for comparison analysis with the natural counterpart. The confirmation of correct disulfide linkage is usually carried out on appropriate enzymatic or chemical digests of the target protein followed by RP-HPLC purification of the fragments and subsequent mass analysis using ESI-MS or MALDI-TOF.

The tertiary structure is the three-dimensional structure of the molecule. The standard methods for tertiary structure determination are NMR (in solution), X-ray diffraction using crystals at high atomic resolution (< 3Å), and near UV circular dicroism. The latter method is very powerful for comparison analysis with the natural counterpart.

The quaternary structure of a protein represents the interaction between individual polypeptide chains (Subunits) resulting in larger assemblies. The standard methods for determination of quaternary structure are HP-SEC, Raman scattering, and light scattering, useful for determination of large macromolecular assemblies.

Posttranslational modifications include chemical modifications of side groups (e.g., oxidation of Met, de-amidation of Asn of Gln), phosphorylation, glycosylation, fatty acid acylation, farnesylation, sialic acid capping, N-methylation, and acetylation. Typical standard methods for the detection of posttranslational modifications are RP-HPLC, HP-IEC, or mass spectrometry. In mass spectrometry, the MW of the molecule can be determined with high accuracy and precision (better than 0.01 per cent). This performance is normally sufficient to identify modifications such as missing residues or additional groups. Peptide mapping, using specific enzymes and subsequent HP-RPC purification of the fragments prior to MW detection is also used, for example, for determination of glycosylation patterns. Heterologous glycosylated products are often identified by their isoelectric focusing slab gel pattern or more rarely by 2D-electrophoresis. A thorough carbohydrate structural analysis may include glycosylation site(s), carbohydrate chain structure, the oligosaccharide pattern, and the content of neutral sugars, amino sugars, and sialic acids.

The extinction coefficient can be determined from a known protein quantity (protein concentration) and the absorbance at 277 to 280 nm. In chromatographic evaluation, the target protein retention time using HP-IEC or RP-HPLC can be used as an identity marker. For biomolecular interaction analysis, the method uses surface plasmon resonance to detect biomolecular interactions.

Biological Activity

The biological activity (Potency) describes the ability of the drug substance to achieve a defined biological effect. Examples

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of procedures used to measure the biological activity include animal based biological assays; cell culture-based biological assays, biochemical assays, and ligand binding assays. A biological assay may be replaced by physicochemical tests provided sufficient information and correlation between the bioassay and the said tests can be demonstrated and there exists a well-established manufacturing history (ICH Harmonized Tripartite Guideline. Specifications: Test Procedures and Acceptance Criteria for Biotechnological Products (Q6B).

Structural variants of therapeutic proteins in general and recombinant proteins in particular which include isoforms (e.g., variations in post translational modifications, varying degrees of processing of C- and N- terminal substitutions etc) often show variations in biological activity. Thus, the batch release of the product should be shown to possess the expected degree of biological activity using relevant bioassays. In order to use the same dosing protocol as a reference medicinal product, the content and specific activity of the clinically comparable product should be compared to that of one or more reference medicinal products, or to an international reference standard to which the reference product is calibrated. (WHO Guideline for abbreviated licensing pathways for certain therapeutic proteins)

Purity

Protein purity has been historically linked to the specific biological activity in terms of units of biological activity per mass unit of the product. The purest product is that of the highest specific biological activity. In contrast to drugs based on small molecules, which could be controlled on the drug product level, protein-based pharmaceuticals are closely linked to the process itself due to the complexity of the active pharmaceutical ingredient and the lack of proper characterization of the final product. With the introduction of recombinant technology and modern analytical methods, a much better drug substance/product characterization has become possible resulting in the well-characterized protein concept and the widespread use of comparability studies. The importance of a stronger focus on presence of adventitious agents and specific impurities are also recognized, as the presence of even minor amounts of toxic, immunogenic, or adventitious compounds proved to have severe side effects. The acceptable level of impurities depends on the nature of the drug product and the dose.

Impurity Profile

Product related impurities

Des-amido Forms. Des-amido forms are target protein derivatives in which one or several of the glutaminyl or asparagyl amino acid residues are converted to the corresponding acids (glutamyl and asparagyl). Des-amido forms are detected by analytical HP-IEC, HP-RPC, native PAGE, IEF, MS, or CE. The content accepted depends on the nature of the drug product and the dose.

Oxidized Forms. Oxidized forms are target protein derivatives in which one or several Met, Cys, His, Trp, or Tyr residues have been oxidized. The oxidation of cystinyl residues results in formation of a disulfide bond (cystinyl residue). Oxidized forms are detected by analytical HP-IEC, RP-HPLC, native PAGE, IEF, MS, or CE. The content accepted depends on the nature of the drug product and the dose.

Scrambled Forms. Scrambled forms are target protein molecules with a disulfide bond pattern different from that of the native molecule. Scrambled forms are typically formed during *in vitro* folding of proteins, but disulfide bond shuffling at neutral pH or above also occurs. This requires studies on control of protein stability during downstream processing. The formation of scrambled forms is closely linked to the folding procedure and is protein specific, which are detected by analytical HP-IEC, HP-RPC, CE, or peptide mapping. The content accepted depends on the nature of the drug product and the dose.

Cleaved Forms. Cleaved forms are those where a peptide bond is cleaved, resulting in loss of a N- or C-terminal site or when an internal peptide bond is cleaved while at the same time the resulting fragments are kept together by means of disulfide bonds. These forms are detected by N- and C- terminal amino acid analysis.

Aggregates. Aggregates are target protein derivatives in which two or more molecules are linked together either by covalent inter-disulfide bonds or by hydrophobic interaction. Target protein aggregates are formed as a result of hydrophobic intermolecular reactions or because of intermolecular disulfide bond formation under oxidizing conditions. Aggregates are very often antigenic, resulting in formation of antibodies against the target protein. Proteins exposed to even mildly denaturing conditions may partially unfold, resulting in the exposure of hydrophobic residues to the aqueous-solvent, favoring aggregation. It is generally believed that the aggregation process is controlled by the initial dimerization step in a second order reaction. Consequently, high protein concentrations will increase the aggregation rate. Intermolecular disulfide bond formation between cystinyl residues takes place at alkaline pH under oxidizing conditions. Proteins with reactive free thiol groups should be purified under reducing conditions (typically 1 to 10 mM reducing agent) in the presence of EDTA. Even proteins with disulfide bonds may participate in intermolecular disulfide bond reactions due to disulfide bond shuffling at neutral and alkaline pH. The aggregation reaction based on intermolecular disulfide bond formation is prevented at pH < 6 and under reducing conditions. The hydrophobic aggregation compounds, enzymes, detergents, and stabilizers must be accounted for and their removal validated. Large amounts of hydrophobic reagents may affect hydrophobic interaction chromatography. Analytical HP-IEC, RP-HPLC, native PAGE, IEF, MS, or CE detects oxidized forms. The content accepted depends on the nature of the drug product and the dose. Non-reducing 1D-SDS, HP-SEC, MS, or CE can detect disulfide-based aggregates. Hydrophobic aggregates may be detected by HP-SEC. The content accepted depends on the nature of the drug product and the dose as specified in the product monographs.

Process Related Impurities

Pyrogens and Endotoxins

Pyrogens are a group of chemically diverse substances, including endotoxins of bacteria and debris of dead bacterial cells that cause fever and shock in severe cases. The most important pyrogenic substances in pharmaceutical industry are bacterial endotoxins. Endotoxins come from Gram-negative bacteria (e.g., *E. coli*), if it is used as the expression system. Presence of endotoxins indicates bacterial contamination in raw materials, columns, water, and buffers. There are two methods of detection: the pyrogen test, which is based upon the measurement of body temperature of rabbits before and after injection of the specimen, and the Limulus Amebocyte Lysate (LAL) test, which is based upon the clotting reaction of an enzyme complex of cells of the horseshoe crab together with bacterial endotoxins (*in vitro* test).

Nucleic acids

Nucleic acid contamination comes from host cell DNA/RNA or retroviral RNA. Nucleic acids are detected by monitoring absorption of light at 260 nm. The residual content in drug substance (DS) or drug product (DP) is usually measured by PCR or amplification techniques. The maximum allowable content of nucleic acid per dose remains under continuous evaluation by regulatory agencies. "Lot-to-lot" testing for DNA content in biological products produced in cell lines should be performed and lot release limits established which reflects a level of purity that can be achieved reasonably and consistently.

Host cell proteins

Host cell proteins (HCP) come from the host organism and constitute a major purification problem due to variability structure and surface properties. The amount released into the culture medium depends on the expression system used, the culture conditions and the process related cell lysis. Extraneous proteins may be introduced in the downstream process also. Several analytical methods have been used to monitor HCP including SDS-PAGE, 2D-electrophoresis (IEF in combination with SDS-PAGE), Western blot (WB), and immunoassays. However, Generic HCP assays are preferred for lot release. If the assays are based on competently produced antibodies raised against cell lysates, the quality may be adequate

Viruses

Virus contamination comes from the host cell, the culture medium, and infections during manufacture. The host cell may contain a genomic virus or virus vectors used to transform the cell line. The type of viral genome or vector depends on the cell line history. Chronic or latent viruses may be present in continuous cell lines, and the retroviruses associated with continuous cell lines are non-infectious, but oncogenic. Epstein-Barr virus or Sendai virus is often used for cell transformations. Contaminants such as BVDV, IBR, reovirus, PI-3, bovine leukemia virus, and bovine polyoma virus should be expected from serum-supplemented media. The cell line history reveals all information on the origin and identity of the cell line and the host genome vectors used to establish the cell line

Prions

Prions come from transmissible spongiform encephalopathies (TSE), The major source of contamination of a recombinant product is the use of animal-derived raw materials, which could harbor bovine prions (BSE agent). Currently, there are no assays that are sensitive or specific enough to test raw materials or sources, and the only reliable prevention is to include barriers, such as avoidance of animal or human raw materials (e.g., trypsin, serum, transferin, bovine/human serum albumin, protein supplements, peptones).

Microbial agents including Mycoplasma

Microbial agents and fungi come from infection of the bioreactor during cell culture. Other sources are contaminated water, buffers, raw materials, chromatographic columns, and equipment. Fermentation and cell culture bioreactors are prone to microbial infections. Viable cells can be identified by spread out of the cell suspension or sample solution on agar plates.

Mycoplasma

Mycoplasmas have for long been recognized as a contaminant of continuous cell cultures caused by an infection of the cell line or bioreactor. Working in closed systems under GMP will reduce the *risk of infection*. The end of production test includes screening for mycoplasmas. Mycoplasmas are difficult to detect, the only reliable way of demonstrating infection is by agar plating, fluorescent dying of DNA, or by PCR

Quantity

Protein content

Quantitative estimation of protein is carried out by several methods (*Kjeldahl analysis, Lowry assay, Biuret assay,*

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Bicinchoninic acid (BCA) assay, Bradford assay, UV absorbance and amino acid analysis). It is desirable to confirm the quantity by more than one method.

Stability

Formulation development of biopharmaceutical therapeutic protein will provide a final dosage form that offers *ex vivo* stability during processing, handling, and long-term storage under specified conditions (e.g., temperature, humidity etc). It is also expected to provide adequate *in vivo* bioavailability that meets the desired pharmacokinetic/pharmacodynamic (PK/PD) properties. Therapeutic proteins do not survive terminal sterilization procedures commonly employed for small therapeutic molecule. Therefore, formulation components and excipients should also undergo microbiological tests including analysis for endotoxins and pyrogens in addition to biological, chemical and physical functions.

Therapeutic Monoclonal Antibodies for Human Use

Monoclonal antibodies for human use are preparations of an immunoglobulin or a fragment of an immunoglobulin, for example, F(ab')2, with defined specificity, produced by a single clone of cells. They may be conjugated to other substances, including for radiolabelling. They can be obtained from immortalized B lymphocytes that are cloned and expanded as continuous cell lines or from rDNA-engineered cell lines.

Currently available rDNA-engineered antibodies include the following:

Chimeric monoclonal antibodies. The variable heavy and light chain domains of a human antibody are replaced by those of a non-human species, which possess the desired antigenic specificity.

Humanised monoclonal antibodies. The three short hyper variable sequences (the complementarity determining regions) of non-human variable domains for each chain are engineered into the variable domain framework of a human antibody; other sequence changes may be made to improve antigen binding.

Recombinant human monoclonal antibodies. The variable heavy and light chain domains of a human antibody are combined with the constant region of a human antibody.

This monograph applies to monoclonal antibodies for therapeutic usages. It does not apply to monoclonal antibodies used as reagents in the manufacture of medicinal products, *in vivo* diagnostics or in prophylactic usage for which requirements are decided by the competent authority.

This monograph is intended to provide guidance on monoclonal antibody development and manufacturing, covers following aspects:

1. General Guidance
2. Product Development
2.1 Cloning and Cell Line Development
2.2 Process Development
2.3 Analytical Method Development
2.4 Process Characterization
2.5 Analytical Characterization
3. Non-clinical and Clinical Studies
4. Manufacturing
4.1 At-scale Manufacturing and Process Validation (Scale up)
4.2 Lot Release Testing (Final Lot)
4.3 Reference Preparation
4.4 Shelf-Life and Storage
4.5 Stability

1. General Guidance

For nomenclature of monoclonal antibodies, 'International Nonproprietary Names (INN) for monoclonal antibodies' is followed (Refer, WHO guideline)

2. Product Development

2.1 Cloning and Cell Line Development:

In order to produce a recombinant monoclonal antibody, the protein of interest is sequenced and the same is cloned into desired vector, then a preferred specific clone is developed.

Source Cells

Source cells include fusion partners, lymphocytes, myeloma cells, feeder cells and host cells for the expression of the recombinant monoclonal antibody.

The origin and characteristics of the parental cell are documented, including information on the health of the donors, and on the fusion partner used (for example, myeloma cell line, human lymphoblastoid B-cell line).

Wherever possible, source cells undergo suitable screening for extraneous agents and endogenous agents. The choice of viruses for the tests is dependent on the species and tissue of origin.

Cell lines producing the monoclonal antibody

The suitability of the cell line producing the monoclonal antibody is demonstrated by documentation on the history of

the cell line including description of the cell fusion, immortalisation or transfection and cloning procedure; characterisation of the cell line using techniques such as gene copy number, barcoding etc.(for example, phenotype, isoenzyme analysis etc.,); characterisation of relevant features of the antibody; stability of antibody secretion with respect to the characteristics of the antibody and level of expression and glycosylation up to or beyond the population doubling level or generation number used for routine production; for recombinant DNA products, stability of the host/vector genetic and phenotypic characteristics up to or beyond the population doubling level or generation number used for routine production.

Host cell line characterization

The host cell line engineered to express the desired antibody molecule must be subjected to appropriate analyses at genomic level to establish stable integration of the foreign gene. Typically, this analysis should be initiated the moment lead clone is identified. Appropriate analytical tools to be used for this purpose.

Cell banks

The master cell bank is a homogeneous suspension of the cell line producing the monoclonal antibody, distributed in equal volumes in a single operation into individual containers for storage. A working cell bank is a homogeneous suspension of the cell material derived from the master cell bank at a finite passage level, distributed in equal volumes in a single operation into individual containers for storage. Post-production cells are cells cultured up to or beyond the population doubling level or generation number used for routine production.

The following tests are performed on the master cell bank: viability, identity, (bacteria, fungiand mycoplasma), characterisation of the monoclonal antibody produced. Non-endogenous viral contamination is tested with a suitable range of *in vivo* and *in vitro* tests. Retrovirus and other endogenous viral contamination are tested using a suitable range of in vitro tests.

The following tests are performed on the working cell bank: viability, identity, absence of microbes such as bacteria, fungi, and mycoplasma. Adventitious viral contamination is tested with a suitable range of *in vivo* and *in vitro* tests. For the first working cell bank, these tests are performed on post-production cells, generated from that working cell bank; for working cell banks subsequent to the first working cell bank, a single *in vitro* and *in vivo* test can be done either directly on the working cell bank or on the post-production cells.

For the master cell bank and working cell bank, tests for specific viruses are carried out when potentially contaminated biological material has been used during preparation of the cell banks, taking into account the species of origin of this material. This may not be necessary when this material is inactivated using validated procedures.

The following tests are performed on the post-production cells: sterility (bacteria, fungi and mycoplasma). Virus tests are performed on cells or cell culture supernatants. For this, non-endogenous viral contamination is tested with a suitable range of *in vivo* and *in vitro* tests. Retrovirus and other endogenous viral contamination is tested using a suitable range of in vitro tests.

2.2 Process Development

In order to achieve product quality and titer as per expectations, upstream process in terms media, feed, process parameters and as needed, bioreactor designing to be completed. Further, based on product quality downstream process, where purification is achieved to be developed. A typical monoclonal antibody process is of three chromatography steps where capture, polish and further purification steps are performed.

Culture and Harvest

Production at finite passage level (single harvest). Cells are cultivated up to a defined maximum number of passages or population doublings, or up to a fixed harvest time (in accordance with the stability of the cell line). Product is harvested in a single operation.

Continuous-culture production (multiple harvest). Cells are continuously cultivated for a defined period (in accordance with the stability of the system and production consistency). Monitoring is necessary throughout the life of the culture; the required frequency and type of monitoring will depend on the nature of the production system.

Each harvest is tested for antibody content, bioburden, endotoxin and mycoplasmas. General or specific tests for adventitious viruses are carried out at a suitable stage depending on the nature of the manufacturing process and the materials used. For processes using production at finite passage level (single harvest), at least 3 harvests are tested for adventitious viruses using a suitable range of *in vitro* methods.

The acceptance criteria for harvests for further processing are clearly defined and linked to the schedule of monitoring applied. If any adventitious viruses are detected, the process is carefully investigated to determine the cause of the contamination and the harvest is not further processed. Harvests in which an endogenous virus has been detected are not used for purification unless an appropriate action plan has been defined to prevent transmission of infectious agents.

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Purification

Harvests or intermediate pools may be pooled before further processing. The purification process includes steps that remove and/or inactivate non-enveloped and enveloped viruses. A validated purification process, for which removal and/or inactivation of infectious agents and removal of product- and process-related impurities has been demonstrated, is used. Defined steps of the process lead to a purified monoclonal antibody (active substance) of consistent quality and biological activity.

2.3 Analytical Methods Development

Analytical methods to be developed that are capable of establishing sufficient information of product quality attributes. Methods required for characterization of product may be extensive than that of methods required for releasing a product from lot to lot. Analytical methods used for product characterization should minimally established suitability, accuracy of the method whereas methods used for in routine lot release should be validated prior to clinical studies.

Wherever possible, compendial procedures should be adapted after establishing suitability. Particular attention to the methods suitability for usage should be shown in safety (microbial) related methods.

2.4 Process Characterization

Once the process is developed, in order to understand the capability of the process, a detailed process characterization is necessary. This will be important to understand the manufacturing process and to address various issues that may arise during routine commercial processing. Also, these studies will provide insight into design of various attributes used during development.

Product Characterization

Monoclonal antibody drug molecule should be characterized at various stages of development using combinations of orthogonal techniques to ascertain and ensure purity, physico-chemical characteristics, conformational integrity including quaternary structure and biological activity.

The product is characterized to obtain adequate information including: structural integrity, isotype, amino-acid sequence, secondary structure, carbohydrate moiety, disulphide bridges, conformation, specificity, affinity, specific biological activity and heterogeneity (characterisation of isoforms). A battery of suitable analytical techniques used includes chemical, physical, immunochemical and biological tests (for example, peptide mapping, N- and C- terminal amino-acid sequencing, amino acid composition, mass spectrometry, chromatographic, electrophoretic and spectroscopic techniques). For those products that are modified by fragmentation or conjugation, the influence of the method used on the antibody needs to be characterized.

2.5 Analytical characterization

Various analytical tools are to be used to characterize the products, some of them are listed below:

(a) **Purity and identity of mAb product.** Purity analysis is typically done by SDS-PAGE; this can be considered the first step of physio-chemical characterization, in this technique, purity of heavy and light chain bands under reducing conditions as well as band pattern under non-reducing conditions is analyzed. SDS-PAGE is followed by western blot analysis to establish the identity of the observed bands using primary antibodies specific to human IgG. Further, impurity analysis is done by HPLC based size exclusion chromatography (SEC); this technique is very useful in detecting presence of dimers and aggregates in the purified product. The analysis is done on the basis of differences in time of elution of contaminating products from the main peak. Other methods like Capillary Electrophoresis etc. can be employed where they are relevant.

(b) **Sequence and structural integrity.** Primary sequence of antibody heavy and light chains is recognized by N terminal sequencing using Edman degradation method. In this technique, though sequence of only first ten amino acids can be properly identified, it gives an indication that the protein has been accurately translated in frame as per the gene design. N terminal sequencing coupled with amino acid analysis, in which acid hydrolysis of individual chains followed by capillary electrophoresis (CE)-HPLC is performed, helps in initial identification of the protein. Primary sequence can be further obtained and verified by 'de novo' sequencing using mass spectrometry based techniques like ESI/MALDI- TOF/ TOF etc. Secondary structure analysis can be done by circular dichroism (Far-UV CD- spectroscopy) for alpha and beta content analysis. Spectrofluorometric analysis based on fluorescence of hydrophobic amino acid residues is used for detecting improper folding in the recombinant protein.

(c) **Characterization of Charge micro-variants.** Charge related heterogeneity in monoclonal antibodies is examined by IE-HPLC or isoelectric focusing (IEF). Since, most human IgGs have a basic isoelectric point, cation exchange (CEX) - HPLC is typically used. Variants that elute before the main peak are less positively charged and are referred as acidic, whereas, species that elute after the main peak are termed as basic. In IEF, where a mixture of ampholytes is used to establish a pH gradient,

antibody molecules migrate to pH equivalent to their pI. As compared to CEX, IEF is generally used for monitoring of variants rather than their characterization, as variants with different surface-charge distribution can merge together during separation due to similar pI.

All human IgGs contain a lysine residue at C-terminus of heavy chain, lysine and arginine residues at carboxyl terminus are removed by cellular enzymes called carboxy peptidases. Usually the removal is incomplete and two variants with lysine on either one or both heavy chains clipping can be expected. Loss of lysine makes the molecule acidic as there is loss of an amino group and can be detected by CEX, also loss of single lysine would result in loss of mass equivalent to 128 Da which can be examined by mass spectrometry. Similarly, C-terminal amidation results in loss of terminal carboxyl group making molecule more basic, amidation leads to a loss of mass by 1 Da.

Another familiar micro-variant involves formation of N-terminal pyroglutamate. In this variant, N- terminal glutamine (Q) or glutamic acid (E) can undergo cyclization forming a pyroglutamic acid, here an amine is converted to neutral amide and Q /E cyclization is detected as acidic variants on CEX. Conversion of N-terminal Q is almost 99 per cent, whereas E conversion is only 3 per cent. The mechanism is not fully understood for this conversion, although cellular glutaminylcyclase has been implicated in this reaction. Pyroglutamate formation has been observed with elevated temperatures and can be associated with process or storage conditions. Pyroglutamate residue interferes with N-terminal sequencing of heavy chain and N-terminal blocking leads to loss of 17 -18 Da mass.

Oxidation is commonly observed in proteins, and in monoclonal antibodies two methionines located in Fc domain are prone to oxidation. This process is seen in formulated products following incubation at elevated temperatures. Full length antibodies with oxidation on both methionines can be eluted as basic shoulders by CEX.

A very common charge variant is generated by deamidation of asparagine (N) and isomerization of aspartate (D) residues. Usually N or D residues, in complementarity determining regions (CDRs) which are surface exposed regions, are prone to these modifications, and are the main sources of degradation under mild conditions. Both reactions proceed through a common cyclic imide intermediate-succinimide, which hydrolyzes into a mixture of isoaspartate and aspartate in 3:1 ratio. Usually N and D followed by glycine in a sequence are prone to this modification. Deamidated species elutes as acidic species in CEX, whereas, succinimide intermediate and isoaspartate variant elute as basic forms. Deamidation leads to loss of 1 Da mass detected by MS, whereas, succinimide intermediate shows loss of mass by 17 Da. Asparagine deamidation and aspartate isomerization can occur throughout the entire life time of the antibody, including in vivo after administration.

Chemicals present in culture media or in formulation can react with antibodies and can lead to formation of chemical adduct. Non-enzymatic addition of sugars (glycation) is commonly observed in monoclonal antibodies. Glycation occurs by reaction of reducing sugars like glucose and lactose (in culture media) with amine group of lysine or the primary amine in N-terminus. Similar reaction can also occur if a sugar is present in the formulation buffer. Glycation can be observed as an acidic shift in IEF and also detected by mass spectrometry as gain of mass by 162 Da.

Any of the variants that are shown to be present different from that of reference standard either in terms of new species or quantity, it is necessary to understand the impact of these variants on product activity in terms of potency.

(d) **N-linked Glycosylation.** In human IgG, asparagine residue 297 in Fc region is the site for N-linked glycosylation. The most common glycoforms G0F, G1F and G2F are bi-antennary blocks of 8-11 carbohydrate residues comprising of fucose 'F' (n=1), N-acetylglucosamine 'GlcNac' (n=2), mannose (N=3), galactose'G' (N= 0-2) residues, respectively. Individual moieties of glycan chain are neutral carbohydrates, except sialic acid residues which can be added to terminal galactose residues. In antibodies expressed in human and in the CHO cell lines, the level of sialylation is low. Various glycoforms can be detected efficiently by mass spectrometry after removal of entire glycan chain by peptide N-glycosidase F (PNGase F). Typically, a G0F form has a mass of 1.4 KDa with each additional hexose adding 162 Da. The glycosylation profile needs to be monitored critically because it can influence the binding of the Fc fragment to receptors that mediate ADCC and CDC. Further, glycosylation profile is prone to changes depending on cell line and fermentation conditions.

Variations in the content/ quantity of glycosylation from that of reference product may be acceptable, provided there is no impact on the product activity.

In addition to the N-glycosylation, there may be specific molecules that have O-glycosylation present on Serine/ Threonine amino acids of monoclonal antibody. Sufficient characterization of the same is required.

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Biological characterization

ELISA is a very important technique for establishing the binding specificity of antibody to its target antigen. However, strength of binding i.e. affinity can be measured only by surface plasmon resonance method. Availability of purified target antigen in correctly folded form is an absolute requirement for both the techniques and obtaining targets which are cell surface receptors in pure form is a challenging task. Binding to cell surface receptors can be established by FACS analysis, which is another reliable technique complementing ELISA. *In vitro* cell based assays give convincing evidence for efficacy of the monoclonal antibody against their targets, and must be standardized to ensure its reproducibility and robustness.

3. Non-clinical and Clinical Studies

As part of the development studies, once the product of interest is developed various non-clinical and clinical studies to be conducted. These studies are to provide study product toxicity in animals, clearance rate in humans and immunogenicity/ safety/efficacy in patients. Specific requirements of these studies are prescribed in relevant guidelines.

4. Manufacturing

Production is based on a seed-lot system using a master cell bank and, if applicable, a working cell bank derived from the cloned cells. The production process of the cell bank is executed in a manner to ensure safety during development studies, to prevent transmission of infectious agents by the final product. All biological materials and cells used in the production are characterized and are in compliance withthe limits prescribed by the competent authority to minimize the risk of transmitting animal spongiformencephalopathy agents via human and veterinary medicinal products. Where monoclonal antibodies for human use are manufactured using materials of human or animal origin,it should comply with viral safety limits, as prescribed by competent authority. Where an immunogen is used it is characterized and the method of immunization is documented.

While planning for product commercialization, process development/characterization, process validation and process monitoring is of vital importance. A robust system needs to be in place to ensure process consistency over the entire lifecycle of the product.

4.1 Scale UP

The scale-up of processes from bench to commercial scale is challenging because of uncertainties about the physio-chemical environment experienced by the cells at large scale. However, there are some features of mAb that make the scale-up task significantly easier than for other proteins like, firstly, mAbs are generally very stable molecules that can tolerate relatively harsh treatments, e.g. extremes of pH, shear, etc and secondly, a highly specific affinity ligand, protein A, is available that binds to most classes of therapeutically relevant human antibodies. Process platforms in both upstream cell culture and downstream purification have become widely established in industry for production of large quantities of pharmaceutical-grade mAbs at moderate costs. Large scale production of mAb utilizes mammalian production systems followed by cell removal and purification through sequential chromatographic and membrane filtration steps, to consistently reduce product- (e.g. protein variants) and non-product related variants (e.g. host cell proteins) impurities to acceptable levels.

Process Validation

During development studies, the production method is validated for the following aspects:

- consistency of the production process including cell-culture/fermentation, purification and, where applicable, fragmentation method;
- removal or inactivation of infectious agents;
- adequate removal of product- and process-related impurities (for example, host-cell protein and DNA, protein A, antibiotics, cell-culture components);
- specificity and biological activity of the monoclonal antibody;
- absence of non-endotoxin pyrogens, where applicable;
- reusability of purification components (for example, column material), limits or acceptance criteria being set as a function of the validation;
- methods used for conjugation, where applicable.

Process intermediates

Where process intermediates are stored, an expiry date or a storage period justified by stability data is established for each.

Active Substance

The test programme for the active substance depends on the validation of the process, on demonstration of consistency and on the expected level of product- and process-related impurities. The active substance is tested for appearance, identity, bioburden and bacterial endotoxins, product-related substances, product- and process-related impurities including tests for host-cell-derived proteins and host-cell- and vector-derived DNA, as well as structural integrity, protein content and biological activity by suitable analytical methods, comparing with the reference preparation where necessary. When the active substance is a conjugated or transformed antibody, appropriate tests must be performed before and after

the antibody conjugation/modification. If storage of intermediates is intended, adequate stability of these preparations and its impact on quality or shelf-life of the finished product are evaluated.

FINAL BULK

One or more batches of active substance may be combined to produce the final bulk. Suitable stabilizers and other excipients may be added during preparation of the final bulk. The final bulk must be stored under validated conditions with respect to bioburden and stability.

4.2 FINAL LOT

The final bulk is sterile-filtered and distributed under aseptic conditions into sterile containers, which may subsequently be freeze-dried.

As part of the in-process control each container (vial, syringe or ampoule) is inspected after filling to eliminate containers that contain visible particles. During development of the product it must be demonstrated that either the process will not generate visible proteinaceous particles in the final lot or such particles are reduced to a low level as justified and authorised.

Identification

The identity is established by suitable validated methods comparing the product with the reference standard, where appropriate. Establishment of identity needs to be shown by more than one method, for ex., peptide mapping as finger print, western blot, biological assay etc.

Tests

Appearance. Liquid or reconstituted freeze-dried preparations comply with the limits approved for the particular product with regard to degree of opalescence and degree of coloration (2.4.1). They are without visible particles, unless otherwise justified and authorised.

Solubility. Freeze-dried preparations dissolve completely in the prescribed volume of reconstituting liquid, within a defined time, as approved for the particular product.

pH (2.4.24). It complies with the limits approved for the particular product.

Osmolality (2.4.23). Minimum 240 mosmol/kg, unless otherwise justified and authorised.

Extractable volume. It complies with the test for extractable volume.

Total protein (2.3.30). It complies with the limits approved for the particular product.

Molecular-size distribution. Molecular-size distribution is determined by a suitable method, for example size-exclusion chromatography (2.4.16). It complies with the limits approved for the particular product.

Molecular identity and structural integrity. Depending on the nature of the monoclonal antibody, its microheterogeneity and isoforms, a number of different tests can be used to demonstrate molecular identity and structural integrity. These tests may include peptide mapping, isoelectric focusing, ion-exchange chromatography, hydrophobic interaction chromatography, oligosaccharide mapping, monosaccharide content and mass spectrometry.

Purity. Tests for process- and product-related impurities are carried out by suitable validated methods. Provided that tests for process-related impurities have been carried out on the active substance or on the final bulk with satisfactory results, they may be omitted on the final lot.

Stabiliser. Where applicable, it complies with the limits approved for the particular product.

Water (2.3.43). Freeze-dried products comply with the limits approved for the particular product.

Sterility (2.2.11). It complies with the test for sterility.

Bacterial endotoxins (2.2.3). It complies with the limits approved for the particular product.

Tests applied to modified antibodies. Suitable tests are carried out depending on the type of modification.

Biological Assay

Carry out a suitable biological assay compared to the reference preparation. Design of the assay and calculation of the results are made according to the usual principles.

4.3 Reference preparation

A reference standard obtained from source recognized by national regulatory authority (NRA). If recognized reference standard is not available an "in-house reference preparation" should be established. A properly qualified in-house reference standard for mAbs with known characteristics, specificity, and potency, and that is stored under appropriate conditions and periodically tested to ensure its integrity, should be used for lot-to-lot comparisons. A batch shown to be stable and shown to be suitable in clinical trials, or a batch representative thereof, is used can be as a reference preparation for the identification, tests and assay. The reference preparation is appropriately characterized as defined under Product characterization, except that it is not necessary to examine cross-reactivity for each batch of reference preparation.

Definition of a batch. Definition of a batch is required throughout the process.

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4.4 Shelf Life and Storage. As stated on the label.

Expiry date. The expiry date is calculated from the date of sterile filtration, the date of filling (for liquid preparations) or the date of freeze-drying (where applicable) or as established based on stability data.

Labelling. The label states.

— amount of monoclonal antibody per milliliter, where applicable;

— as applicable, total quantity of protein per container;

For liquid preparations:

— volume of the preparation in the container

For freeze-dried preparations:

— Name and the volume of the reconstitution liquid to be added, where applicable;

— Period of time within which the monoclonal antibody is to be used after reconstitution;

— Dilution to be made before use of the product, where applicable.

4.5 Stability

The development of a proper long term stability program is critical to the successful development of a commercial product and hence adequate stability studies form an essential part of the product development process and they should be designed to help setting up the shelf life of product. The stability testing protocol should include all stability indicating parameters which provide assurance that change in identity, purity and potency of the product will be detected. Some important tests such as molecular size, potency, residual moisture, protein content, preservative can be considered for evaluation. The product should retain its specifications within established limits for safety, purity and potency throughout the proposed shelf life.

If purified bulk material is to be stored after manufacture but prior to formulation and final manufacturing, atleast 3 batches should be placed on stability at the recommended storage temperature and should be representative of the manufacturing scale of production. The stability of intermediate products (as applicable) before mixing into the final bulk should be demonstrated. The quality of drug substance placed into stability program should be representative of the quality of material used in preclinical and clinical studies. The drug substance entered into stability program should be stored in containers which properly represent the actual holding containers used during manufacture. Containers of reduced size is acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of containers closure system. Manufacturer is also recommended to consider end of stability of the drug substance should be formulated and the product manufactured thereof should be place on stability till the product claim.

The stability of the product in its final form and at the recommended storage temperatures should be demonstrated using containers from at least three lots of final product made from different independent purified batches of monoclonal antibodies. In addition, real time stability study should be conducted on at least one final container lot produced per year. Wherever possible, the final product containers should be representative of the quality of the material used in the preclinical and clinical studies.

Accelerated stability studies may provide additional supporting evidence of the stability of the product but cannot replace real time studies. When changes are made in production process or in the primary packaging that may affect the stability of the product, the product manufactured by the new change should demonstrate stability. These will also applicable to different presentations/strengths of the same product, but bracketing can be done if required.

The statements concerning storage temperature and expiry date appearing on the label should be based on experimental evidence which should be submitted to NRA for approval.

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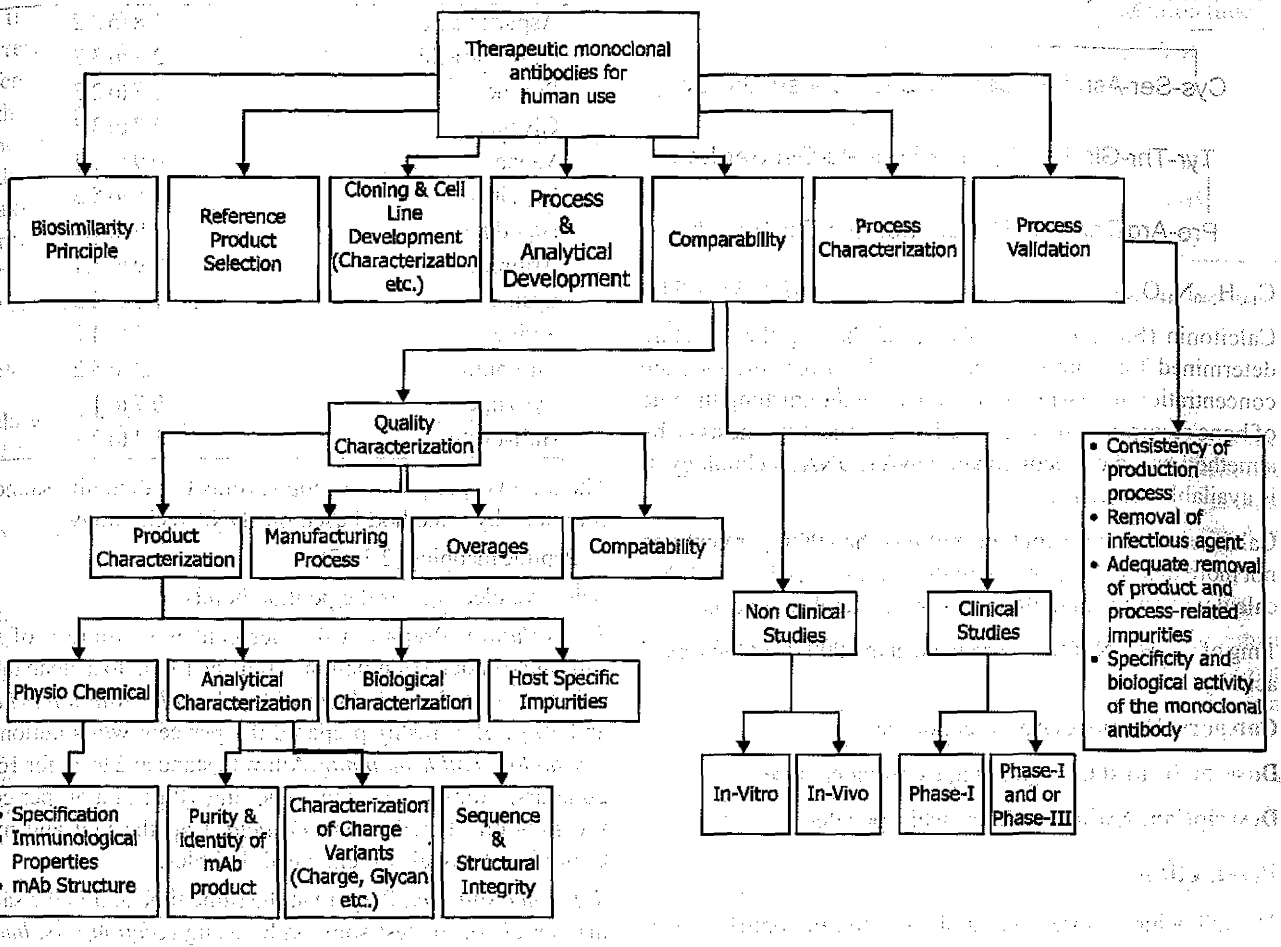

Table :- Summary for Monoclonal Antibodies Development

(A brief overview of general steps in monoclonal antibody development is presented in the table above, it may not necessarily consolidate all the steps)

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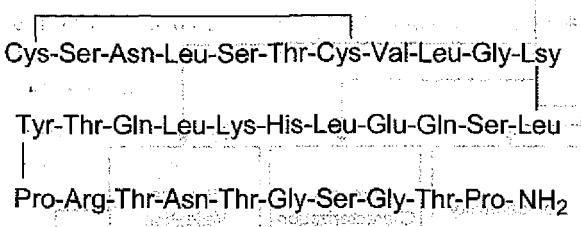
Calcitonin (Salmon)



$C_{145}H_{240}N_{44}O_{48}S_2$ Mol. Wt. 3431.9

Calcitonin (Salmon) is a polypeptide having the structure determined for salmon calcitonin I. It lowers the calcium concentration in plasma of mammals by diminishing the rate of bone resorption. It is obtained by chemical synthesis or by a method based on recombinant DNA (rDNA) technology. It is available as acetate.

Calcitonin (Salmon) contains not less than 90.0 per cent and not more than 105.0 per cent of the peptide $C_{145}H_{240}N_{44}O_{48}S_2$, calculated on the anhydrous and acetic acid-free basis.

1 mg of $C_{145}H_{240}N_{44}O_{48}S_2$ is equivalent to 6000 IU of biological activity.

Category. Hormone (Antiosteoporotic).

Dose. 50 IU to 400 IU, in a single or divided doses.

Description. A white or almost white powder.

Production

The following requirements apply only to calcitonin (salmon) produced by a method based on rDNA technology.

Prior to release the following tests are carried out on each batch of final bulk product unless exemption has been granted by the competent authority.

Host-cell-derived proteins. Not more than 100 ppm.

Host-cell or vector-derived DNA. Not more than 10 ng per dose.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

The following requirement applies only to calcitonin (salmon) obtained by chemical synthesis.

B. Amino acid analysis (2.2.19).

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids taking as equivalent to the sum, divided by 20, of the number of moles of aspartic acid, glutamic acid, proline, glycine, valine, leucine, histidine, arginine and lysine.

The values fall within the following limit.

Aspartic acid	1.8 to 2.2
Glutamic acid	2.7 to 3.3
Proline	1.7 to 2.3
Glycine	2.7 to 3.3
Valine	0.9 to 1.1
Leucine	4.5 to 5.3
Histidine	0.9 to 1.1
Arginine	0.9 to 1.1
Lysine	1.8 to 2.2
Serine	3.2 to 4.2
Threonine	4.2 to 5.2
Tyrosine	0.7 to 1.1
Half-cystine	1.4 to 2.1

The following requirement applies only to calcitonin (salmon) produced by a method based on rDNA technology.

C. Peptide mapping (2.3.47).

Selective cleavage of the peptide bonds.

Test solution. Prepare a 0.1 per cent w/v solution of the substance under examination. Transfer 1.0 ml to a clean tube. Add 100 µl of *1 M tris-hydrochloride buffer solution pH 8.0* and 20 µl of a freshly prepared 0.1 per cent w/v solution of *trypsin for peptide mapping*. Allow to stand at 2 to 8° for 16 to 20 hours. Stop the reaction by adding 10 µl of a 50 per cent v/v solution of *trifluoroacetic acid*. Cap the vial and mix. Centrifuge the vials to remove air bubbles.

Reference solution. Prepare at the same time and in the same manner as for the test solution by using *calcitonin (salmon) IPRS*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- mobile phase: A. a mixture of 1 volume of *trifluoroacetic acid* and 1000 volumes of *water*,
  B. a mixture of 0.85 volume of *trifluoroacetic acid*, 200 volumes of *water* and 800 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1.2 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
50	65	35
60	40	60
60.1	0	100
65.1	40	60
65.2	100	0
80	100	0

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Inject the reference solution and the test solution. The profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution. The retention times of the fragment peaks in the chromatogram obtained with the test solution are within 5 per cent of the retention times of the fragments obtained with the reference solution. The peak area ratios of the fragment peaks in the chromatogram obtained with the test solution, normalised to the area of peak T_2, are within 5 per cent of the corresponding peak ratios in the chromatogram obtained with the reference solution.

Tests

Related substances. Determine by liquid chromatography (2.4.14).

A. The following requirement applies to calcitonin (salmon), produced by chemical synthesis.

Test solution. Dissolve 25 mg of the substance under examination under examination in 25.0 ml of the mobile phase.

Reference solution (a). A 0.1 per cent w/v solution of *calcitonin (salmon) IPRS* in mobile phase A.

Reference solution (b). Dissolve the contents of a vial of *N-acetyl-cys[1]-calcitonin IPRS* in 400 µl of mobile phase A and add 100 µl of the test solution.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 65°,
- mobile phase: A. a mixture of a buffer solution prepared by dissolving 3.26 g of *tetramethylammonium hydroxide* in 900 ml of *water*, adjust the pH to 2.5 with *orthophosphoric acid* and 100 ml of *acetonitrile*,

  B. a mixture of a buffer solution prepared by dissolving 1.45 g of *tetramethylammonium hydroxide* in 400 ml of *water*, adjust the pH to 2.5 with *orthophosphoric acid* and 600 ml of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	72	28
30	48	52
32	72	28
55	72	28

Name	Relative retention time
Calcitonin impurity B[1]	0.8
Calcitonin impurity C[2]	0.9
Calcitonin (salmon) (Retention time: about 20 minutes)	1.0
Calcitonin impurity D[3]	1.05
Calcitonin impurity A[4]	1.15

[1] [9-D-leucine]calcitonin (salmon),

[2] des-22-tyrosine-calcitonin (salmon),

[3] O-*acetylated calcitonin* (salmon),

[4] acetylcalcitonin (salmon).

Inject reference solution (b). The test is not valid unless the resolution between the peaks corresponding to calcitonin (salmon) and calcitonin (salmon) impurity A is not less than 5.0 and the tailing factor of calcitonin (salmon) impurity A is not more than 2.5.

Inject the test solution. The area of the peak corresponding to calcitonin (salmon) impurities A, B, C and D is not more than 3.0 per cent. The sum of areas of all the secondary peaks is not more than 5.0 per cent. Ignore any peak with an area less than 0.1 per cent, calculated by area normalization.

B. The following requirement applies only to calcitonin (salmon) produced by a method based on rDNA technology.

Test solution. Dissolve 50 mg of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase. To 1.0 ml of this solution, add 100 µl of *0.25 M citrate buffer pH 3.0*, prepared by dissolving 4.8 g of *citric acid* in 80 ml of *water*, adjust the pH to 3.0 with *1 M sodium hydroxide* and dilute to 100.0 ml with *water*.

Reference solution. Dissolve 0.1 g of the substance under examination in the mobile phase and dilute to 100.0 ml with the mobile phase. Prepare a 1 mg per ml solution of the substance to be examination. Mix 1 volume of this solution and 1 volume of *calcitonin-Gly IPRS*. To 1.0 ml of this solution, add 100 µl of *0.25 M citrate buffer solution pH 3.0*.

Chromatographic system

- a stainless steel column 20 cm x 4.6 mm, packed with polysulfoethylaspartamide ion-exchange gel (5 µm),
- mobile phase: A. a mixture of 15 volumes of *acetonitrile* and 85 volumes of a buffer solution prepared by dissolving 2.72 g of *potassium dihydrogen phosphate* in 1000 ml *water*, adjusted to pH 5.0 with a 60 per cent w/v solution of *potassium hydroxide*,

  B. a mixture of 15 volumes of *acetonitrile* and 85 volumes of a solution containing 0.27 per cent w/v solution of *dihydrogen phosphate* and 2.92 per cent w/v of *sodium chloride*, adjusted to pH 4.6 with a 60 per cent w/v solution of *potassium hydroxide*,

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– a gradient programme using the conditions given below,
– flow rate: 1.2 ml per minute,
– spectrophotometer set at 220 nm,
– injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
10	0	100
15	0	100
15.1	100	0
22.1	100	0

Name	Relative retention time
Calcitonin Impurity G[1]	0.4
Calcitonin Impurity F[2]	0.6
Calcitonin Impurity E[3]	0.9
Calcitonin (salmon) (Retention time: about 9 minutes)	1.0

[1][1,7-bis(3-sulfo-L-alanine)]calcitoninylglycine (salmon),

[2][1,7-bis(3-sulfo-L-alanine)]calcitonin (salmon),

[3]salmon calcitoninylglycine.

Inject the reference solution. The test is not valid unless the resolution between the peaks corresponding to calcitonin (salmon) impurity E and calcitonin (salmon) is not less than 3.0.

Inject the test solution. The area of the peak corresponding to calcitonin (salmon) impurity E is not more than 0.6 per cent, the area of the peak corresponding to calcitonin (salmon) impurities F and G is not more than 0.2 per cent, calculated by area normalization.

Acetic acid. 4.0 per cent to 15.0 per cent.

Determine by liquid chromatography (2.4.14).

Solvent mixture. 5 volumes of mobile phase B and 95 volumes of mobile phase A.

Test solution. Dissolve 10 mg of the substance under examination in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution. A 0.01 per cent w/v solution of *glacial acetic acid* in the solvent mixture.

Chromatographic system
– a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
– mobile phase: A. dilute 0.7 ml of *orthophosphoric acid* to 1000 ml with *water*, adjusted to pH 3.0 with *sodium hydroxide solution*,
B. *methanol*,
– flow rate: 1.2 ml per minute,
– spectrophotometer set at 210 nm,
– injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
5	95	5
10	50	50
20	50	50
22	95	5
30	95	5

The retention time of acetic acid peak is about 3 to 4 minutes.

Inject the reference solution and the test solution.

Calculate the content of acetic acid.

Water (2.3.43). Not more than 10.0 per cent, using method 3.

Acetic acid and water. Not more than 20 per cent, calculated by adding together the percentage contents of acetic acid and water determined by the methods described above.

Assay. Determine by liquid chromatography (2.4.14) as described in the Related substances with the following modifications.

Use method A for calcitonin (salmon) obtained by chemical synthesis and method B for calcitonin (salmon) obtained by a method based on rDNA technology.

Calculate the content of calcitonin (salmon), $C_{145}H_{240}N_{44}O_{48}S_2$.

Calcitonin intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 25 Endotoxin Units per mg.

Storage. Store protected from light at a temperature between 2° to 8°, if the substance is sterile, store in a sterile container protected from moisture.

Labelling. The label states the calcitonin peptide content and the origin: synthetic or rDNA technology.

Calcitonin (Salmon) Injection

Calcitonin (Salmon) Injection is a sterile solution of Calcitonin (Salmon) in Water for Injections.

Calcitonin (Salmon) Injection contains not less than 90.0 per cent and not more than 115.0 per cent of the stated amount of peptide $C_{145}H_{240}N_{44}O_{48}S_2$.

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Usual strengths. 50 IU per ml; 100 IU per ml; 200 IU per ml.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 3.9 to 4.5.

Calcitonin C. Determine by liquid chromatography (2.4.14).

Solvent mixture. 0.1 M sodium dihydrogen orthophosphate, adjusted to pH 4.0 with *orthophosphoric acid.*

Test solution. Dilute the volume of injection, if necessary with the solvent mixture to obtain 10 µg per ml of calcitonin (salmon).

Reference solution (a). A 0.001 per cent w/v solution of *calcitonin (salmon) IPRS* in the solvent mixture.

Reference solution (b). Heat the injection at 75° for 15 hours and, if necessary, dilute to obtain 10 µg per ml of calcitonin (salmon).

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) (Such as Vydac C18, 300 A wide pore column for proteins and peptides),
- column temperature: 40°,
- mobile phase: A. a mixture of 100 volumes of a 0.363 per cent w/v solution of *tetramethylammonium hydroxide pentahydrate* and 150 volumes of *acetonitrile*, adjusted to pH 2.5 with *orthophosphoric acid*,
  B. a mixture of 50 volumes of *acetonitrile* and 450 volumes of a 0.402 per cent w/v solution of *tetramethylammonium hydroxide pentahydrate*, adjusted to pH 2.5 with *orthophosphoric acid*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 200 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	35	65
21	57	43
22	35	65
30	35	65

The relative retention time with reference to calcitonin (salmon) for calcitonin C is about 0.5.

Inject reference solution (b). The test is not valid unless the resolution between the peaks corresponding to calcitonin C and calcitonin (salmon) is not less than 3.0.

Inject the test solution. The area of any peak corresponding to calcitonin C is not more than 7.0 per cent, calculated by area normalization.

Related peptides. Determine by liquid chromatography (2.4.14).

Test solution. Dilute the injection, if necessary with mobile phase A to obtain 10 µg per ml of calcitonin (salmon).

Reference solution. Dissolve the contents of a vial of *N-acetyl-cys[1] calcitonin IPRS* in 0.4 ml of mobile phase A, dilute to 40 ml with mobile phase A and add 0.1 ml of the test solution.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 65°,
- mobile phase: A. dissolve 3.26 g of *tetramethyl ammonium hydroxide pentahydrate* in 900 ml of *water*, adjusted to pH 2.5 with *orthophosphoric acid* and mix with 100 ml of *acetonitrile*,
  B. dissolve 1.45 g of *tetramethyl ammonium hydroxide pentahydrate* in 400 ml of *water*, adjusted to pH 2.5 with *orthophosphoric acid* and mix with 600 ml of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 200 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	72	28
30	48	52
32	72	28
55	72	28

The relative retention time with reference to calcitonin (salmon) for N-acetylcys[1] calcitonin is about 1.15.

Inject the reference solution. The test is not valid unless the resolution between the peaks corresponding to calcitonin and N-acetyl-cys[1] calcitonin is not less than 5.0 and the tailing factor for the N-acetyl-cys[1] calcitonin peak is not more than 2.5.

Inject the test solution. The area of any secondary peak is not more than 3.0 per cent of the total area of all the peaks. The sum of the areas of any such peaks is not more than 5.0 per cent of the total area of all the peaks, calculated by area normalisation. Ignore any peak with an area less than 0.1 per cent of that of the principal peak.

Bacterial endotoxins (2.2.3). Not more than 0.625 Endotoxin Unit per unit of calcitonin (salmon).

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

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Assay. Determine by liquid chromatography (2.4.14) as described in the test for Calcitonin C.

Calculate the content of calcitonin (salmon) in the injection.

Storage. Store protected from light, at a temperature of 2° to 8°.

Labelling. The label stated the strength as the number of IU (units) per ml. The label also states the equivalent number of micrograms of peptide per ml.

Erythropoietin Concentrated Solution

APPRLICDSR	VLERYLLEAK	EAENITTGCA
EHCSLNENIT	VPDTKVNFYA	WKRMEVGQQA
VEVWQGLALL	SEAVLRGQAL	LVNSSQPWEP
LQLHVDKAVS	GLRSLTTLLR	ALGAQKEAIS
PPDAASAAPL	RTITADTFRK	LFRVYSNFLR
GKLKLYTGEA	CRTGD	

Mol. Wt. 30,600 Da (approx.)

Erythropoietin Concentrated Solution contains a family of closely related glycoproteins which are not different from the naturally occurring human erythropoietin (urinary erythropoietin) in terms of the amino acid sequence (165 amino acids) and average glycosylation pattern, at a concentration of 0.5 mg per ml to 10 mg per ml. It has a potency of not less than 100000 IU per mg of active substance determined using the conditions described under assay and in the test for protein.

Erythropoietin is produced in rodent cells *in vitro* by a method based on recombinant DNA technology.

Prior to release, the following tests are carried out on each batch of the final bulk product, unless the regulatory authority has granted exemption.

Host cell-derived proteins (HCP). Not more than 100 ppm.

Host cell and vector-derived DNA. Not more than 10 ng per dose.

Description. A clear or slightly turbid and colourless to slightly yellowish solution.

Identification

A. It gives the appropriate response when examined using the conditions described under Assay.

B. Determine by isoelectric focusing (2.4.33).

Test solution. Dilute the preparation under examination in *water*, if necessary to produce 1 mg per ml and desalt the preparation, using a membrane filtration system suitable for desalting proteins.

Reference solution. Dissolve *erythropoietin IPRS* in *water* to produce a solution containing 1 mg per ml and desalt as above.

The isoelectric focusing procedure may be carried out using a 0.5 mm thick polyacrylamide slab gel containing ampholytes covering the pH of range 3 to 10.

Dilute the test solution to 0.5 mg per ml with *water*. Apply to the gel 10 µl of each solution. Carry out focusing for a further 30 minutes at the same power supply settings. Take the gel out of the focusing chamber, and transfer the proteins onto a membrane suitable for immobilization of proteins (such as Polyvinylidene Fluoride (PVDF) or Nitrocellulose), using commercially available electrotransfer equipment and following the manufacturer's instructions. After electrotransfer, incubate the membrane in a neutral isotonic buffer containing a suitable blocking agent (for example, dried milk powder, 50 g per litre), for 1 hour, followed by incubation in the same blocking solution with a suitable dilution of a polyclonal anti-erythropoietin antibody. Detect the erythropoietin-bound antibody using a suitable enzyme- or radiolabelled antibody (for example, an alkaline phosphatase-conjugated second antibody). The precise details of blocking agents, concentrations and incubation times should be optimized using the principles set out in Immunochemical methods.

The test is not valid unless the distribution of bands in the electrophoretogram obtained with the reference solution contains at least 6 well separated bands. If necessary, the voltage settings and duration may be altered to optimize the separation of the isoforms.

In the electrophoretogram obtained with the test solution, the pI range of the bands observed corresponds to that of the electrophoretogram obtained with the reference solution. The predominant bands correspond to isoforms 4, 5, 6 and 7. Additional, fainter bands corresponding to isoforms 1, 2, 3 and 8 may also be present. Other bands are present in no more than trace amounts.

C. Determine by capillary electrophoresis (capillary zone electrophoresis) (2.4.32).

All the solutions should be filtered through a 0.45 µm membrane filter before use.

Test solution. Dilute the substance under examination with *water* or concentrate it to obtain a concentration of 1 mg per ml. Desalt 0.25 ml of the solution by passage through a micro-concentrator cartridge provided with a membrane with a molecular mass cut-off not more than 10 kDa. Add 0.2 ml of *water* to the sample and desalt again. Repeat the desalting procedure once more. Dilute the sample with *water*, determine its protein concentration as described under Tests and adjust to a concentration of approximately 1 mg per ml with *water*.

Reference solution. Prepare 1 mg per ml solution of *erythropoietin IPRS*. Desalt the sample as described for the test solution.

Capillary system
- material: uncoated fused silica,
- size: effective length = about 100 cm, internal diameter = 50 µm,
- temperature: 35°,
- spectrophotometer set at 214 nm,
- injection: under pressure or vacuum.

CZE buffer concentrate (0.1 M sodium chloride, 0.1 M tricine, 0.1 M sodium acetate). Dissolve 0.584 g of *sodium chloride*, 1.792 g of *tricine* and 0.820 g of *anhydrous sodium acetate* in *water* and dilute to 100 ml with the same solvent.

1 M putrescine solution. Dissolve 0.882 g of *putrescine* in 10 ml of *water*. Distribute in 0.5 ml aliquots.

CZE buffer (0.01 M tricine, 0.01 M sodium chloride, 0.01 M sodium acetate, 7 M urea, 2.5 mM putrescine). Dissolve 21 g of *urea* in 25 ml of *water* by warming in a water-bath at 30°. Add 5.0 ml of *CZE buffer concentrate* and 125 ml of *1 M putrescine solution*. Dilute to 50 ml with *water*. Using *dilute acetic acid*, adjust the pH to 5.55 at room temperature and filter through a 0.45 µm membrane filter.

Set the auto sampler to store the samples at 4° during analysis.

Preconditioning of the capillary. Rinse the capillary for 60 minutes with *0.1 M sodium hydroxide* filtered through a 0.45 µm membrane filter and for 60 minutes with *CZE buffer*. Apply voltage for 12 hours (20 kV).

Between-run rinsing. Rinse the capillary for 10 minutes with *water*, for 5 minutes with *0.1 M sodium hydroxide* filtered through a 0.45 µm membrane filter and for 10 minutes with *CZE buffer*.

Migration. Apply a field strength of 143 V/cm (15.4 kV for capillaries of 107 cm total length) for 80 min, using *CZE buffer* as the electrolyte in both buffer reservoirs.

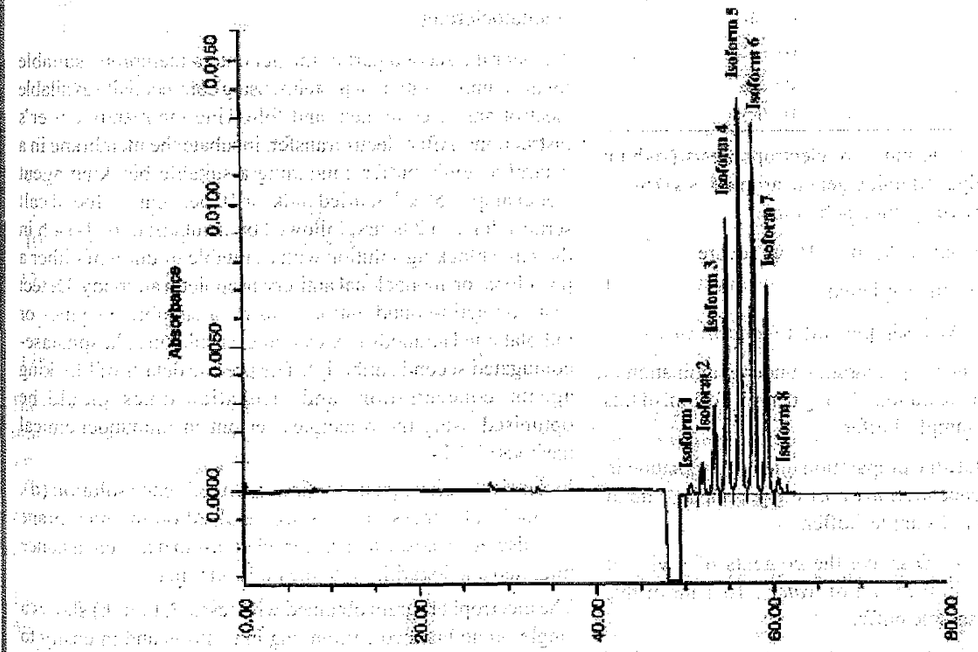

Fig.: Reference electropherogram of Erythropoietin

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In the electropherogram obtained with the reference solution, a pattern of well-separated peaks corresponding to the peaks in the reference electropherogram of *erythropoietin IPRS* is seen, and the largest peak is at least 50 times greater than the baseline noise. If necessary, adjust the sample load to give peaks of sufficient height. Identify the peaks corresponding to isoforms 1 to 8. The peak corresponding to isoform 8 is detected; the resolution between the peaks corresponding to isoforms 5 and 6 is not less than 1. Repeat the separation at least 3 times. The baseline is stable, showing little drift, and the distribution of peaks is qualitatively and quantitatively similar to the distribution of peaks in the *reference electropherogram of erythropoietin IPRS*. The relative standard deviation of the migration time of the peak corresponding to isoform 2 equal to or is less than 2 per cent.

Identify the peaks corresponding to isoforms 1 to 8 in the electropherogram obtained with the test solution by comparison with the electropherogram obtained with the reference solution. Calculate the percentage content of each isoform from the corresponding peak area.

The percentages are within the following ranges:

Isoform Number	Content (per cent)
1	0 – 15
2	0 – 15
3	1 – 20
4	10 – 35
5	15 – 40
6	10 – 35
7	5 – 25
8	0 – 15

D. Immunoblotting. Determine by electrophoresis (sodium dodecyl sulphate polyacrylamide gel electrophoresis) (SDS-PAGE) (2.4.12) followed by immunoblotting.

Gel dimensions.0.75 mm thick, about 16 cm square.

Resolving gel. 12 per cent acrylamide.

Sample buffer. SDS-PAGE sample buffer *(concentrated)*.

Test solution (a). Dilute the preparation under examination in *water* to obtain a concentration of 1 mg per ml. To 1 ml of this solution add 1 ml of sample buffer.

Test solution (b). Dilute the preparation under examination in *water* to obtain a concentration of 0.1 mg per ml. To 1 ml of this solution add 1 ml of sample buffer.

Reference solution (a). Dissolve the contents of a vial of *erythropoietin IPRS* in 0.25 ml of *water* . To 1 ml of this solution add 1 ml of sample buffer.

Reference solution (b). Dissolve the contents of a vial of *erythropoietin IPRS* in *water* and dilute with the same solvent to obtain a concentration of 0.1 mg per ml. To 1 ml of this solution add 1 ml of sample buffer.

Reference solution (c). A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 10-70 kDa.

Reference solution (d). A solution of pre-stained molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 10-70 kDa and suitable for the electrotransfer to an appropriate membrane.

Sample treatment. Boil for 2 minutes.

Application. 20 µl, in the following order: reference solution (c), reference solution (a), test solution (a), empty well, reference solution (b), test solution (b), reference solution (d).

At the end of the separation, remove the gel-cassette from the apparatus and cut the gel into 2 parts: the first part containing reference solution (c), reference solution (a) and test solution (a); the second part containing reference solution (b), test solution (b) and reference solution (d).

Detection. By Coomassie staining on the first part of the gel.

In the electropherogram obtained with *Reference solution (c)* the validation criteria are met.

The electropherogram obtained with test solution (a) shows a single diffuse band corresponding in position and intensity to the single band seen in the electropherogram obtained with reference solution (a).

Immunoblotting

Transfer the second part of the gel onto a membrane suitable for the immobilisation of proteins, using commercially available electrotransfer equipment and following the manufacturer's instructions. After electrotransfer, incubate the membrane in a neutral isotonic buffer containing a suitable blocking agent (for example, 50 g/l of dried milk or 10 per cent *v/v* foetal calf serum), for 1 to 2 hours, followed by incubation for 1-14 h in the same blocking solution with a suitable dilution of either a polyclonal or monoclonal anti-erythropoietin antibody. Detect erythropoietin-bound antibody using a suitable enzyme- or radiolabelled antibody (for example, an alkaline phosphatase-conjugated second antibody). The precise details of blocking agents, concentrations and incubation times should be optimised using the principles set out in immunochemical methods (2.2.14).

In the electropherogram obtained with reference solution (d), the molecular mass markers are resolved on the membrane into discrete bands, with a linear relationship between distance migrated and logarithm$_{10}$ of the molecular mass.

The electropherogram obtained with test solution (b) shows a single broad band corresponding in position and intensity to the single band seen in the electropherogram obtained with reference solution (b).

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E. Determine by Peptide mapping (2.3.47).

Test solution. Dilute the substance under examination in *tris-acetate buffer solution pH 8.5* to a concentration of 1 mg per ml. Equilibrate the solution in *tris-acetate buffer solution pH 8.5* using a suitable procedure (such as dialysis against *tris-acetate buffer solution pH 8.5*, or membrane filtration using the procedure described under Identification C, but reconstituting the desalted sample with *tris-acetate buffer solution pH 8.5*). Transfer the dialyzed solution to a polypropylene centrifuge tube. Freshly prepare a solution of *trypsin for peptide mapping* at a concentration of 1 mg per ml in *water*, and add 5 µl to 0.25 ml of the dialysed solution. Cap the tube and place in a water-bath at 37° for 18 hours. Remove the sample from the water-bath and stop the reaction immediately by freezing.

Reference solution. Prepare reference solution using *erythropoietin IPRS* as per the procedure given for the test solution, ensuring that all procedures are carried out simultaneously, and under identical conditions.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with butylsilyl silica gel (5-10 µm),
- mobile phase: A. 0.06 per cent v/v solution of *trifluoroacetic acid*,
  B. to 100 ml of *water* add 0.6 ml of *trifluoroacetic acid* and dilute to 1000 ml with *acetonitrile*,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 214 nm,
- injection volume: 50 µl.

Time (in min)	Flow rate (ml/min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0 – 10	0.75	100	0
10 – 125	0.75	100 → 39	0 → 61
125 – 135	1.25	39 → 17	61 → 83
135 – 145	1.25	17 → 0	83 → 100
145 – 150	1.25	100	0

The chromatogram obtained with each solution is qualitatively similar to the reference chromatogram of *erythropoietin IPRS* digest. The profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

F. Determine by *N*-terminal sequence analysis

The first 15 amino acids are: Alanine - Proline - Proline - Arginine - Leucine - Isoleucine - (no recovered peak) - Aspartic acid - Serine - Arginine - Valine - Leucine - Glutamic acid - Arginine - Tyrosine.

Perform the Edman degradation using an automated solid-phase sequencer, operated in accordance with the manufacturer's instructions.

Desalt the equivalent of 50 µg of erythropoietin. For example, dilute a volume of the substance under examination containing 50 µg of the active substance in 1 ml of a 0.1 per cent v/v solution of *trifluoroacetic acid*. Pre-wash C18 reverse-phase sample preparation cartridge according to the instructions supplied by the manufacturer and equilibrate the cartridge in a 0.1 per cent v/v solution of *trifluoroacetic acid*. Apply the sample to the cartridge, and wash successively with a 0.1 per cent v/v solution of *trifluoroacetic acid* containing 0 per cent, 10 per cent and 50 per cent v/v of *acetonitrile* according to the manufacturer's instructions. Lyophilise the 50 per cent v/v *acetonitrile* eluate.

Re-dissolve the desalted sample in 50 µl of a 0.1 per cent v/v solution of *trifluoroacetic acid* and couple to a sequencing cartridge using the protocol provided by the manufacturer. Run 15 sequencing cycles, using the reaction conditions for proline when running the second and third cycles.

Identify the phenylthiohydantoin (PTH)-amino acids released at each sequencing cycle by reverse-phase liquid chromatography. The procedure may be carried out using the column and reagents recommended by the manufacturer of the sequencing equipment for the separation of PTH-amino-acids.

The separation procedure is calibrated using:

- the mixture of PTH-amino acids provided by the manufacturer of the sequencer, with the gradient conditions adjusted as indicated to achieve optimum resolution of all amino acids,
- a sample obtained from a blank sequencing cycle obtained as recommended by the equipment manufacturer.

Tests

Protein. 80 per cent to 120 per cent of the stated amount.

Test solution. Dilute the substance under examination in a 0.4 per cent w/v solution of *ammonium hydrogen carbonate or the appropriate blank solution.*

When examined in the range of 250 nm to 400 nm (2.4.7), the solution shows an absorption maximum between 276 nm and 280 nm. After correction of any light scattering measured up to 400 nm, calculate the content of erythropoietin taking the specific absorbance to be 7.43.

Dimers and related substances of higher molecular mass

A. Determine by size-exclusion chromatography (2.4.16).

Test solution. Dilute the substance under examination in the mobile phase to obtain a concentration of 0.2 mg per ml.

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Reference solution. To 0.02 ml of the test solution add 0.98 ml of the mobile phase (2 per cent).

Chromatographic system

- a stainless steel column 60 cm x 7.5 mm, packed with hydrophilic silica gel, of a grade suitable for fractionation of globular proteins in the molecular mass range of 20000 to 200000,
- mobile phase: dissolve 1.15 g of *anhydrous disodium hydrogen phosphate*, 0.2 g of *potassium dihydrogen phosphate* and 23.4 g of *sodium chloride* in 1 liter of *water (1.5 mM potassium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate, 0.4 M sodium chloride, pH 7.4)*; adjusted to pH 7.4, if necessary,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 100 µl.

Inject the test solution and the reference solution. Continue the chromatography for 1 hour. The area of the principal peak in the chromatogram obtained with the reference solution is 1.5 to 2.5 per cent of the area of the principal peak in the chromatogram obtained with the test solution.

The total area of any peaks eluted before the principal peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (2 per cent).

B. Determine by electrophoresis (sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12).

Separating gel. 12 per cent

Samples include:

1.	Test sample	10 µg
2.	*Reference standard IPRS*	10 µg
3.	Marker for non-reducing gel	20 µl

The dye front is run for at least 80 per cent of the total gel length. Molecular weight markers are resolved on the gel into discrete bands, with a linear relationship between distance migrated and logarithm 10 of the molecular mass. The single diffuse band of the test solution and of the reference solution match in position and intensity.

Sialic acids. Not less than 10 moles of Sialic acids (calculated as *N*-acetylneuraminic acid) per mole of erythropoietin, determined in the following manner.

Test solution (a). Dilute the preparation under examination in the mobile phase used in the test for dimers and related substances of higher molecular mass to obtain a concentration of 0.3 mg per ml.

Test solution (b). To 0.5 ml of test solution (a) add 0.5 ml of the mobile phase used in the test for dimers and related substances of higher molecular mass.

Reference solution (a). Dissolve a suitable amount of *N-acetylneuraminic acid* in *water* to produce a solution containing 0.1 mg per ml.

Reference solution (b). To 0.8 ml of reference solution (a) add 0.2 ml of *water*.

Reference solution (c). To 0.6 ml of reference solution (a) add 0.4 ml of *water*.

Reference solution (d). To 0.4 ml of reference solution (a) add 0.6 ml of *water*.

Reference solution (e). To 0.2 ml of reference solution (a) add 0.8 ml of *water*.

Reference solution (f). Use *water*.

Carry out the test in triplicate. Transfer 100 µl each of the test and reference solutions to 10 ml glass test tubes. To each tube add 1 ml of *resorcinol reagent*. Stopper the tubes and incubate at 100° for 30 minutes. Cool on ice. To each tube, add 2 ml of a mixture of 12 volumes of *butanol* and 48 volumes of *butyl acetate*. Mix vigorously, and allow the 2 phases to separate. Ensuring that the upper phase is completely clear, remove the upper phase, taking care to exclude completely any of the lower phases. Measure the absorbance of all samples at 580 nm (2.4.7).

Using the calibration curve generated by the reference solutions, determine the content of sialic acids in each of the two test solutions and calculate the mean. Calculate the number of moles of sialic acids per mole of erythropoietin assuming that the relative molecular mass of erythropoietin is 30,600 and that the relative molecular mass of *N*-acetylneuraminic acid is 309.

The individual replicates agree to within ±10 per cent of each other; the value obtained from reference solution (a) is between 1.5 and 3.3 times that obtained with test solution (a).

Bacterial endotoxins (2.2.3). Not more than 20 Endotoxin Units in the volume that contains 100 000 IU of erythropoietin.

Assay

The activity of the preparation is compared with that of *erythropoietin IPRS* and expressed in International Units (IU).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The fiducial limits of error of the estimated potency ($P = 0.95$) are not less than 64 per cent and not more than 156 per cent of the stated potency.

Determine by Method A or Method B.

A. *In polycythaemic mice*

The activity of the preparation is estimated by examining, under given conditions, its effect in stimulating the

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incorporation of ^{59}Fe into circulating red blood cells of mice made polycythaemic by exposure to reduced atmospheric pressure.

The following schedule, using treatment in a hypobaric chamber, has been found to be suitable.

Induce polycythaemia in female mice of the same strain, weighing 16-18 g. Place the mice in a hypoxic chamber and reduce the pressure to 0.6 atmospheres. After 3 days at 0.6 atmospheres, further reduce the pressure to 0.4-0.5 atmospheres and maintain the animals at this pressure for a further 11 days (the partial vacuum is interrupted daily for a maximum of 1 hour at about 11:00 A.M., in order to clean the cages and feed the animals). At the end of the specified period, return the mice to normal atmospheric conditions. Randomly distribute the mice into cages, each containing 6 animals, and mark them.

Test solution (a). Dilute the substance to be examined in *phosphate-albumin buffered saline pH 7.2* to obtain a concentration of 0.2 IU per ml.

Test solution (b). Mix equal volumes of test solution (a) and *phosphate-albumin buffered saline pH 7.2.*

Test solution (c). Mix equal volumes of test solution (b) and *phosphate-albumin buffered saline pH 7.2.*

Reference solution (a). Dissolve *erythropoietin IPRS* in *phosphate-albumin buffered saline pH 7.2* to obtain a concentration of 0.2 IU per ml.

Reference solution (b). Mix equal volumes of reference solution (a) and *phosphate- albumin buffered saline pH 7.2.*

Reference solution (c). Mix equal volumes of reference solution (b) and *phosphate albumin buffered saline pH 7.2.*

Radiolabelled ferric [^{59}Fe] chloride solution, concentrated. Use a commercially available solution of [^{59}Fe]ferric chloride (approximate specific activity: 100-1000 MBq per mg of Fe).

Radiolabelled [^{59}Fe]ferric chloride solution. Dilute the concentrated radiolabelled [^{59}Fe]ferric chloride solution in *sodium citrate buffer solution pH 7.8* to obtain a solution with an activity of 3.7×10^4 Bq per ml.

The concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

3 days after returning the animals to atmospheric pressure, inject each animal subcutaneously with 0.2 ml of one of the solutions. The 6 animals in each cage must each receive one of the 6 different treatments (3 test solutions and 3 reference solutions), and the order of injection must be separately randomised for each cage. A minimum of 8 cages is recommended. 2 days after injection of the test or reference solution, inject each animal intraperitoneally with 0.2 ml of radiolabelled [^{59}Fe] ferric chloride solution. The order of the injections must be the same as that of the erythropoietin injections, and the time interval between administration of the erythropoietin and the radiolabelled ferric chloride solution must be the same for each animal. After a further 48 h, anaesthetise each animal by injection of a suitable anaesthetic, record body weights and withdraw blood samples (0.65 ml) into haematocrit capillaries from the bifurcation of the aorta. After determining the packed cell volume for each sample, measure the radioactivity.

Calculate the response (percentage of iron-59 in total circulating blood) for each mouse using the expression:

$$\frac{A_s \times M \times 7.5}{A_t \times V_s}$$

Where, A_s = radioactivity in the sample,
A_t = total radioactivity injected,
7.5 = total blood volume as per cent body weight,
M = body weight, in grams,
V_s = sample volume.

Calculate the potency by the usual statistical methods for a parallel line assay. Eliminate from the calculation any animal where the packed cell volume is less than 54 per cent, or where the body weight is more than 24 g.

B. *In normocythaemic mice*

The assay is based on the measurement of stimulation of reticulocyte production in normocythaemic mice.

Test solution (a). Dilute the substance under examination in *phosphate-albumin buffered saline pH 7.2* to obtain a concentration of 80 IU per ml.

Test solution (b). Mix equal volumes of test solution (a) and *phosphate-albumin buffered saline pH 7.2.*

Test solution (c). Mix equal volumes of test solution (b) and *phosphate-albumin buffered saline pH 7.2.*

Reference solution (a). Dissolve *erythropoietin IPRS* in *phosphate-albumin buffered saline pH 7.2* to produce a solution containing 80 IU per ml.

Reference solution (b). Mix equal volumes of reference solution (a) and *phosphate-albumin buffered saline pH 7.2.*

Reference solution (c). Mix equal volumes of reference solution (b) and *phosphate-albumin buffered saline pH 7.2.*

The exact concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

At the beginning of the assay procedure, randomly distribute mice of a suitable age and strain (8-week old B6D2F1 mice are suitable. Other strains like Swiss Albino, Balb/C of suitable

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age can also be used) into 6 cages. A minimum of 8 mice per cage is recommended. Inject each animal subcutaneously with 0.5 ml of the appropriate treatment (one solution per cage) and put the animal in a new cage. Combine the mice in such a way that each cage housing the treated mice contains one mouse out of the 6 different treatments (3 test solutions and 3 reference solutions, 6 mice per cage). 4 days after the injections, collect blood samples from the animals and determine the number of reticulocytes using the following procedure.

The volume of blood, dilution procedure and fluorescent reagent may need to be modified to ensure maximum development and stability of fluorescence.

Colorant solution, concentrated. Use a solution of thiazole orange suitable for the determination of reticulocytes. Prepare at a concentration twice that necessary for the analysis.

Proceed with the following dilution steps. Dilute whole blood 500-fold in the buffer used to prepare the colorant solution. Dilute this solution 2-fold in the concentrated colorant solution. After staining for 3-10 min, determine the reticulocyte count microfluorometrically in a flow cytometer. The percentage of reticulocytes is determined using a biparametric histogram: number of cells per red fluorescence (620 nm).

Calculate the potency by the usual statistical methods for a parallel line assay.

Storage. Store in an airtight container at a temperature below -20°. Avoid repeated freezing and thawing.

Labeling. The label states (1) the erythropoietin content in mg per ml; (2) the activity in International units per millilitre; (3) the name and the concentration of any other excipients.

Erythropoietin for Injection

Erythropoietin for Injection is a freeze dried sterile preparation prepared from erythropoietin concentrated solution. It is supplied in a sealed container.

Where the product contains human serum albumin, it does not comply with the test for Dimers and related substances of higher molecular weight; however the manufacturing process is validated to show that aggregation does not occur.

Usual strengths. 1000 IU per container, 2000 IU per container, 3000 IU per container, 4000 IU per container, 5000 IU per container, 6000 IU per container and 10,000 IU per container.

Identification

A. It gives the appropriate response when examined using the conditions described under Assay.

B. Determine by polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12) followed by Immunoblotting (2.2.14) using slab gels 0.75 mm thick or as appropriate and about 16 cm square (or 7 cm x 8 cm) and 12 per cent *acrylamide* as the resolving gel.

Test solution. Dissolve the injection under examination in *water* to give a solution containing 2000 IU per ml in *water* and then add 1 volume of SDS-PAGE sample buffer (concentrated) or 100-150 ng.

Reference solution (a). Dissolve the contents of a vial of *erythropoietin IPRS* in *water* to give a solution containing 3012 IU per ml and then add 1 volume of SDS- PAGE *sample buffer (concentrated).*

Reference solution (b). A solution of pre- stained molecular weight markers suitable for calibrating SDS-polyacrylamide gels in the range of 10 to 70 kDa and suitable for the electrotransfer to an appropriate membrane.

Boil reference solution and test solutions and reference solution (a) for 2 minutes. Apply 20 µl (or 15 µl for 7 cm x 8 cm slab gels) of the solutions separately to the surface of the gel in the following order: reference solution, reference solution (a) and test solution at the end of the separation, remove the gel cassette from the apparatus.

Immunoblotting is carried out as follows. Transfer the gel on to a membrane suitable for immobilization of proteins, using commercially available electrotransfer equipment following the manufacturer's instructions. After electrotransfer, incubate the membrane in a neutral isotonic buffer containing a suitable blocking agent (for example, 50 g per litre of dried milk or 10 per cent v/v foetal calf serum), for 1 to 2 hours, followed by incubation for 1 to 14 hours in the same blocking solution with a suitable dilution of either a polyclonal or monoclonal anti-erythropoietin antibody. Detect erythropoietin-bound antibody using a suitable enzyme or radiolabelled antibody or any other suitable method (for example, an alkaline phosphatase-conjugated second antibody). The precise details of blocking agents, concentrations and incubation times should be optimized using the principles set out in immunochemical methods (2.2.14).

The test is not valid unless, in the electropherogram obtained with reference solution (b), the molecular weight markers are resolved on the membrane into discrete bands, with a linear relationship between distance migrated and logarithm$_{10}$ of the molecular weight.

The electropherogram obtained with test solution shows a single broad band corresponding in position and intensity to the single band seen in the electropherogram obtained with reference solution (a).

Tests

pH (2.4.24). 6.4 to 7.4

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Dimers and related substances of higher molecular mass. Determine by using method A or B or C.

A. Determine by size-exclusion chromatography (2.4.16)

Test solution. Dissolve preparation under examination in the mobile phase to give solution containing 1000 IU of erythropoietin per ml

Reference solution. Dissolve 0.02 ml of the test solution in 0.98 ml of the mobile phase (2 per cent).

Chromatographic system

- a stainless steel column 60 cm x 7.5 mm, packed with hydrophilic silica gel, of a grade suitable for fractionation of globular proteins in the molecular mass range of 20000 to 200000,
- mobile phase: dissolve 1.15 g of *anhydrous disodium hydrogen phosphate*, 0.2 g of *potassium dihydrogen phosphate* and 23.4 g of *sodium chloride* in 1000 ml of *water* (*1.5 mM potassium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate, 0.4 M sodium chloride*, pH 7.4); adjusted to pH 7.4, if necessary,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 100 µl.

Inject the test solution and the reference solution. Continue the chromatography for 1 hour. The area of the principal peak in the chromatogram obtained with the reference solution is 1.5 to 2.5 per cent of the area of the principal peak in the chromatogram obtained with the test solution.

The total area of any peaks eluted before the principal peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (2 per cent).

B. Determine by size exclusion chromatography (2.4.16).

Citrate buffered saline. Dissolve 11.76 of *trisodium citrate* in 1800 ml of water, adjust to pH 7.0 with *1M hydrochloric acid* and add sufficient *water* to produce 2000 ml.

Test solution. Dilute the preparation under examination with the mobile phase to give a solution containing 1000 IU of erythropoietin per ml.

Reference solution (a). Dissolve 0.02 ml of test solution to 1 ml with the mobile phase.

Reference solution (b). Prepare aggregated erythropoietin by heating a 0.1 per cent w/v solution of *erythropoietin IPRS* in *citrate buffered saline* for 14 days at 55°. Dilute 0.1 ml to 1 ml with the mobile phase.

Chromatographic system

- a stainless steel column 60 cm x 7.5 mm, packed with hydrophilic silica gel for chromatography of a grade suitable for fractionation of globular proteins in the molecular weight range of 20000 to 200000,
- mobile phase: 0.115 per cent w/v of *anhydrous dihydrogen phosphate*, 0.02 per cent w/v of *potassium dihydrogen phosphate* and 2.34 per cent w/v of *sodium chloride* in *water*; if necessary, adjusted to pH 7.4,
- flow rate: 0.5 ml per minute,
- fluorimetric detection with an excitation wavelength of 280 nm and an emission wavelength of 340 nm,
- injection volume: 100 µl.

Inject reference solution (b). The test is not valid unless, in the chromatogram obtained with reference solution (b) aggregates are present, the resolution between the aggregate and monomer peaks is not less than 0.8 and relative standard deviation is not more than 10 per cent.

Inject reference solution (a) and test solution. In the chromatogram obtained with reference solution (a) the area of the principal peak is 1.5 to 2.5 per cent of the area of the principal peak in the chromatogram obtained with the test solution. In the chromatogram obtained with reference solution (a) the total area of any peaks eluting before the principal peak is not more than the area of the principal peak in the chromatogram obtained with test solution (2.0 per cent).

C. Determine by size exclusion chromatography (2.4.16).

Citrate buffered saline. Dissolve 11.76 of *trisodium citrate* in 1800 ml of *water*, adjusted to pH 7.0 with *1M hydrochloric acid* and add sufficient *water* to produce 2000 ml.

Solution A. Mix 2.28 per cent w/v *dipotassium hydrogen orthophosphate*, 1.49 per cent w/v *potassium chloride* adjusted pH to 6.9 with 85 per cent *orthophosphoric acid.*

Solution B. Mix 0.43 per cent w/v *sodium chloride*, 0.103 per cent w/v *sodium dihydrogen orthophosphate*, 0.223 per cent w/v *disodium hydrogen phosphate dehydrate*, 0.5 per cent w/v *glycine* and 0.03 per cent *polysorbate 80*, adjusted to pH 6.9.

Sample dilution buffer. The sample dilution buffer contains 2 volume of solution A and 1 volume of solution B.

Test solution. Dissolve the preparation under examination with the mobile phase to give a solution containing 1000 IU of erythropoietin per ml.

Reference solution (a). Dissolve 0.02 ml of test solution to 1 ml with the sample dilution buffer.

Reference solution (b). Prepare aggregated erythropoietin by heating a 0.1 per cent w/v solution of *erythropoietin IPRS* in *citrate buffered saline* for 14 days at 55°. Dilute 0.1 ml with the sample dilution buffer.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm packed with hydrophilic silica gel for chromatography of a grade suitable for fractionation of globular proteins in the molecular weight range of 20000 to 200000,
- mobile phase: 25 per cent v/v of *propan-2-ol* in solution A,

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– flow rate: 0.3 ml per minute,
– fluorimetric detection with an excitation wavelength of 280 nm and an emission wavelength of 340 nm,
– injection volume: 100 µl.

Inject reference solution (b). The test is not valid unless, the chromatogram obtained with reference solution, aggregates are present and the resolution factor between the aggregate and monomer peak is not less than 0.8.

Inject reference solution (a) and test solution. In the chromatogram obtained with test solution the total area of any peaks eluting before the principal peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent).

Water (2.3.43). Not more than 4.0 per cent w/w.

Bacterial endotoxins (2.2.3). Not more than 20 Endotoxin Units in a volume containing 10,000 IU of erythropoietin.

Other tests. Complies with the requirements for powder for injection or infusions stated under Parenteral Preparations.

Assay

In vivo assay

The activity of the preparation is compared with that of *erythropoietin IPRS* and expressed in International Units (IU).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The fiducial limits of error of the estimated potency ($P = 0.95$) are not less than 64 per cent and not more than 156 per cent of the stated potency.

Determine by Method A or Method B.

A. *In polycythaemic mice*

The activity of the preparation is estimated by examining, under given conditions, its effect in stimulating the incorporation of ^{59}Fe into circulating red blood cells of mice made polycythaemic by exposure to reduced atmospheric pressure.

The following schedule, using treatment in a hypobaric chamber, has been found to be suitable.

Induce polycythaemia in female mice of the same strain, weighing 16-18 g. Place the mice in a hypoxic chamber and reduce the pressure to 0.6 atmospheres. After 3 days at 0.6 atmospheres, further reduce the pressure to 0.4-0.5 atmospheres and maintain the animals at this pressure for a further 11 days (the partial vacuum is interrupted daily for a maximum of 1 hour at about 11:00 A.M., in order to clean the cages and feed the animals). At the end of the specified period, return the mice to normal atmospheric conditions. Randomly distribute the mice into cages, each containing 6 animals, and mark them.

Test solution (a). Dilute the substance under examination in *phosphate-albumin buffered saline pH 7.2* to obtain a concentration of 0.2 IU per ml.

Test solution (b). Mix equal volumes of test solution (a) and *phosphate-albumin buffered saline pH 7.2.*

Test solution (c). Mix equal volumes of test solution (b) and *phosphate-albumin buffered saline pH 7.2.*

Reference solution (a). Dissolve *erythropoietin IPRS* in *phosphate-albumin buffered saline pH 7.2* to obtain a concentration of 0.2 IU per ml.

Reference solution (b). Mix equal volumes of reference solution (a) and *phosphate-albumin buffered saline pH 7.2.*

Reference solution (c). Mix equal volumes of reference solution (b) and *phosphate-albumin buffered saline pH 7.2.*

Radiolabelled ferric [^{59}Fe] chloride solution, concentrated. Use a commercially available solution of [^{59}Fe]ferric chloride (approximate specific activity: 100-1000 MBq per mg of Fe).

Radiolabelled [^{59}Fe]ferric chloride solution. Dilute the *concentrated radiolabelled [^{59}Fe]ferric chloride solution* in *sodium citrate buffer solution pH 7.8* to obtain a solution with an activity of 3.7×10^4 Bq per ml.

The concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

3 days after returning the animals to atmospheric pressure, inject each animal subcutaneously with 0.2 ml of one of the solutions. The 6 animals in each cage must each receive one of the 6 different treatments (3 test solutions and 3 reference solutions), and the order of injection must be separately randomised for each cage. A minimum of 8 cages is recommended. 2 days after injection of the test or reference solution, inject each animal intraperitoneally with 0.2 ml of *radiolabelled [^{59}Fe] ferric chloride solution.* The order of the injections must be the same as that of the erythropoietin injections, and the time interval between administration of the erythropoietin and the radiolabelled ferric chloride solution must be the same for each animal. After a further 48 h, anaesthetise each animal by injection of a suitable anaesthetic, record body weights and withdraw blood samples (0.65 ml) into haematocrit capillaries from the bifurcation of the aorta. After determining the packed cell volume for each sample, measure the radioactivity.

Calculate the response (percentage of iron-59 in total circulating blood) for each mouse using the expression:

$$\frac{A_s \times M \times 7.5}{A_t \times V_s}$$

Where, A_s = radioactivity in the sample,

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A_t = total radioactivity injected,
7.5 = total blood volume as per cent body weight,
M = body weight, in grams,
V_s = sample volume.

Calculate the potency by the usual statistical methods for a parallel line assay. Eliminate from the calculation any animal where the packed cell volume is less than 54 per cent, or where the body weight is more than 24 g.

B. *In normocythaemic mice*

The assay is based on the measurement of stimulation of reticulocyte production in normocythaemic mice.

Test solution (a). Dilute the substance under examination in *phosphate-albumin buffered saline pH 7.2* to obtain a concentration of 80 IU per ml.

Test solution (b). Mix equal volumes of test solution (a) and *phosphate-albumin buffered saline pH 7.2.*

Test solution (c). Mix equal volumes of test solution (b) and *phosphate-albumin buffered saline pH 7.2.*

Reference solution (a). Dissolve *erythropoietin IPRS* in *phosphate-albumin buffered saline pH 7.2* to produce a solution containing 80 IU per ml.

Reference solution (b). Mix equal volumes of reference solution (a) and *phosphate-albumin buffered saline pH 7.2.*

Reference solution (c). Mix equal volumes of reference solution (b) and *phosphate-albumin buffered saline pH 7.2.*

The exact concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

At the beginning of the assay procedure, randomly distribute mice of a suitable age and strain (8-week old B6D2F1 mice are suitable. Other strains like Swiss Albino, Balb/C of suitable age can also be used) into 6 cages. A minimum of 8 mice per cage is recommended. Inject each animal subcutaneously with 0.5 ml of the appropriate treatment (one solution per cage) and put the animal in a new cage. Combine the mice in such a way that each cage housing the treated mice contains one mouse out of the 6 different treatments (3 test solutions and 3 reference solutions, 6 mice per cage). 4 days after the injections, collect blood samples from the animals and determine the number of reticulocytes using the following procedure.

The volume of blood, dilution procedure and fluorescent reagent may need to be modified to ensure maximum development and stability of fluorescence.

Colourant solution, concentrated. Use a solution of thiazole orange suitable for the determination of reticulocytes. Prepare at a concentration twice that necessary for the analysis.

Proceed with the following dilution steps. Dilute whole blood 500-fold in the buffer used to prepare the colourant solution. Dilute this solution 2-fold in the concentrated colourant solution. After staining for 3-10 min, determine the reticulocyte count microfluorometrically in a flow cytometer. The percentage of reticulocytes is determined using a biparametric histogram: number of cells/red fluorescence (530 nm).

Calculate the potency by the usual statistical methods for a parallel line assay.

Storage. Erythropoietin injection prepared by dissolving the contents of a sealed container in the liquid stated on the label should be used immediately after preparation but, in any case, within the period recommended by the manufacturer when prepared and stored strictly in accordance with the manufacturer's instructions.

Labeling. The label states (1) the erythropoietin content in IU or IU per ml; (2) the activity in International units per millilitre; (3) the name and the concentration of any other excipients.

Erythropoietin Injection

Erythropoietin Injection is a sterile solution of erythropoietin concentrated solution in a suitable liquid. It is either supplied as a ready-to-use solution.

Where the product contains human serum albumin, it does not comply with the test for dimers and related substances of higher molecular weight; however the manufacturing process is validated to show that aggregation does not occur.

Usual strengths. 1000 IU per container, 2000 IU per container, 3000 IU per container, 4000 IU per container, 5000 IU per container, 6000 IU per container and 10,000 IU per container.

Description. A clear and colourless solution virtually free from particles.

Identification

A. It gives the appropriate response when examined using the conditions described under Assay.

B. Determine by polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12) followed by Immunoblotting (2.2.14) using slab gels 0.75 mm thick or as appropriate and about 16 cm square (or 7 cm x 8 cm) and 12 per cent *acrylamide* as the resolving gel.

Test solution. Concentrate or dilute, if necessary, the injection under examination to give a solution containing 2000 IU per ml in water and then add 1 volume of SDS-PAGE sample buffer (concentrated) or 100-150 ng.

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Reference solution (a). Dissolve the contents of a vial of *erythro-poietin IPRS* in water to give a solution containing 3012 IU per ml and then add 1 volume of SDS- PAGE *sample buffer (concentrated).*

Reference solution (b). A solution of pre- stained molecular weight markers suitable for calibrating SDS-polyacrylamide gels in the range of 10 to 70 kDa and suitable for the electrotransfer to an appropriate membrane.

Boil reference solution and test solutions and reference (a) for 2 minutes. Apply 20 µl (or 15 µl for 7 cm x 8 cm slab gels) of the solutions separately to the surface of the gel in the following order: reference solution, reference solution (a) and test solution at the end of the separation, remove the gel cassette from the apparatus.

Immunoblotting is carried out as follows. Transfer the gel on to a membrane suitable for immobilization of proteins, using commercially available electrotransfer equipment following the manufacturer's instructions. After electrotransfer, incubate the membrane in a neutral isotonic buffer containing a suitable blocking agent (for example, 50 g per litre of dried milk or 10 per cent v/v foetal calf serum), for 1 to 2 hours, followed by incubation for 1 to 14 hours in the same blocking solution with a suitable dilution of either a polyclonal or monoclonal anti-erythropoietin antibody. Detect erythropoietin- bound antibody using a suitable enzyme or radiolabelled antibody or any other suitable method (for example, an alkaline phosphatase-conjugated second antibody). The precise details of blocking agents, concentrations and incubation times should be optimized using the principles set out in immunochemical methods (2.2.14).

The test is not valid unless, in the electropherogram obtained with reference solution (b), the molecular weight markers are resolved on the membrane into discrete bands, with a linear relationship between distance migrated and logarithm$_{10}$ of the molecular weight.

The electropherogram obtained with test solution shows a single broad band corresponding in position and intensity to the single band seen in the electropherogram obtained with reference solution (a).

Tests

pH (2.4.24). 6.4 to 7.4

Dimers and related substances of higher molecular mass. Determine by using method A or B or C.

A. Determine by size-exclusion chromatography (2.4.16)

Test solution. Dilute the preparation under examination in the mobile phase to obtain a concentration of 1000 IU per ml

Reference solution. Dissolve 0.02 ml of the test solution in 0.98 ml of the mobile phase (2 per cent).

Chromatographic system

- a stainless steel column 60 cm x 7.5 mm, packed with hydrophilic silica gel, of a grade suitable for fractionation of globular proteins in the molecular mass range of 20000 to 200 000,
- mobile phase: dissolve 1.15 g of *anhydrous disodium hydrogen phosphate*, 0.2 g of *potassium dihydrogen phosphate* and 23.4 g of *sodium chloride* in 1000 ml of *water* (*1.5 mM potassium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate, 0.4 M sodium chloride*, pH 7.4); adjusted to pH to 7.4, if necessary,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 100 µl.

Inject the test solution and the reference solution. Continue the chromatography for 1 hour. The area of the principal peak in the chromatogram obtained with the reference solution is 1.5 to 2.5 per cent of the area of the principal peak in the chromatogram obtained with the test solution.

The total area of any peaks eluted before the principal peak is not more than the area of the principal peak in the chromatogram obtained with the reference solution (2 per cent).

B. Determine by size exclusion chromatography (2.4.16).

Citrate buffered saline. Dissolve 11.76 of *trisodium citrate* in 1800 ml of *water*, adjusted to pH 7.0 with *1M hydrochloric acid* and add sufficient *water* to produce 2000 ml.

Test solution. Dilute the preparation under examination, if necessary, with the mobile phase to give a solution containing 1000 IU of erythropoietin per ml.

Reference solution (a). Dissolve 0.02 ml of test solution (a) to 1 ml with the mobile phase.

Reference solution (b). Prepare aggregated erythropoietin by heating a 0.1 per cent w/v solution of *erythropoietin IPRS* in *citrate buffered saline* for 14 days at 55°. Dilute 0.1 ml to 1 ml with the mobile phase.

Chromatographic system

- a stainless steel column 60 cm x 7.5 mm, packed with hydrophilic silica gel for chromatography of a grade suitable for fractionation of globular proteins in the molecular weight range of 20,000 to 200,000,
- mobile phase: 0.115 per cent w/v of *anhydrous dihydrogen phosphate*, 0.02 per cent w/v of *potassium dihydrogen phosphate* and 2.34 per cent w/v of *sodium chloride* in *water*; if necessary, adjusted to pH 7.4,
- flow rate: 0.5 ml per minute,
- fluorimetric detection with an excitation wavelength of 280 nm and an emission wavelength of 340 nm,
- injection volume: 100 µl.

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Inject reference solution (b). The test is not valid unless, in the chromatogram obtained with reference solution (b) aggregates are present, the resolution between the aggregate and monomer peaks is at least 0.8 and relative standard deviation is less than 10 per cent.

Inject reference solution (a) and test solution. In the chromatogram obtained with reference solution (a) the area of the principal peak is 1.5 to 2.5 per cent of the area of the principal peak in the chromatogram obtained with the test solution. In the chromatogram obtained with reference solution (a) the total area of any peaks eluting before the principal peak is not more than the area of the principal peak in the chromatogram obtained with test solution (2.0 per cent).

C. Determine by size exclusion chromatography (2.4.16).

Citrate buffered saline. Dissolve 11.76 g of *trisodium citrate* in 1800 ml of *water*, adjust to pH 7.0 with *1 M hydrochloric acid* and add sufficient *water* to produce 2000 ml.

Solution A. Mix 2.28 per cent w/v *dipotassium hydrogen orthophosphate*, 1.49 per cent w/v *potassium chloride* adjusted pH to 6.9 with 85 per cent *orthophosphoric acid.*

Solution B. Mix 0.43 per cent w/v *sodium chloride*, 0.103 per cent w/v *sodium dihydrogen orthophosphate*, 0.223 per cent w/v *disodium hydrogen phosphate dehydrate*, 0.5 per cent w/v *glycine* and 0.03 per cent *polysorbate 80* adjusted pH 6.9.

Sample dilution buffer. The sample dilution buffer contains 2 volume of solution A and 1 volume of solution B.

Test solution. Dilute the preparation under examination as given in the table below using the following solutions

Sample solution IU per ml	µg per ml	Dilution	Diluent	Final concentration of Polysorbate 80 (w/v) (per cent)	Injection volume in µl
2000	16.67	Undiluted	Not applicable	0.03	50
4000	33.33	Undiluted	Not applicable	0.03	50
10,000	83.33	1:3	Solution A	0.01	100
40,000	333.33	1:6	Sample dilution buffer	0.01	50

Reference solution (a). Dilute 0.02 ml of test solution (a) to 1 ml with sample dilution buffer.

Reference solution (b). Prepare aggregated erythropoietin by heating a 0.1 per cent w/v solution of *erythropoietin IPRS* in *citrate buffered saline* for 14 days at 55°. Dilute 0.1 ml with the sample dilution buffer.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm packed with hydrophilic silica gel for chromatography of a grade suitable for fractionation of globular proteins in the molecular weight range of 20,000 to 200,000,
- mobile phase: 25 per cent v/v of *propan-2-ol* in solution A,
- flow rate: 0.3 ml per minute,
- fluorimetric detection with an excitation wavelength of 280 nm and an emission wavelength of 340 nm,
- injection volume: 100 µl.

Inject reference solution (b). The test is not valid unless, the chromatogram obtained with reference solution, aggregates are present and the resolution factor between the aggregate and monomer peak is at least 0.8.

Inject reference solution (a) and test solution. In the chromatogram obtained with test solution the total area of any peaks eluting before the principal peak is not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent).

Bacterial endotoxins (2.2.3). Not more than 20 Endotoxin Units in a volume containing 10,000 IU of erythropoietin.

Other tests. Complies with the tests stated under Parenteral Preparations (Injections)

Assay

In vivo assay

The activity of the preparation is compared with that of *erythropoietin IPRS* and expressed in International Units (IU).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The fiducial limits of error of the estimated potency ($P = 0.95$) are not less than 64 per cent and not more than 156 per cent of the stated potency.

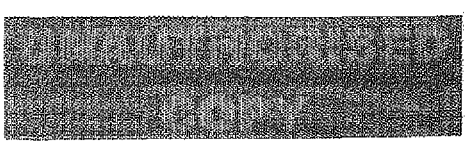
Determine by Method A or Method B.

A. *In polycythaemic mice*

The activity of the preparation is estimated by examining, under given conditions, its effect in stimulating the incorporation of ^{59}Fe into circulating red blood cells of mice made polycythaemic by exposure to reduced atmospheric pressure.

The following schedule, using treatment in a hypobaric chamber, has been found to be suitable.

Induce polycythaemia in female mice of the same strain, weighing 16-18 g. Place the mice in a hypoxic chamber and reduce the pressure to 0.6 atmospheres. After 3 days at 0.6 atmospheres, further reduce the pressure to 0.4-0.5 atmospheres and maintain the animals at this pressure for a further 11 days (the partial vacuum is interrupted daily for a maximum of 1 hour at about 11:00 AM, in order to clean the



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cages and feed the animals). At the end of the specified period, return the mice to normal atmospheric conditions. Randomly distribute the mice into cages, each containing 6 animals, and mark them.

Test solution (a). Dilute the substance under examination in *phosphate-albumin buffered saline pH 7.2* to obtain a concentration of 0.2 IU per ml.

Test solution (b). Mix equal volumes of test solution (a) and *phosphate-albumin buffered saline pH 7.2.*

Test solution (c). Mix equal volumes of test solution (b) and *phosphate-albumin buffered saline pH 7.2.*

Reference solution (a). Dissolve *erythropoietin IPRS* in *phosphate-albumin buffered saline pH 7.2* to obtain a concentration of 0.2 IU per ml.

Reference solution (b). Mix equal volumes of reference solution (a) and *phosphate- albumin buffered saline pH 7.2.*

Reference solution (c). Mix equal volumes of reference solution (b) and *phosphate- albumin buffered saline pH 7.2.*

Radiolabelled ferric [^{59}Fe] chloride solution, concentrated. Use a commercially available solution of [^{59}Fe]ferric chloride (approximate specific activity: 100-1000 MBq per mg of Fe).

Radiolabelled [^{59}Fe]ferric chloride solution. Dilute the concentrated radiolabelled [^{59}Fe]ferric chloride solution in *sodium citrate buffer solution pH 7.8* to obtain a solution with an activity of 3.7×10^4 Bq per ml.

The concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

3 days after returning the animals to atmospheric pressure, inject each animal subcutaneously with 0.2 ml of one of the solutions. The 6 animals in each cage must each receive one of the 6 different treatments (3 test solutions and 3 reference solutions), and the order of injection must be separately randomised for each cage. A minimum of 8 cages is recommended. 2 days after injection of the test or reference solution, inject each animal intraperitoneally with 0.2 ml of radiolabelled [^{59}Fe] ferric chloride solution. The order of the injections must be the same as that of the erythropoietin injections, and the time interval between administration of the erythropoietin and the radiolabelled ferric chloride solution must be the same for each animal. After a further 48 hours, anaesthetise each animal by injection of a suitable anaesthetic, record body weights and withdraw blood samples (0.65 ml) into haematocrit capillaries from the bifurcation of the aorta. After determining the packed cell volume for each sample, measure the radioactivity.

Calculate the response (percentage of iron-59 in total circulating blood) for each mouse using the expression:

$$\frac{A_s \times M \times 7.5}{A_t \times V_s}$$

Where, A_s = radioactivity in the sample,
A_t = total radioactivity injected,
7.5 = total blood volume as per cent body weight,
M = body weight, in grams,
V_s = sample volume.

Calculate the potency by the usual statistical methods for a parallel line assay. Eliminate from the calculation any animal where the packed cell volume is less than 54 per cent, or where the body weight is more than 24 g.

B. *In normocythaemic mice*

The assay is based on the measurement of stimulation of reticulocyte production in normocythaemic mice.

Test solution (a). Dilute the substance under examination in *phosphate-albumin buffered saline pH 7.2* to obtain a concentration of 80 IU per ml.

Test solution (b). Mix equal volumes of test solution (a) and *phosphate-albumin buffered saline pH 7.2.*

Test solution (c). Mix equal volumes of test solution (b) and *phosphate-albumin buffered saline pH 7.2.*

Reference solution (a). Dissolve *erythropoietin IPRS* in *phosphate-albumin buffered saline pH 7.2* to produce a solution containing 80 IU per ml.

Reference solution (b). Mix equal volumes of reference solution (a) and *phosphate-albumin buffered saline pH 7.2.*

Reference solution (c). Mix equal volumes of reference solution (b) and *phosphate-albumin buffered saline pH 7.2.*

The exact concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

At the beginning of the assay procedure, randomly distribute mice of a suitable age and strain (8-week old B6D2F1 mice are suitable. Other strains like Swiss Albino, Balb/C of suitable age can also be used) into 6 cages. A minimum of 8 mice per cage is recommended. Inject each animal subcutaneously with 0.5 ml of the appropriate treatment (one solution per cage) and put the animal in a new cage. Combine the mice in such a way that each cage housing the treated mice contains one mouse out of the 6 different treatments (3 test solutions and 3 reference solutions, 6 mice per cage). 4 days after the injections, collect blood samples from the animals and determine the number of reticulocytes using the following procedure.

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The volume of blood, dilution procedure and fluorescent reagent may need to be modified to ensure maximum development and stability of fluorescence.

Colourant solution, concentrated. Use a solution of thiazole orange suitable for the determination of reticulocytes. Prepare at a concentration twice that necessary for the analysis.

Proceed with the following dilution steps. Dilute whole blood 500-fold in the buffer used to prepare the colourant solution. Dilute this solution 2-fold in the concentrated colourant solution. After staining for 3 to10 minutes, determine the reticulocyte count microfluorometrically in a flow cytometer. The percentage of reticulocytes is determined using a biparametric histogram: number of cells/red fluorescence (530 nm).

Calculate the potency by the usual statistical methods for a parallel line assay.

Storage. Erythropoietin injection should be stored at 2° to 8° protected from light.

Labeling. The label states (1) the erythropoietin content in IU or IU per ml; (2) the activity in International units per millilitre; (3) the name and the concentration of any other excipients.

Filgrastim Concentrated Solution

Granulocyte Colony Stimulating Factor Solution

MTPLGPASSL PQSFLLKCLE QVRKIQGDGA ALQEKLCATY KLCHPEELVL LGHSLGIPWA
PLSSCPSQAL QLAGCLSQLH SGLFLYQGLL QALEGISPEL GPTLDTLQLD VADFATTIWQ
QMEELGMAPA LQPTQGAMPA FASAFQRRAG GVLVASHLQS FLEVSYRVLR HLAQP

$C_{845}H_{1339}N_{223}O_{243}S_9$ Mol. Wt. 18799

Filgrastim Concentrated Solution is a solution of a protein having the primary structure of the granulocyte-colony stimulating factor (G-CSF), produced and secreted by various human blood cell types and contains an additional amino acid, an N-terminal methionine (r-met HU G-CSF). The natural form is glycosylated while the recombinant form is non-glycosylated. The protein stimulates the differentiation and proliferation of leukocytes stem cells into mature granulocytes. It is produced by a method based on rDNA technology, using bacteria as host cells.

Filgrastim Concentrated Solution contains not less than 0.9 mg of protein per ml, and not less than 1.0×10^8 IU of filgrastim per mg of protein, contains not less than 80 per cent and not more than 125 per cent of the stated potency.

Prior to release, the following tests are carried out on each batch of the final bulk product, unless the regulatory authority has granted exemption.

Host cell derived proteins (HCP). Not more than 100 ppm.

Host cell or vector derived DNA. Not more than 10 ng per dose.

Category. Haemopoietic growth factor.

Description. A clear, colourless to slightly yellowish liquid.

Identification

A. It shows the biological activity as described under Assay.

B. Determine by isoelectric focusing (2.4.33).

In the test for Impurities with charges different from that of filgrastim the principal band in the electropherogram obtained with the test solution is similar in position to the principal band in the electropherogram obtained with the reference solution (a).

C. Determine by size-exclusion chromatography (2.4.16).

In the test for dimers and related substances of higher molecular mass, the retention time of the principal peak obtained with the test solution is similar to that of the principal peak obtained with the reference solution.

D. Determine by polyacrylamide gel electrophoresis under both reducing and non-reducing conditions (2.4.12).

In the test for impurities with molecular masses differing from that of rG-CSF under both reducing and non-reducing conditions, the principal band in the electropherogram obtained with test solution (a) is similar in position to the principal band in the electropherogram obtained with reference solution (b).

E. Determine by peptide mapping (2.3.47).

Test solution. Introduce 50 µl of a *0.05M sodium phosphate buffer pH 8.0* into a polypropylene tube. Add a volume of the substance under examination corresponding to 25 µg of protein, add 25 µl of a 0.1 mg per ml solution of glutamyl endopeptidase; dilute to100 µl with *water,* stopper the tube and incubate at about 37° for 18 hours. Add 125 µl of a 76.4 per cent w/v solution of *guanidine hydrochloride* and mix well. Add 10 µl of a 15.42 per cent w/v solution of *dithiothreitol* and mix well. Place the capped tube in boiling water for 1 minute. Allow to cool to room temperature.

Reference solution. Prepare at the same time and in the same manner as for the test solution but use *filgrastin IPRS* instead of the test preparation under examination.

Chromatographic system

- a stainless steel column 10 cm x 2.1 mm, packed with octadecylsilyl silica gel (5 µm) with a pore size of 20 nm,
- column temperature: 60°,
- mobile phase: A. dilute 0.5 ml of *trifluoroacetic acid* to 950 ml with *water*; add 50 ml of *acetonitrile*,

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B. dilute 0.5 ml of *trifluoroacetic acid* to 50 ml with *water*, add 950 ml of *acetonitrile*,

- flow rate: 0.2 ml per minute,
- A linear gradient programme using the conditions given below,
- spectrophotometer set at 215 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	97	3
8	94	6
25	66	34
40	10	90
45	10	90
46	97	3
65	97	3

Inject the test solution and the reference solution. The profile of chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

Tests

Impurities with molecular masses differing from that of Filgrastim. Determine by electrophoresis (sodium Dodecyl sulphate Polyacrylamide gel electrophoresis) (SDS-PAGE) (2.4.12) under both reducing and non-reducing conditions.

Resolving gel. 14 per cent Acrylamide

Sample buffer A (non-reducing conditions). Mix equal volumes of water and concentrated SDS-PAGE sample buffer.

Sample buffer B (reducing conditions). Mix equal volumes of water and concentrated SDS-PAGE sample buffer for reducing conditions containing 2-mercaptoethanol as the reducing agent.

Test solution (a). Dilute the preparation under examination with sample buffer A (non-reducing conditions) and sample buffer B (reducing conditions) to obtain a protein concentration of 100 µg per ml.

Test solution (b). To 0.20 ml of test solution (a) add 0.20 ml of sample buffer A or B.

Test solution (c). Dilute 0.20 ml of test solution (b) to 1.0 ml with sample buffer A or B.

Test solution (d). Dilute 0.20 ml of test solution (c) to 1.0 ml with sample buffer A or B.

Test solution (e). To 0.20 ml of test solution (d) add 0.20 ml of sample buffer A or B.

Reference solution (a). Solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 14.4-94 kDa.

Reference solution (b). Dilute filgrastim *IPRS* with sample buffer (A or B) to obtain a concentration of 100 µg per ml.

Sample treatment. boil for 5 minutes.

Apply 20 µl of each reduced and non-reduced solutions to separate gels.

Detection: Silver staining

The test is not valid unless the proteins of the molecular weight marker are distributed along 80 per cent of the gel and over the required separation range (the range covering the product and its dimer or the product and its related impurities); a band is seen in the electropherogram obtained with test solution (e), and a gradation of intensity is seen in the electropherogram obtained with test solutions (a) to (e).

In the electropherogram obtained with the test solution (a), no band other than the principal band is more intense than the principal band in the electropherogram obtained with test solution (d) (2 per cent).

Impurities with charges differing from that of Filgrastim. Determine by isoelectric focusing (2.4.33).

Test solution. Dilute the preparation under examination to produce a solution containing 0.3 mg per ml.

Reference solution (a). A solution of *filgrastim IPRS* containing 0.3 mg per ml.

Reference solution (b). A solution of *filgrastim IPRS* containing 0.03 mg per ml.

Reference solution (c). Use an isoelectric point (pI) calibration solution, in the pI range of 2.5-6.5, prepared according to manufacturer's instructions.

Focusing:

- pH gradient: 4.5 - 8.0,
- catholyte: *1 M sodium hydroxide*,
- anolyte: *0.04 M glutamic acid* in a 0.0025 per cent v/v solution of *phosphoric acid*,
- application: 20 µl.

Detection. Proceed as described in Isoelectric Focusing (2.4.33).

Detect the product and its related impurities.

In the electropherogram obtained with reference solution (c), relevant isoelectric point markers are distributed along the entire length of the gel. In the electropherogram obtained with reference solution (a), the pI of the principal band is 5.7 to 6.3.

In the electropherogram obtained with test solution no band other than the principal band is more intense than the principal

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band in the electropherogram obtained with reference solution (b) (10.0 per cent).

Related Proteins. Determine by method A or method B.

A. Determine by liquid chromatography (2.4.14)

Test solution. Dilute the preparation under examination with mobile phase A to obtain a concentration of 0.3 mg per ml

Reference solution (a). Dilute *filgrastim IPRS* with the same mobile phase to obtain a concentration of 0.3 mg per ml.

Reference solution (b). To 570 µl of reference solution (a), add 6.8 µl of a 0.45 per cent v/v solution of *hydrogen peroxide*; mix and incubate at 25° for 1 hour, then add 2.5 mg of *methionine IPRS*.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 65°,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 500 ml with *water* and add 499 ml of *acetonitrile*,
  B. dilute 1 ml of *trifluoroacetic acid* to 950 ml with *acetonitrile* and add 49 ml of *water*,
- flow rate: 1 ml per minute,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 215 nm,
- injection volume: 50 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	92	8
4	92	8
19	72	28
19.1	0	100
21	0	100
21.1	92	8
25	92	8

Inject reference solution (b). The profile of the chromatogram is similar to that of the chromatogram of oxidized filgrastim supplied with *filgrastim IPRS*. The chromatogram shows two peaks corresponding to oxidized filgrastim 1 and oxidized filgrastim 2 that elute before the principal peak, the second peak not being completely separated from the principal peak.

Inject reference solution (a) and test solution, in the chromatogram obtained with the test solution, the area of any peak other than the principal peak is not more than 2.0 per cent of the total area of all the peaks. The sum of the areas of all the peaks other than the principal peak is not more than 3.5 per cent of the total area of all of the peaks. Relative retention with reference to filgrastim (retention time is about 12 minutes) for oxidized filgrastim 2 is about 0.95.

B. Determine by liquid chromatography (2.4.14). Use the normalisation procedure.

Solution A. Dissolve 0.1 mg per ml of *polysorbate 80* and 50 mg per ml of *sorbitol in 0.1M sodium acetate buffer solution pH 4.0*.

Test solution. Dilute the preparation under examination with solution A to obtain a concentration of 0.2 mg per ml.

Reference solution (a). Dilute *filgrastim IPRS* with solution A to obtain a concentration of 0.2 mg per ml.

Reference solution (b). Add 2.0 µl of a 0.45 per cent solution of hydrogen peroxide to 500 µl of reference solution (a). Mix and incubate at 25° for 30 min, then add 1.5 mg of *L-methionine R*.

Chromatographic system

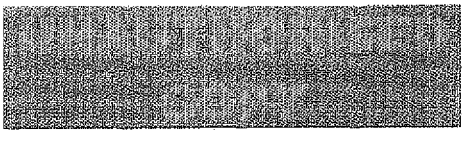
- a stainless steel column 25 cm x 4.6 mm, packed with butylsilyl silica gel (5 µm),
- column temperature: 60°,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 900 ml with *water* and add 100 ml of *acetonitrile*,
  B. dilute 1 ml of *trifluoroacetic acid* to 200 ml with *water* and add 800 ml of *acetonitrile*,
- flow rate: 0.6 ml per minute,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 215 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	34	66
35	27	73
50	10	90
60	34	66

Inject reference solution (b), the profile of the chromatogram obtained is similar to that of the chromatogram of oxidized filgrastim supplied with *filgrastim IPRS*. The chromatogram shows two peaks corresponding to oxidized filgrastim 1 and oxidized filgrastim 2 with the resolution of 1.5 between the peaks.

Inject reference solution (a) and test solution. In the chromatogram obtained with the test solution, the area of any peak other than the principal peak is not more than 2.0 per cent of the total area of all the peaks. The sum of the areas of all the peaks other than the principal peak is not more than 3.5 per cent of the total area of all of the peaks. Relative retention with reference to filgrastim (retention time is about 28 minutes) for oxidised form 1 is about 0.85, oxidised form 2 is about 0.95 and deamidated forms is about 1.1.

Dimers and Related Substance of Higher Molecular Mass. Determine by size-exclusion chromatography (2.4.16).



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Solution A. Dissolve 4.1 g of *sodium acetate* in 400 ml of *water*, adjust to pH 4.0 with *acetic acid* and dilute to 500 ml with *water*.

Test solution. Dilute the preparation under examination with solution A to obtain a concentration of 0.4 mg per ml.

Reference solution (a). Dilute *filgrastim IPRS* with solution A to obtain a concentration of 0.4 mg per ml.

Reference solution (b). Mix a sample of the reference solution for about 30 seconds using a vortex mixer.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm, packed with hydrophilic silica gel, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10,000 to 5,00,000,
- column temperature: 30°,
- mobile phase: dissolve 7.9 g of *ammonium hydrogen carbonate* in 1000 ml of *water* and adjust to pH 7.0 with *phosphoric acid*, dilute to 2000 ml with *water*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 20 µl.

Inject reference solution (a) and test solution. Relative retention times with reference to filgrastim monomer (retention time is about 19 minutes): aggregates is about 0.60, filgrastim oligomer 1 is about 0.75, filgrastim oligomer 2 is about 0.80 and filgrastim dimer is about 0.85.

Inject the reference solution (b). The retention time of filgrastim monomer is 17 minutes to 20 minutes. The resolution between the peaks due to filgrastim dimer and filgrastim monomer is not less than 3.0.

Calculate the content of dimer, oligomers and aggregates. The sum of the peaks with retention times less that that of the principal peak is not more than 2.0 per cent.

Bacterial endotoxin (2.2.3). Not more than 2.0 Endotoxin Units per mg of protein.

Assay

A. *Protein.* Determine by liquid chromatography (2.4.14) as described under the test for related proteins.

Inject the test solution and reference solution (a).

Calculate the content of Filgrastim ($C_{845}H_{1339}N_{223}O_{243}S_9$) from the declared content of $C_{845}H_{1339}N_{223}O_{243}S_9$ in *filgrastim IPRS*.

B. *Potency.* Determination of the biological activity of filgrastim concentrated solution is based on the stimulation of M-NFS-60 cells (murine Myeloblastic cell line) or any other suitable Myeloblastic cell line by filgrastim.

The following method uses the conversion of tetrazolium bromide (MTT or any other suitable dye) as a staining method. Alternative methods of quantifying cell proliferation, such as measurement of intracellular ATP by luciferase bioluminescence have also been found suitable, and may be used as the assay readout, subject to appropriate validation.

M-NFS-60 cells (murine Myeloblastic cell line) (ATCC No. CRL-1838) or any other suitable Myeloblastic cell line are incubated with varying dilution of test and reference preparations of filgrastim. They are then incubated with a solution of MTS, MTT, XTT or any other suitable dye. This cytochemical stain is converted by cellular dehydrogenase to a purple formazan product. The formazan is then measured spectrophoto-metrically. The potency of the test preparation is determined by comparison of the dilutions of the test preparation with the dilutions of the appropriate International Standard of filgrastim or with a reference preparation calibrated in International Units, which yield the same response (50 per cent maximal stimulation).

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International standard is stated by the World Health Organization.

Add 50 µl of the dilution medium to all wells of a 96-well microtitre plate. Add an additional 50 µl of this solution to the wells designed for blanks. Add 50 µl of each solution to be tested in triplicate (test preparation and reference preparation at a concentration of about 800 IU per ml, plus a series of 10 two fold dilutions to obtain a standard curve). Prepare a suspension of NFS-60 cells (murine Myeloblastic cell line) or any other suitable Myeloblastic cell line containing 7 x 10^5 cells per ml immediately before use, add 2-mercaptoethanol to a final concentration of 0.1 mM, and add 50 µl of the prepared cell suspension to each well, maintaining the cells in a uniform suspension during addition.

Incubate the plate at 37° ± 1° for a minimum of 46 ± 2 hours in a humidified incubator using 6 ± 1 per cent CO_2. Add 20 µl of a 5.0 g per litre sterile solution of Tetrazolium bromide to each well and re-incubate for 4 hours. Estimate the quantity of formazan produced using a microtitre well plate reader at 490 nm.

Analyze the data by fitting a sigmoidal dose-response curve to the data obtained and by using a suitable statistical method, for example the 4-parameter or parallel line models.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits (P=0.95) of the estimated potency are not less than 74 per cent and not more than 136 per cent of the stated potency.

Storage. Store protected from light in a refrigerator (2° to 8°).

Labelling. The label states the content, in mg of protein per ml; the potency, in IU per mg of protein.

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Filgrastim Injection

Filgrastim Injection is a sterile solution of a protein having the primary structure of the granulocyte colony-stimulating factor (G-CSF), produced and secreted by various human blood cell types and contains an additional amino acid, an N-terminal methionine (r-met HU G-CSF). The natural form is glycosylated while the recombinant form is non-glycosylated. The protein stimulates the differentiation and proliferation of leukocytes stem cells into mature granulocytes. It is produced by a method based on rDNA technology, using bacteria as host cells.

Filgrastim injection contains not less than 80 per cent and not more than 125 per cent of the stated amount of filgrastim (not less than 1.0×10^8 IU per mg of protein).

Usual strengths. 150 µg per ml; 300 µg per 0.5 ml; 300 µg per ml; 480 µg per ml

Description. A clear, colourless liquid, free from particles.

Identification

A. It shows the biological activity as described under Assay.

B. Determine by Size-exclusion chromatography (2.4.16).

In the test for dimers and related substances of higher molecular mass, the retention time of the principal peak obtained with the test solution is similar to that of the principal peak obtained with the reference solution.

C. Determine by Polyacrylamide gel electrophoresis under both reducing and non-reducing conditions (2.4.12).

In the test for impurities with molecular masses differing from that of filgrastim under both reducing and non-reducing conditions, the principal band in the electropherogram obtained with test solution (a) is similar in position to the principal band in the electropherogram obtained with reference solution (b).

Tests

pH (2.4.24). 3.5 to 4.5.

Impurities with molecular masses differing from that of Filgrastim. Determine by electrophoresis (sodium dodecyl sulphate polyacrylamide gel electrophoresis) under both reducing and non-reducing conditions (2.4.12).

Resolving gel. 14 per cent Acrylamide.

Sample buffer A (Non-reducing conditions). Mix equal volumes of water and concentrated SDS-PAGE sample buffer.

Sample buffer B (reducing conditions). Mix equal volumes of water and concentrated SDS-PAGE sample buffer for reducing conditions containing 2-mercaptoethanol as the reducing agent.

Test solution (a). Dilute the preparation under examination with sample buffer A for non-reducing conditions or sample buffer B for reducing conditions to obtain a protein concentration of 100 µg per ml.

Test solution (b). To 0.20 ml of test solution (a) add 0.20 ml of sample buffer A or B.

Test solution (c). Dilute 0.20 ml of test solution (b) to 1.0 ml with sample buffer A or B.

Test solution (d). Dilute 0.20 ml of test solution (c) to 1.0 ml with sample buffer A or B.

Test solution (e). To 0.20 ml of test solution (d) add 0.20 ml of sample buffer A or B.

Reference solution (a). Solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 14.4-94 kDa.

Reference solution (b). Dilute *filgrastim IPRS* with sample buffer (A or B) to obtain a concentration of 100 µg per ml.

Sample treatment. Boil for 5 minutes.

Apply 20 µl of each reduced and non-reduced solutions to separate gels.

Detection. Silver staining.

The test is not valid unless the proteins of the molecular weight marker are distributed along 80 per cent of the gel and over the required separation range (the range covering the product and its dimer or the product and its related impurities); a band is seen in the electropherogram obtained with test solution (e), and a gradation of intensity is seen in the electropherogram obtained with test solutions (a) to (e).

In the electropherogram obtained with the test solution (a), no band other than the principal band is more intense than the principal band in the electropherogram obtained with test solution (d).

Related Proteins. Determine by method A or method B.

A. Determine by liquid chromatography (2.4.14)

Solution A. Dissolve 0.1 mg per ml of *polysorbate 80* and 50 mg per ml of *sorbitol* in *0.1 M sodium acetate buffer solution pH 4.0*.

Test solution. Dilute the preparation under examination with solution A to obtain a concentration of 0.3 mg per ml.

Reference solution (a). Dilute *filgrastim IPRS* with the same solution A to obtain 0.3 mg per ml solution.

Reference solution (b). To 570 µl of reference solution (a), add 6.8 µl of a 0.45 per cent v/v solution of *hydrogen peroxide*; mix and incubate at 25° for 1 hour, then add 2.5 mg of *methionine IPRS*.

Chromatographic system

– a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm)

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- column temperature: 65°,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 500 ml with *water* and add 499 ml of *acetonitrile*,
  B. dilute 1 ml of *trifluoroacetic acid* to 950 ml with *acetonitrile* and add 49 ml of *water*,
- a linear gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	92	8
4	92	8
19	72	28
19.1	0	100
21	0	100
21.1	92	8
25	92	8

Inject reference solutions (a) and (b) and the test solution.

In the chromatogram obtained with reference solution (b) is similar to that of the chromatogram of oxidized filgrastim supplied with *filgrastim IPRS*. The chromatogram shows two peaks corresponding to oxidized filgrastim 1 and oxidized filgrastim 2 that elute before the principal peak, the second peak not being completely separated from the principal peak. Relative retention with reference to filgrastim (retention time is about 12 minutes) for oxidized filgrastim 2 is about 0.95.

In the chromatogram obtained with the test solution, the area of any peak other than the principal peak is not greater than 2.0 per cent of the total area of all the peaks. The sum of the areas of all the peaks other than the principal peak is not greater than 3.5 per cent of the total area of all of the peaks.

B. Determine by liquid chromatography (2.4.14) using normalisation procedure.

Solution A. Dissolve 0.1 mg per ml of *polysorbate 80* and 50 mg per ml of *sorbitol* in *0.1M sodium acetate buffer solution pH 4.0*.

Test solution. Dilute the preparation under examination with solution A to obtain a concentration of 0.2 mg per ml.

Reference solution (a). Dilute *filgrastim IPRS* with solution A to obtain a concentration of 0.2 mg per ml.

Reference solution (b). Add 2 µl of a 0.45 per cent solution of hydrogen peroxide to 500 µl of reference solution (a). Mix and incubate at 25° for 30 min, then add 1.5 mg of *L-methionine*.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with butylsilyl silica gel (5 µm) with pore size of 30 nm,
- column temperature: 60°,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 900 ml with *water* and add 100 ml of *acetonitrile*,
  B. dilute 1 ml of *trifluoroacetic acid* to 200 ml with *water* and add 800 ml of *acetonitrile*,
- a linear gradient programme using the conditions given below,
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	34	66
35	27	73
50	10	90
60	34	66

Inject reference solutions (a) and (b) and the test solution.

Relative retention with reference to filgrastim (retention time is about 28 min) for oxidised form 1 is about 0.85, oxidised form 2 is about 0.95 and deamidated forms is about 1.1.

The profile of the chromatogram obtained with reference solution (b) is similar to that of the chromatogram of oxidized filgrastim supplied with *filgrastim IPRS*. The chromatogram shows two peaks corresponding to oxidized filgrastim 1 and oxidized filgrastim 2 with the resolution of 1.5 between the peaks.

In the chromatogram obtained with the test solution, the area of any peak other than the principal peak is not more than 2.0 per cent of the total area of all the peaks. The sum of the areas of all the peaks other than the principal peak is not more than 3.5 per cent of the total area of all of the peaks.

Dimers and Related Substance of Higher Molecular Mass. Determine by size-exclusion chromatography (2.4.16).

Solution A. Dissolve 4.1 g of *sodium acetate* in 400 ml of *water*, adjust to pH 4.0 with *acetic acid* and dilute to 500 ml with *water*.

Test solution. Dilute the preparation under examination (if necessary) with solution A to obtain a protein concentration of 20 µg.

Reference solution (a). Dilute *filgrastim IPRS* with solution A to obtain a protein concentration of 20 µg.

Reference solution (b). Mix a sample of the reference solution for about 30 seconds using a vortex mixer.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm, packed with hydrophilic silica gel, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10,000 to 5,00,000,

- column temperature: 30°,
- mobile phase: dissolve 7.9 g of *ammonium hydrogen carbonate* in 1000 ml of *water* and adjust to pH 7.0 with *phosphoric acid*; dilute to 2000 ml with *water*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 15 µg.

Inject reference solution (a) and the test solution. Relative retention times with reference to filgrastim monomer (retention time is about 19 minutes) for aggregates is about 0.60, filgrastim oligomer 1 is about 0.75, filgrastim oligomer 2 is about 0.80 and filgrastim dimer is about 0.85.

Inject the reference solution (b). The retention time of filgrastim monomer is 17 minutes to 20 minutes. The resolution between the peaks due to filgrastim dimer and filgrastim monomer is not less than 3.0.

Calculate the content of dimer, oligomers and aggregates. The sum of the peaks with retention times before the principal peak is not more than 2.0 per cent.

Bacterial endotoxin (2.2.3). Less than 2 Endotoxin Units per mg of protein.

Other tests. Complies with the tests stated under Parenteral Preparations (Injections).

Assay

A. *Protein.* Determine by liquid chromatography (2.4.14) as described under the test for related proteins. Inject the test solution and reference solution (a). Calculate the content of Filgrastim ($C_{845}H_{1339}N_{223}O_{243}S_9$) from the declared content of $C_{845}H_{1339}N_{223}O_{243}S_9$ in *filgrastim IPRS*.

B. *Potency.* Determination of the biological activity of filgrastim injection is based on the proliferation of M-NFS-60 cells or any other suitable myeloblastic cell line by filgrastim injection.

The following method uses the conversion of Tetrazolium salt (MTS or any other suitable dye) as a staining method. Alternative methods of quantifying cell proliferation, such as measurement of intracellular ATP by luciferase bioluminescence have also been found suitable, and may be used as the assay readout, subject to appropriate validation.

M-NFS 60 (ATCC No. CRL-1838) or any other suitable myeloblastic cell line are incubated with varying dilution of test and reference preparations of filgrastim. After a timed incubation of cells with reference and test preparation, add MTS into assay plates. Cellular dehydrogenase enzyme converts MTS into a formazan product that is soluble in cell culture medium. The formazan is then measured spectrophotometrically. The absorbance value is directly proportional to the number of viable cells in culture and hence to the concentration of filgrastim applied. The biological activity of the test preparation is determined by comparison of the dilutions of the test preparation with the dilutions of the reference preparation.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International standard is stated by the World Health Organization.

Add 50 µl of each solution to be tested in triplicate (test preparation and reference preparation at a concentration of about 800 IU per ml, plus a series of 10 two fold dilutions to obtain a standard curve. Number of doses and concentration of doses can be adjusted). Prepare a cell suspension containing 7×10^5 cells per ml (adjust if necessary) immediately before use, add equal volume of *2-mercaptoethanol* to a final concentration of 0.1 mM and add 50 µl of the prepared cell suspension to each well, maintaining the cells in a uniform suspension during addition. Add 150 µl of media in wells designated for media blank and 50 µl of media plus 100 µl of cell suspension in wells selected for cell control.

Incubate the plate at 37° ± 1° for a minimum of 46 ± 2 hours (adjust if necessary) in a humidified incubator using 6 ± 1 per cent CO_2. Add 20 µl of a sterile MTS solution (0.092 per cent PMS and 0.5 per cent MTS in a ratio of 1:8 to each well and re-incubate for 4 hours. Estimate the quantity of formazan produced using a microtitre well plate reader at 490 nm.

Analyze the data by fitting a sigmoidal dose-response curve to the data obtained and by using a suitable statistical method, for example the 4-parameter or parallel line models.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits (P = 0.95) of the estimated potency are not less than 74 per cent and not more than 136 per cent of the stated potency.

Storage. Store protected from light in a refrigerator (2 ° to 8 °).

Labelling. The label states (1) the Filgrastim content in IU per ml or mcg per ml; (2) the activity in International units per milliliter; (3) the name and the concentration of any other excipient.

Follicle Stimulating Hormone

Follitropin

α-subunit

APDVQDCPECTLQENPFFSQPGAPILQCMG
CCFSRAYPTP 40

L*IPRS*KKTMLVQKNVTSESTCCVAKSYNRVTV
MGGFKVENHT 80

ACHCSTCYYHKS 92

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β-subunit

NSCELTNITIAIEKEECRFC ISINTTWCAG
YCYTRDLVYK 40

DPARPKIQKTCTFKELVYETVRVPGCAHHA
DSLYTYPVAT 80

QCHCGKCDSDSTDCTVRGLGPSYCSFGEMKE 111

Glycosylation sites:

Asn-52 (α-subunit), Asn-78 (α-subunit), Asn-7 (β-subunit), Asn-24 (β-subunit)

Disulfide bridges:

α-subunit : 7-31, 10-60, 28-82, 32-84, 59-87.

β-subunit : 3-51, 17-66, 20-104, 28-82, 32-84, 87-94.

Mol. Wt. approx. 30,000-40,000.

Follitropin is a freeze-dried preparation of a heterodimeric glycoprotein having the structure of human follicle-stimulating hormone (FSH). It consists of 2 subunits: a 92 amino acid α-chain common to other glycoprotein hormones and a specific 111 amino acid β-chain.

Follitropin is produced by a method based on recombinant DNA (rDNA) technology using mammalian cells as host cells. Follitropin has the potency of not less than 9000 IU and not more than 17 000 IU per milligram of protein.

Host cell derived proteins. Not more than 100 ppm.

Host cell or vector derived DNA. Not more than 10 ng per dose.

Category. Treatment of infertility in both men and women

Description. White or almost white powder.

Identification

A. It complies with the biological activity as described under Assay.

B. Determine by Isoelectric focusing (2.4.33)

Test solution. Dissolve the preparation under examination in *water* to obtain a concentration of about 2 mg per ml, then desalt and concentrate using a suitably validated procedure. Dissolve the recovered material in *water* to obtain a concentration of 5 mg per ml.

Reference solution. Dissolve the contents of a vial of *follitropin IPRS* in *water.* Desalt and concentrate using a suitably validated procedure. Dissolve the recovered material in *water* to obtain a concentration of 5 mg per ml.

Focusing:

- pH gradient: a combination of ampholytes and electrode buffers giving a functional separation in the isoelectric point pI range of 3.5-5.5 is selected as defined by the system suitability criteria; where pre-cast gels are employed, proprietary electrode solutions may be used in conjunctions; otherwise suitable dilute mineral or organic acids and bases are employed at pH levels respectively lower and higher than the functional range of ampholytes,
- catholyte: 2 per cent w/v solution of glycine,
- anolyte: a mixture containing 0.34 per cent w/v solution of *aspartic acid* and 0.36 per cent w/v solution of *glutamic acid*, adjusted to pH 2.8-3.8,
- application: 10 µl.

Detection. Proceed as described in Isoelectric Focusing (2.4.33).

In the electropherogram obtained with the reference solution, the number of bands seen in the pI region 3.5-5.5 corresponds to that shown in the electropherogram supplied with follitropin *IPRS*; the distribution of bands in the pI region 3.5-5.5 is qualitatively similar to that shown in the electropherogram supplied with *follitropin IPRS.*

The principal band in the electropherogram obtained with the test solution is similar in position to the principal band in the electropherogram obtained with the reference solution.

C. Examine the chromatograms in the test for follitropin oligomers.

In the chromatogram, the principal peak obtained with the test solution corresponds to the principal peak obtained with the reference solution.

D. Determine by Peptide mapping (2.3.47).

Separation of α and β subunits by Liquid Chromatography (2.4.14).

Test solution. Dissolve the preparation under examination in mobile phase A to obtain a concentration of about 0.4 mg per ml.

Reference solution. Dissolve the content of *follitropin IPRS* in mobile phase A to obtain a concentration of about 0.4 mg per ml.

Chromatographic system

- a stainless steel pre-column 2 cm x 4.0 mm, packed with butylsilyl silica gel (5 µm),
- a stainless steel column 25 cm x 4.6 mm, packed with butylsilyl silica gel (5 µm) with a pore size of 30 nm,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 1000 ml with *water*;
  B. a mixture of 0.9 volumes of *trifluoroacetic acid*, 50 volumes of *water* and 950 volumes of *acetonitrile*;
- a gradient programme using the conditions given below;
- flow rate: 1 ml per minute;

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– spectrophotometer set at 226 nm,
– injection volume: 800 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
2	100	0
8	76	24
17	76	24
36	70	30
41	25	75
46	25	75
47	100	0
57	100	0

Inject reference solution and test solution. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution. The retention time of β-subunit is about 14 minutes and α-subunit is about 30 minutes.

Collect the fractions containing the α- and β-subunits and freeze-dry them.

Reduction, Modification and Desalting of the Purified Subunits

Reduction and modification

Solution (a). Dilute 10 µl of *tributylphosphine* to 2 ml with *propanol.* Saturate with nitrogen.

Solution (b). Dilute 20 µl of *4-vinylpyridine* to 200 µl with *propanol.* Saturate with nitrogen.

Test solution. Dissolve each of the α- and β-subunit fractions obtained from the test solution in the previous step in 300 µl of *guanidine-tris (hydroxymethyl) aminomethane-EDTA buffer solution pH 8.5* and incubate at 37° for 60 minutes in a thermostatically controlled water-bath. Add 100 µl of solution (a), mix and saturate with nitrogen. Incubate at 37° for 90 minutes. Add 10 µl of solution (b), mix and saturate with nitrogen. Incubate at 37° for 45 minutes. Add 100 µl of a 10 per cent v/v solution of *trifluoroacetic acid* and mix.

Reference solution. Prepare at the same time as for the test solutions. Dissolve each of the α- and β-subunit fractions obtained from the reference solution in the previous step in 300 µl of *guanidine-tris (hydroxymethyl) aminomethane-EDTA buffer solution pH 8.5* and incubate at 37° for 60 minutes in a thermostatically controlled water-bath. Add 100 µl of solution (a), mix and saturate with nitrogen. Incubate at 37° for 90 minutes. Add 10 µl of solution (b), mix and saturate with nitrogen. Incubate at 37° for 45 minutes. Add 100 µl of a 10 per cent v/v solution of *trifluoroacetic acid* and mix.

Desalting

Dilute the α- and β-subunits obtained with the test solution and reference solutions each to 840 µl with mobile phase A.

Chromatographic system
– a stainless steel column 2 cm x 4.6 mm, packed with butylsilyl silica gel (5 µm),
– mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 1000 ml with *water,*
B. a mixture of 1 volume of *trifluoroacetic acid,* 300 volumes of *water* and 700 volumes of *acetonitrile,*
– a gradient programme using the conditions given below,
– flow rate: 1 ml per minute,
– spectrophotometer set at 226 nm,
– injection volume: 800 µl,
– retention time: *α-subunit solution*: monovinylpyridine-modified α-subunit = about 15 minutes; *β-subunit solution*: monovinylpyridine-modified β-subunit = about 16 minutes.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
7	100	0
27	0	100
27.01	100	0
32	100	0

Inject the reference solution and test solution. For each solution the chromatogram shows a principal peak due to the monovinylpyridine-modified subunit and several minor peaks due to the di- and oligovinylpyridine-modified subunits. The retention time of monovinylpyridine-modified α-subunit peak obtained with α-subunit solution is about 15 minutes and monovinylpyridine-modified β-subunit obtained with β-subunit solution is about 16 minutes.

Only the fraction containing the monovinylpyridine-modified subunits is used for digestion in the following step.

Collect the fractions containing the monovinylpyridine-modified subunits and freeze-dry them.

Selective Cleavage of the Peptide Bonds

Solution (c). Dissolve 480 g of *urea* in 600 ml of *water* and dilute to 1000 ml with the same solvent. Add about 3-5 g of mixed-bed resin and stir for about 1 hour. Filter through a glass filter before use.

Solution (d). Dissolve 15.8 g of *ammonium hydrogen carbonate* and 8.3 g of sodium *edetate* in 800 ml of *water.* Adjust to pH 7.8 with an 80 g per litre solution of *sodium hydroxide* and dilute to 1000 ml with *water.*

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Test solutions. Dissolve each of the modified α- and β-subunits obtained from the test solutions in the previous step in 42.5 µl of solution (c) and incubate at room temperature for 30 minutes. Add 42.5 µl of solution (d) and mix. To 42.5 µl of these solutions add 35 µl of a solution containing about 23 mU per µl of *endoproteinase Lys-C* and mix. Incubate at 37° for 4 hours, then add 35 µl of the same *endoproteinase Lys-C solution* and mix. Incubate at 37° overnight, then dilute to 420 µl with mobile phase A.

Reference solutions: Prepare at the same time as for the test solutions. Dissolve each of the modified α- and β-subunits obtained from the reference solutions in the previous step in 42.5 µl of solution (c) and incubate at room temperature for 30 minutes. Add 42.5 µl of solution (d) and mix. To 42.5 µl of these solutions add 35 µl of a solution containing about 23 mU per µl of endoproteinase Lys-C and mix. Incubate at 37° for 4 hour, then add 35 µl of the same endoproteinase Lys-C solution and mix. Incubate at 37° overnight, then dilute to 420 µl with mobile phase A.

Determine the subunits by chromatographic separation using Liquid chromatography (2.4.14).

Chromatographic system

- a stainless steel pre-column 2 cm x 4.6 mm, packed with octadecylsilyl silica gel (5 µm),
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilyl silica gel (5 µm) with a pore size of 30 nm,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 1000 ml with *water*,

  B. a mixture of 1 volume of *trifluoroacetic acid*, 300 volumes of *water* and 700 volumes of *acetonitrile*.
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 400 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
7	100	0
77	30	70
82	0	100
87	0	100
92	100	0
107	100	0

Inject the reference solution. The test is not valid unless (i) for *α-subunit*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of follitropin α-subunit digest supplied with *follitropin IPRS*; both chromatograms show peaks due to the L4, L6, L3, L5 and L1-2/L1 fragments; (ii) retention times obtained with the test and reference solutions differ by not more than 5 per cent for fragments L4, L6 and L3, not more than 3 per cent for fragment L5 and not more than 2 per cent for fragments L1-2/L1; (iii) for *β-subunit*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of follitropin β-subunit digest supplied with *follitropin IPRS*; both chromatograms show peaks due to the L5, L7, L6, and L1-4 fragments; (iv) retention times obtained with the test and reference solutions differ by not more than 5 per cent for fragment L5, not more than 2 per cent for fragments L7 and L6 and not more than 1 per cent for fragments L1-4.

Inject the test solution. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the corresponding reference solution.

E. Glycan analysis (2.2.21). Determine the glycan content by either method A or method B.

Method A

Protein Denaturation

Test solution. Dissolve 500 µg of the preparation under examination in 60 µl of *0.05 M phosphate buffer solution pH 7.5*. Add 6 µl of a 10 mg per ml solution of *sodium dodecyl sulphate* and 35 µl of a 1 per cent *v/v* solution of *2-mercaptoethanol*. Mix using a vortex mixer, centrifuge and incubate at 37° for 15 minutes.

Reference solution. Prepare at the same time of preparing test solution. Dissolve the contents of *follitropin IPRS* in 60 µl of *0.05 M phosphate buffer solution pH 7.5*. Add 6 µl of a 10 mg per ml solution of *sodium dodecyl sulphate* and 35 µl of a 1 per cent *v/v* solution of *2-mercaptoethanol*. Mix using a vortex mixer, centrifuge and incubate at 37° for 15 minutes.

Selective release of the glycans

Test solution. To the test solution obtained in the previous step add 0.75 µl of octylphenyl-polyethylene glycol and mix using a vortex mixer. Add 25 mU of *peptide N-glycosidase F*, mix using a vortex mixer and centrifuge. Incubate at 37° for 24 hours. Remove the protein fraction using a suitable, validated procedure. The following method has been found to be appropriate. Add 600 µl of *anhydrous ethanol*, previously cooled at -20° for 45 minutes. Mix using a vortex mixer and centrifuge. Precipitate the proteins at -20° for 15 minutes, then centrifuge at 10600 g at 4° for 5 minutes. Transfer the supernatant to a separate tube and evaporate the ethanol for 15 minutes. Add 1000 ml of particle-free *water* and resume evaporating until the remaining volume is about 500-800 µl, then freeze-dry.

Label the liberated glycans contained in the sample with *2-aminobenzamide*. The procedure employs a combination of reagents optimized and validated for the efficient labelling of

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glycans, and for the subsequent extraction and recovery of the labeled glycans from the reaction. Recover the sample in 1.5 ml of particle-free *water*.

Reference solution. Prepare at the same time as for the test solution. To the reference solution obtained in the previous step add 0.75 µl of octylphenyl-polyethylene glycol and mix using a vortex mixer. Add 25 mU of *peptide N-glycosidase F*, mix using a vortex mixer and centrifuge. Incubate at 37° for 24 hours. Remove the protein fraction using a suitable, validated procedure. Add 600 µl of *anhydrous ethanol*, previously cooled at -20° for 45 minutes. Mix using a vortex mixer and centrifuge. Precipitate the proteins at -20° for 15 minutes, then centrifuge at 10600 g at 4° for 5 minutes. Transfer the supernatant to a separate tube and evaporate the ethanol for 15 minutes. Add 1000 ml of *water* and resume evaporating until the remaining volume is about 500-800 µl, then freeze-dry.

Label the liberated glycans contained in the sample with 2-aminobenzamide. The procedure employs a combination of reagents optimized and validated for the efficient labelling of glycans, and for the subsequent extraction and recovery of the labeled glycans from the reaction. Recover the sample in 1.5 ml of particle-free water.

Determine the glycan content by chromatographic separation using Liquid chromatography (2.4.14).

Chromatographic system
- a stainless steel column 7.5 cm x 7.5 mm, packed with weak anion-exchange resin (10 µm),
- column temperature: 30°,
- mobile phase: A. *acetonitrile*,
  B. *0.5 M ammonium acetate buffer solution pH 4.5*,
  C. *water*,
- a gradient programme using the conditions given below,
- flow rate: 0.4 ml per minute,
- fluorimeter set at 330 nm for excitation and at 420 nm for emission,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)	Mobile phase C (per cent v/v)
0	20	0	80
5	20	0	80
21	20	4	76
61	20	25	55
62	20	50	30
71	20	50	30
72	20	0	80
117	20	0	80

Inject the reference solution. The test is not valid unless the chromatogram obtained is qualitatively similar to the chromatogram supplied with *follitropin IPRS*. Identify the peaks due to neutral, mono-, di-, tri- and tetra-sialylated forms by comparison with the chromatogram supplied with *follitropin IPRS*.

Inject the test solution. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution.

Determine the area of each peak and express it as a percentage of the total. Calculate the *Z* number using the following expression:

$$= (A_0 \times 0) + (A_1 \times 1) + (A_2 \times 2) + (A_3 \times 3) + (A_4 \times 4)$$

where, A_0 = peak area percentage due to the neutral form;
A_1 = peak area percentage due to the mono-sialylated form;
A_2 = peak area percentage due to the di-sialylated form;
A_3 = peak area percentage due to the tri-sialylated form;
A_4 = peak area percentage due to the tetra-sialylated form.

The Z number obtained for the reference solution and the test solution is not less than 177 and not more than 233.

Method B

Protein Denaturation

Solution (a). To 1.952 g of *2-[N-morpholino]ethanesulfonic acid* and 57.32 g of *guanidine hydrochloride*, add 1 ml of a 15.4 g per litre solution of *dithiothreitol*, 10 ml of an 18.61 g per litre solution of *sodium edetate* and 20 ml of *water*. Maintain in a water-bath at about 37° for 1 minute to dissolve the components. Adjust to pH 8.1 with 8.0 per cent solution of *sodium hydroxide* and dilute to 100 ml with *water*.

Solution (b). Dissolve 37 mg of iodoacetamide in 1 ml of *water* and mix. Protect from light.

Solution (c). Dissolve 26.7 g of *disodium hydrogen phosphate dihydrate* and 11.2 g of *sodium edetate* in 3 litres of *water* and mix. Adjust to pH 7.5 with a 40 g per litre solution of *sodium hydroxide*.

Test solution Dissolve 1 mg of the preparation under examination in 0.2 ml of solution (a) and incubate in a water-bath at 37 ± 1° for 2 hours. Add 20 µl of freshly prepared solution (b), mix and incubate at 37 ± 1° for a further 2 hours, protected from light. Add 10 µl of *2-mercaptoethanol* and mix. Dialyze against 1000 ml of solution (c). Add 200 µl of solution (c) and mix. Determine the protein content of the solution.

Reference solution (a). Dissolve 1 mg of the *follitropin IPRS* in 0.2 ml of solution (a) and incubate in a water-bath at 37 ± 1°

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for 2 hours. Add 20 µl of freshly prepared solution (b), mix and incubate at 37 ± 1° for a further 2 hours, protected from light. Add 10 µl of *2-mercaptoethanol* and mix. Dialyze against 1000 ml of solution (c). Add 200 µl of solution (c) and mix. Determine the protein content of the solution.

Reference solution (b). Dissolve 1 mg of fetuin in 0.2 ml of solution (a) and incubate in a water-bath at 37 ± 1° for 2 hours. Add 20 µl of freshly prepared solution (b), mix and incubate at 37 ± 1° for a further 2 hours, protected from light. Add 10 µl of *2-mercaptoethanol* and mix. Dialyze against 1000 ml of solution (c). Add 200 µl of solution (c) and mix. Determine the protein content of the solution.

Selective release of the glycans

Test solution. Dilute the test solution obtained in the previous step with solution (c) to obtain a concentration of 1.1 g per litre. Add 1 U of *peptide N-glycosidase F* to 500 µg of the solution, mix and incubate at 37 ± 1° for 24 hours. Place the solution in ice. Precipitate the protein and salts with 3 volumes of ice-cold *anhydrous ethanol* and allow to stand in ice for 10 minutes. Centrifuge at 16000 *g* for about 5 minutes and transfer the supernatant to a separate tube. Add 3 µl of a 1 µg per µl solution of *maltotriose*, then freeze-dry. Dissolve in 100 µl of *water*.

Reference solution (a). Dilute the reference solution obtained in the previous step with solution (c) to obtain a concentration of 1.1 g per litre. Add 1 U of *peptide N-glycosidase F* to 500 µg of the solution, mix and incubate at 37 ± 1° for 24 hours. Place the solution in ice. Precipitate the protein and salts with 3 volumes of ice-cold *anhydrous ethanol* and allow to stand in ice for 10 minutes. Centrifuge at 16000 *g* for about 5 minutes and transfer the supernatant to a separate tube. Add 3 µl of a 1 µg per µl solution of *maltotriose*, then freeze-dry. Dissolve in 100 µl of *water*.

Reference solution (b). Dilute the fetuin solution obtained in the previous step with solution (c) to obtain a concentration of 1.1 g per litre. Add 1 U of *peptide N-glycosidase F* to 500 µg of the solution, mix and incubate at 37 ± 1° for 24 hours. Place the solution in ice. Precipitate the protein and salts with 3 volumes of ice-cold *anhydrous ethanol* and allow to stand in ice for 10 minutes. Centrifuge at 16000 *g* for about 5 minutes and transfer the supernatant to a separate tube. Add 3 µl of a 1 µg per µl solution of *maltotriose*, then freeze-dry. Dissolve in 100 µl of *water*.

Determine the glycan content by chromatographic separation using Liquid chromatography (2.4.14).

Chromatographic system

- a stainless steel pre-column 5 cm x 4.0 mm, packed with strongly basic anion-exchange resin,
- a stainless steel column 25 cm x 4.0 mm, packed with strongly basic anion-exchange resin,
- mobile phase: A. 20 g per litre solution of *sodium hydroxide;* maintain under helium,
  B. *water;* maintain under helium,
  C. dissolve 41 g of anhydrous *sodium acetate* in 800 ml of *water*, dilute to 1000 ml with the same solvent, maintain under helium,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- detector: pulsed amperometric,
- injection volume: 45 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)	Mobile phase C (per cent v/v)
0	20	80	0
0.2	20	80	0
94.0	20	34	46
97.0	20	34	46
97.1	20	80	0
115.0	20	80	0

Inject reference solution (b). The test is not valid unless the chromatogram obtained with the reference solution (b) is qualitatively similar to the chromatogram for fetuin supplied with the *follitropin IPRS.*

Inject the reference solution (a) and test solution. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution (a) identify the peaks due to neutral, mono-, di-, tri- and tetra-sialylated forms in the chromatogram obtained with reference solution (b); determine the area of each peak and express it as a percentage of the total; calculate the Z number using the following expression:

$$= (A_0 \times 0) + (A_1 \times 1) + (A_2 \times 2) + (A_3 \times 3) + (A_4 \times 4)$$

where, A_0 = peak area percentage due to the neutral form;
A_1 = peak area percentage due to the mono-sialylated form;
A_2 = peak area percentage due to the di-sialylated form;
A_3 = peak area percentage due to the tri-sialylated form;
A_4 = peak area percentage due to the tetra-sialylated form.

The Z number obtained for the reference solution (b) is not less than 290 and not more than 325 and for the test solution is not less than 178 and not more than 274.

Tests

Follitropin oligomers. Determine by Size-exclusion chromatography (2.4.16) using the normalisation procedure.

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Solution (a). Dissolve 118 mg of *sodium dihydrogen phosphate*, 1.65 g of *disodium hydrogen phosphate dihydrate*, and 30.0 g of *sucrose* in 40 ml of *water* and dilute to 100 ml with the same solvent.

Solution (b). Dissolve 1 mg of *bovine albumin* in 30 ml of solution (a).

Test solution. Dissolve the preparation under examination in solution (a) to obtain a concentration of 0.25 mg per ml.

Reference solution (a). Dissolve *follitropin IPRS* in solution (a) to obtain a concentration of 0.25 mg per ml.

Reference solution (b). Dissolve the contents of a vial of *follitropin IPRS* in 200 µl of solution (a) and mix with the same volume of solution (b). If necessary, dilute further with solution (a) to obtain a concentration of 0.25 mg per ml.

Chromatographic system
- a stainless steel column 30 cm x 7.8 mm packed with hydrophilic silica gel of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10000 to 500000 (5 µm),
- mobile phase. a buffer solution prepared by dissolving 14.2 g of *anhydrous sodium sulphate* in 1000 ml of *0.1 M phosphate buffer solution pH 6.7*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 100 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to bovine albumin and follitropin is not less than 1.2.

Inject reference solution (a) and the test solution. The chromatogram obtained with the test solution should correspond to the chromatogram obtained with reference solution (a). The sum of the peaks with the retention time less than that of the principal peak is not more than 0.5 per cent of the total peaks area.

Free subunits. Determine by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12) under non-reducing conditions.

Gel dimensions. 1.5 mm thick.

Resolving gel. 12 per cent acrylamide.

Sample buffer. Concentrated SDS-PAGE sample buffer.

Test solution. Dissolve the preparation under examination in water to obtain a concentration of 2 µg per µl. To 55 µl of the solution add 55 µl of the sample buffer. Allow to stand for 4 hours at room temperature.

Reference solution (a). Dissolve the contents of a vial of *follitropin IPRS* in *water* to obtain a concentration of 2 µg per µl. To 25 µl of the solution add 25 µl of the sample buffer. To 40 µl of this solution add 180 µl of the sample buffer and 180 µl of *water*. Allow to stand for 4 hours at room temperature, then boil for 5 minutes.

Reference solution (b). A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 14.4-94 kDa.

Application:

Well	Solution(s)	Volume (µl)
1	Reference solution (a)	40
2	Reference solution (a)	30
3	Reference solution (a)	20
4	Reference solution (a)	15
5	Reference solution (a)	10
6	Reference solution (a)	5
7	Test solution	50
8	Test solution + Reference solution (a)	50 + 25
9	Reference solution (b)	10

Detection. By Coomassie staining.

The test is not valid unless (i) reference solution (b) meets the validation criteria (2.4.12); (ii) the bands corresponding to follitropin heterodimer and subunits obtained with the electropherogram of test solution and reference solution (a) are clearly separated; (iii) reference solution (a) shows no bands corresponding to follitropin heterodimer.

In the electropherogram obtained, the content of *free subunits* is not more than 3 per cent.

Oxidised follitropin. Determine the oxidised follitropin content by chromatographic separation using Liquid chromatography (2.4.14).

Solution (a). Dissolve 3.3 mg of *2,4-dichlorobenzoic acid* in 10.0 ml of *ethanol (96 per cent)*.

Test solution. Dilute the preparation under examination in *water* to obtain the concentration of 300 µg per ml.

Reference solution (a). Dilute the contents of *follitropin IPRS* in *water* to obtain a concentration of 300 µg per ml.

Reference solution (b). Dilute 0.1 ml of strong *hydrogen peroxide solution* to 30 ml with *water*. Dissolve the contents of a vial of *follitropin IPRS* in this solution to obtain a concentration of 300 µg per ml. Incubate for 30 to 45 minutes. Add 10 µl of solution (a) and inject immediately.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with butylsilyl silica gel (5 µm),
- column temperature: 30°,

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– mobile phase: A. *0.2 M phosphate buffer* solution adjusted to pH 2.5,
B. 40 volumes of *water* and 60 volumes of *acetronitrile*,
C. *water*,
– flow rate: 1 ml per minute,
– a gradient programme using the conditions given below,
– spectrophotometer set at 210 nm,
– injection volume: 25 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)	Mobile phase C (per cent v/v)
0	50	25	25
8.4	50	39	11
8.5	50	45	5
15	50	45	5
15.1	50	25	25
25	50	25	25

Inject reference solution (b). The test is not valid unless (i) the peaks due to the oxidised follitropin α- and β-subunits are separated from the peaks due to the non-oxidised follitropin subunits and from the peak due to 2,4-dichlorobenzoic acid; (ii) the chromatogram obtained is similar to the chromatogram supplied with *follitropin IPRS*.

Inject test solution. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with reference solution (b).

Calculate the percentage of oxidation of the follitropin subunits using the following expression:

$$\frac{(A_2 + A_4) \times 100}{A_1 + A_2 + A_3 + A_4}$$

where, A_1 = area of the peak due to the follitropin α-subunit;
A_2 = area of the peaks due to the oxidised follitropin α-subunit;
A_3 = area of the peak due to the follitropin β-subunit;
A_4 = area of the peak due to the oxidised follitropin β-subunit.

The total oxidized forms are not more than 6 per cent of follitropin.

Bacterial Endotoxins (2.2.3). Not more than 0.1 IU per International Unit of follitropin activity.

Assay

A. Determine by Size exclusion chromatography (2.4.16).

Solution (a). Dissolve 100 mg of *poloxamer 188* in 900 ml of *water* and dilute to 1000 ml with the same solvent.

Test solution. Dilute the preparation under examination on solution (a) to obtain a concentration of about 0.03 mg per ml.

Reference solution. Dilute the contents of a vial of *follitropin IPRS* in solution (a) to obtain a concentration of 0.03 mg per ml.

Chromatographic system
– a stainless steel column 30 cm x 7.8 mm packed with hydrophilic silica gel of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10000 to 500000 (5 µm),
– mobile phase: Mix 6.74 ml of *orthophosphoric acid*, 14.2 g of *anhydrous sodium sulphate* and 900 ml of *water*, pH adjusted to 6.7 with a 50 per cent w/v solution of *sodium hydroxide* and dilute to 1000 ml with *water*,
– flow rate: 1 ml per minute,
– spectrophotometer set at 214 nm,
– injection volume: 100 µl.

Inject the reference solution. The test is not valid unless the number of theoretical plates calculated for the peak of follitropin is not less than 1300.

Calculate the content of follitropin taking into account the assigned content of *follitropin IPRS*.

B. *Potency.* The follicle-stimulating activity of follitropin is estimated by comparing under given conditions its effect in enlarging the ovaries of immature rats treated with chorionic gonadotrophin with the same effect of the International Standard preparation of human recombinant follicle-stimulating hormone or of a reference preparation calibrated in International Units. The International Unit of FSH is the activity contained in stated amounts of the International Standard of human recombinant follicle-stimulating hormone. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Use immature female rats of the same strain, 19-28 days old, differing in age by not more than 3 days and having masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 6 equal groups of at least 5 rats. If sets of 6 litter mates are available, assign 1 litter mate from each set to each group and mark according to litter.

Choose three doses of the reference preparation and three doses of the preparation under examination such that the smallest dose produces a positive response in some of the rats and the largest dose does not produce a maximal response in all of the rats. Use doses in geometric progression and as an initial approximation total doses of 1.5 IU, 3.0 IU and 6.0 IU may be tried, although the dose will depend on the sensitivity of the rats used, which may vary widely.

Dissolve separately the total quantities of the preparation under examination and of the reference preparation corresponding to the daily doses to be used in sufficient

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phosphate-albumin buffered saline pH 7.2 such that the daily dose is administered in a volume of about 0.5 ml. The buffer solution shall contain in the daily dose not less than 14 IU of chorionic gonadotrophin to ensure complete luteinisation. Add a suitable antimicrobial preservative such as 4 g per litre of phenol or 0.02 g per liter of thiomersal. Store the solutions at 5 ± 3°.

Inject subcutaneously into each rat the daily dose allocated to its group. Repeat the injection of each dose 24 hours and 48 hours after the first injection. About 24 hours after the last injection, euthanise the rats and remove the ovaries from each rat. Remove any extraneous fluid and tissue from the ovaries and weigh the 2 combined ovaries of each rat immediately. Calculate the results by the usual statistical methods using the mass of the 2 combined ovaries as the response. (The precision of the assay may be improved by a suitable correction of the organ mass with reference to the mass of the rat from which it was taken; an analysis of covariance may be used.)

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits (P = 0.95) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

Storage. Store in an airtight container, at temperature not exceeding -20°.

Labeling. The label states (1) Follicle Stimulating Hormone content in mg per ml; (2) the activity in International Unit per milliliter; (3) the name and the concentration of any other excipient.

Follicle Stimulating Hormone Concentrated Solution

Follitropin Concentrated Solution

α-subunit

APDVQDCPECTLQENPFFSQ PGAPILQCMG CCFSRAYPTP 40

LIPRSKKTMLVQ KNVTSESTCC VAKSYNRVTV MGGFKVENHT 80

ACHCSTCYYH KS 92

β-subunit

NSCELTNITI AIEKEECRFC ISINTTWCAG YCYTRDLVYK 40

DPARPKIQKT CTFKELVYET VRVPGCAHHA DSLYTYPVAT 80

QCHCGKCDSD STDCTVRGLG PSYCSFGEMK E 111

Glycosylation sites:

Asn-52 (α-subunit), Asn-78 (α-subunit), Asn-7 (β-subunit), Asn-24 (β-subunit)

Disulfide bridges:

α-subunit : 7-31, 10-60, 28-82, 32-84, 59-87.

β-subunit : 3-51, 17-66, 20-104, 28-82, 32-84, 87-94.

Mol. Wt. approx. 30,000-40,000.

Follicle Stimulating Hormone Concentrated Solution is a heterodimeric glycoprotein having the structure of follicle stimulating hormone. It consists of 2 subunits: a 92 amino acid α-chain common to other glycoprotein hormones and a specific 111 amino acid β-chain.

Follicle Stimulating Hormone is produced by a method based on recombinant DNA (rDNA) technology using mammalian cells as host cells. Follicle Stimulating Hormone Concentrated Solution contains not less than 0.1 mg and not more than 0.8 mg of protein content per ml of the solution and has the potency of not less than 9000 IU and not more than 17000 IU per mg of protein.

Host cell derived proteins. Not more than 100 ppm.

Host cell or vector derived DNA. Not more than 10 ng per dose.

Category. Treatment of infertility in both men and women

Description. Clear or slightly turbid and colorless solution

Identification

A. It complies with the biological activity as described under Assay.

B. Determine by Isoelectric focusing (2.4.33).

Test solution. Desalt and concentrate the preparation under examination using a suitably validated procedure. Redissolve the recovered material in water to obtain the concentration of 5 mg per ml.

Reference solution. Desalt and concentrate the reference solution using a suitably validated procedure. Redissolve the recovered material in water to obtain the concentration of 5 mg per ml.

Focusing:

– pH gradient: a combination of ampholytes and electrode buffers giving a functional separation in the isoelectric point pI range of 3.5-5.5 is selected as defined by the system suitability criteria; where pre-cast gels are employed, proprietary electrode solutions may be used in conjunctions; otherwise suitable dilute mineral or

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organic acids and bases are employed at pH levels respectively lower and higher than the functional range of ampholytes,
- catholyte: 2 per cent w/v solution of glycine,
- anolyte: a mixture containing 0.34 per cent w/v solution of aspartic acid and 0.36 per cent w/v solution of glutamic acid adjusted to pH 2.8-3.8,
- application: 10 µl.

Detection. Proceed as described in Isoelectric Focusing (2.4.33).

In the electropherogram obtained with the reference solution, the number of bands seen in the pI region 3.5-5.5 corresponds to that shown in the electropherogram supplied with follitropin *IPRS*; the distribution of bands in the pI region 3.5-5.5 is qualitatively similar to that shown in the electropherogram supplied with *follitropin IPRS*.

The principal band in the electropherogram obtained with the test solution is similar in position to the principal band in the electropherogram obtained with the reference solution.

C. Examine the chromatograms in the test for follitropin oligomers.

In the chromatogram, the principal peak obtained with the test solution corresponds to the principal peak obtained with the reference solution.

D. Immunoblotting. Determine by electrophoresis (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) (SDS-PAGE) followed by immunoblotting (2.4.12).

Transfer buffer. Mix 0.3 per cent tris, 1.4 per cent glycine and 20 per cent v/v solution of *methanol*. Perform a SDS-PAGE as described in test for free subunits, except that the gel after electrophoresis is not stained, instead placed in a tray containing transfer buffer. The tray is placed on a shaker for 5-10 minutes and then assembled on an electro blotting cassette.

Assembly of transfer cassette. Soak two pieces of sponge sheets, 6 sheets of Whatman no.1 paper and a nitro cellulose membrane in transfer buffer and place in a cassette with the nitro cellulose membrane covering the gel; place equal numbers of Whatman sheets above and below the gel-membrane setup. Then place this whole set up between two sheets of sponge and lower the whole cassette into the transblot apparatus containing the transfer buffer with the nitro cellulose membrane side facing the anode.

Transfer and development of protein bands. Transfer is done at 100 V for 1 hour at room temperature. Remove the membrane and stain briefly with 0.1 per cent Ponceau S (made in 5 per cent acetic acid) and destain with water; mark the protein bands with a pencil. Block the membrane for 1 hour with 5 per cent non-fat dried milk in *tris buffered saline (TBS)* at room temperature on a shaker. Remove the membrane blot and rinse with TBST. Wash the blot with TBST three times, for 5 minutes each, on a shaker.

Dilute primary antibody solution i.e. anti FSH antibody (from rabbit) to 1:1000 in TBST and incubate the blot with this solution for 1 hour on a shaker at room temperature. Remove the blot and rinse with 3 washes of TBST of 5 minutes each, on a shaker.

Then incubate the blot with the second antibody (i.e. goat anti rabbit, ALP conjugated) at a dilution of 1:10,000 in TBST, for 1 hour at room temperature on a shaker. Wash the blot 3 times with TBST, 5 minutes each, on a shaker.

Then rinse the blot once with water and pour the 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT) substrate solution on to the blot. After the development of bands remove the solution and wash the blot with water and air-dry.

Preparation of substrate solution. Use solution BCIP/NBT dissolved in 10 ml water as a substrate solution.

In the electropherogram obtained with the test solution, the principal band corresponds to the principal band obtained with the reference and no significant additional bands with higher or lower molecular weights are present.

E. Peptide mapping (2.3.47).

Separation of α and β subunits by Liquid Chromatography (2.4.14).

Test solution. Dissolve the preparation under examination in mobile phase A to obtain a concentration of about 0.4 mg per ml.

Reference solution. Dissolve the content of *follitropin IPRS* in mobile phase A to obtain a concentration of about 0.4 mg per ml.

Chromatographic system
- a stainless steel pre-column 2 cm x 4.0 mm, packed with butylsilyl silica gel (5 µm),
- a stainless steel column 25 cm x 4.6 mm, packed with butylsilyl silica gel (5 µm) with a pore size of 30 nm,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 1000 ml with *water*,
  B. a mixture of 0.9 volumes of *trifluoroacetic acid*, 50 volumes of *water* and 950 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 226 nm,
- injection volume: 800 µl.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
2	100	0
8	76	24
17	76	24
36	70	30
41	25	75
46	25	75
47	100	0
57	100	0

Inject reference solution and test solution. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution. The retention time of β-subunit is about 14 minutes and α-subunit is about 30 minutes.

Collect the fractions containing the α- and β-subunits and freeze-dry them.

Reduction, Modification and Desalting of the Purified Subunits

Reduction and modification

Solution (a). Dilute 10 µl of *tributylphosphine* to 2 ml with *propanol.* Saturate with nitrogen.

Solution (b). Dilute 20 µl of *4-vinylpyridine* to 200 µl with *propanol.* Saturate with nitrogen.

Test solution. Dissolve each of the α- and β-subunit fractions obtained from the test solution in the previous step in 300 µl of *guanidine-tris (hydroxymethyl) aminomethane-EDTA buffer solution pH 8.5* and incubate at 37° for 60 minutes in a thermostatically controlled water-bath. Add 100 µl of solution (a), mix and saturate with nitrogen. Incubate at 37° for 90 minutes. Add 10 µl of solution (b), mix and saturate with nitrogen. Incubate at 37° for 45 minutes. Add 100 µl of a 10 per cent v/v solution of *trifluoroacetic acid* and mix.

Reference solution. Prepare at the same time as for the test solutions. Dissolve each of the α- and β-subunit fractions obtained from the reference solution in the previous step in 300 µl of *guanidine-tris (hydroxymethyl) aminomethane-EDTA buffer solution pH 8.5* and incubate at 37° for 60 minutes. Add 100 µl of solution (a), mix and saturate with nitrogen. Incubate at 37° for 90 minutes. Add 10 µl of solution (b), mix and saturate with nitrogen. Incubate at 37° for 45 minutes. Add 100 µl of a 10 per cent v/v solution of *trifluoroacetic acid* and mix.

Desalting

Dilute the α- and β-subunits obtained with the test solution and reference solutions each to 840 µl with mobile phase A.

Chromatographic system

- a stainless steel column 2 cm x 4.6 mm, packed with butylsilyl silica gel (5 µm),
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 1000 ml with *water*,

  B. a mixture of 1 volume of *trifluoroacetic acid*, 300 volumes of *water* and 700 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 226 nm,
- injection volume: 800 µl,
- retention time: *α-subunit solution*: monovinylpyridine-modified α-subunit = about 15 minutes; *β-subunit solution*: monovinylpyridine-modified β-subunit = about 16 minutes.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
7	100	0
27	0	100
27.01	100	0
32	100	0

Inject the reference solution and test solution. For each solution the chromatogram shows a principal peak due to the monovinylpyridine-modified subunit and several minor peaks due to the di- and oligovinylpyridine-modified subunits. The retention time of monovinylpyridine-modified α-subunit peak obtained with α-subunit solution is about 15 minutes and monovinylpyridine-modified β-subunit obtained with β-subunit solution is about 16 minutes.

Only the fraction containing the monovinylpyridine-modified subunits is used for digestion in the following step.

Collect the fractions containing the monovinylpyridine-modified subunits and freeze-dry them.

Selective Cleavage of the Peptide Bonds

Solution (c). Dissolve 480 g of *urea* in 600 ml of *water* and dilute to 1000 ml with the same solvent. Add about 3-5 g of mixed-bed resin and stir for about 1 hour. Filter through a glass filter before use.

Solution (d). Dissolve 15.8 g of *ammonium hydrogen carbonate* and 8.3 g of sodium *edetate* in 800 ml of *water.* Adjust to pH 7.8 with an 80 g per litre solution of *sodium hydroxide* and dilute to 1000 ml with *water.*

Test solutions. Dissolve each of the modified α- and β-subunits obtained from the test solutions in the previous step in 42.5 µl of solution (c) and incubate at room temperature for 30 minutes. Add 42.5 µl of solution (d) and mix. To 42.5 µl of these solutions

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add 35 µl of a solution containing about 23 mU per µl of *endoproteinase Lys-C* and mix. Incubate at 37° for 4 hours, then add 35 µl of the same *endoproteinase Lys-C* solution and mix. Incubate at 37° overnight, then dilute to 420 µl with mobile phase A.

Reference solutions. Prepare at the same time as for the test solutions Dissolve each of the modified α- and β-subunits obtained from the reference solutions in the previous step in 42.5 µl of solution (c) and incubate at room temperature for 30 minutes. Add 42.5 µl of solution (d) and mix. To 42.5 µl of these solutions add 35 µl of a solution containing about 23 mU per µl of *endoproteinase Lys-C* and mix. Incubate at 37° for 4 hour, then add 35 µl of the same *endoproteinase Lys-C solution* and mix. Incubate at 37° overnight, then dilute to 420 µl with mobile phase A.

Determine the subunits by chromatographic separation using Liquid chromatography (2.4.14).

Chromatographic system

- a stainless steel pre-column 2 cm x 4.6 mm, packed with octadecylsilyl silica gel (5 µm),
- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilyl silica gel (5 µm) with a pore size of 30 nm,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 1000 ml with *water*
  B. a mixture of 1 volume of *trifluoroacetic acid*, 300 volumes of *water* and 700 volumes of *acetonitrile.*
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 400 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
7	100	0
77	30	70
82	0	100
87	0	100
92	100	0
107	100	0

Inject the reference solution. The test is not valid unless (i) for *α-subunit*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of follitropin α-subunit digest supplied with *follitropin IPRS*; both chromatograms show peaks due to the L4, L6, L3, L5 and L1-2/L1 fragments; (ii) retention times obtained with the test and reference solutions differ by not more than 5 per cent for fragments L4, L6 and L3, not more than 3 per cent for fragment L5 and not more than 2 per cent for fragments L1-2/L1; (iii) for *β-subunit*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of follitropin β-subunit digest supplied with *follitropin IPRS*; both chromatograms show peaks due to the L5, L7, L6, and L1-4 fragments; (iv) retention times obtained with the test and reference solutions differ by not more than 5 per cent for fragment L5, not more than 2 per cent for fragments L7 and L6 and not more than 1 per cent for fragments L1-4.

Inject the test solution. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the corresponding reference solution.

F. Glycan analysis (2.2.21). Determine the glycan content by either method A or method B.

Method A

Protein Denaturation

Test solution. Dissolve 500 µg of the preparation under examination in 60 µl of *0.05 M phosphate buffer solution pH 7.5*. Add 6 µl of a 10 mg per ml solution of *sodium dodecyl sulphate* and 35 µl of a 1 per cent *v/v* solution of *2-mercaptoethanol*. Mix using a vortex mixer, centrifuge and incubate at 37° for 15 minutes.

Reference solution. Prepare at the same time of preparing test solution. Dissolve the contents of *follitropin IPRS* in 60 µl of *0.05 M phosphate buffer solution pH 7.5*. Add 6 µl of a 10 mg per ml solution of *sodium dodecyl sulphate* and 35 µl of a 1 per cent *v/v* solution of *2-mercaptoethanol*. Mix using a vortex mixer, centrifuge and incubate at 37° for 15 minutes.

Selective release of the glycans

Test solution. To the test solution obtained in the previous step add 0.75 µl of *octylphenyl-polyethylene glycol* and mix using a vortex mixer. Add 25 mU of *peptide N-glycosidase F*, mix using a vortex mixer and centrifuge. Incubate at 37° for 24 hours. Remove the protein fraction using a suitable, validated procedure. The following method has been found to be appropriate. Add 600 µl of *anhydrous ethanol*, previously cooled at -20° for 45 minutes. Mix using a vortex mixer and centrifuge. Precipitate the proteins at -20° for 15 minutes, then centrifuge at 10600 g at 4° for 5 minutes. Transfer the supernatant to a separate tube and evaporate the ethanol for 15 minutes. Add 1000 ml of *water* and resume evaporating until the remaining volume is about 500-800 µl, then freeze-dry.

Label the liberated glycans contained in the sample with 2-aminobenzamide. The procedure employs a combination of reagents optimized and validated for the efficient labelling of glycans, and for the subsequent extraction and recovery of the labeled glycans from the reaction. Recover the sample in 1.5 ml of *water*.

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Reference solution. Prepare at the same time as for the test solution. To the reference solution obtained in the previous step add 0.75 µl of octylphenyl-polyethylene glycol and mix using a vortex mixer. Add 25 mU of *peptide N-glycosidase F*, mix using a vortex mixer and centrifuge. Incubate at 37° for 24 hours. Remove the protein fraction using a suitable, validated procedure. Add 600 µl of *anhydrous ethanol*, previously cooled at -20° for 45 minutes. Mix using a vortex mixer and centrifuge. Precipitate the proteins at -20° for 15 minutes, then centrifuge at 10600 g at 4° for 5 minutes. Transfer the supernatant to a separate tube and evaporate the ethanol for 15 minutes. Add 1000 ml of *water* and resume evaporating until the remaining volume is about 500-800 µl, then freeze-dry.

Label the liberated glycans contained in the sample with 2-aminobenzamide. The procedure employs a combination of reagents optimized and validated for the efficient labelling of glycans, and for the subsequent extraction and recovery of the labeled glycans from the reaction. Recover the sample in 1.5 ml of particle-free water.

Determine the glycan content by chromatographic separation using Liquid chromatography (2.4.14).

Chromatographic system

- a stainless steel column 7.5 cm x 7.5 mm, packed with weak anion-exchange resin (10 µm),
- column temperature: 30°,
- mobile phase: A. *acetonitrile*,
  B. *0.5 M ammonium acetate buffer solution pH 4.5*,
  C. *water*,
- a gradient programme using the conditions given below,
- flow rate: 0.4 ml per minute,
- fluorimeter set at 330 nm for excitation and at 420 nm for emission,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)	Mobile phase C (per cent v/v)
0	20	0	80
5	20	0	80
21	20	4	76
61	20	25	55
62	20	50	30
71	20	50	30
72	20	0	80
117	20	0	80

Inject the reference solution. The test is not valid unless the chromatogram obtained is qualitatively similar to the chromatogram supplied with *follitropin IPRS*. Identify the peaks due to neutral, mono-, di-, tri- and tetra-sialylated forms by comparison with the chromatogram supplied with *follitropin IPRS*.

Inject the test solution. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution.

Determine the area of each peak and express it as a percentage of the total. Calculate the *Z* number using the following expression:

$$= (A_0 \times 0) + (A_1 \times 1) + (A_2 \times 2) + (A_3 \times 3) + (A_4 \times 4)$$

where, A_0 = peak area percentage due to the neutral form;
A_1 = peak area percentage due to the mono-sialylated form;
A_2 = peak area percentage due to the di-sialylated form;
A_3 = peak area percentage due to the tri-sialylated form;
A_4 = peak area percentage due to the tetra-sialylated form.

The Z number obtained for the reference solution and the test solution is not less than 177 and not more than 233.

Method B

Protein Denaturation

Solution (a). To 1.952 g of *2-[N-morpholino]ethanesulfonic acid* and 57.32 g of *guanidine hydrochloride*, add 1 ml of a 15.4 g per litre solution of *dithiothreitol*, 10 ml of an 18.61 g per litre solution of *sodium edetate* and 20 ml of *water*. Maintain in a water-bath at about 37° for 1 minute to dissolve the components. Adjust to pH 8.1 with 8.0 per cent solution of *sodium hydroxide* and dilute to 100 ml with *water*.

Solution (b). Dissolve 37 mg of iodoacetamide in 1 ml of *water* and mix. Protect from light.

Solution (c). Dissolve 26.7 g of *disodium hydrogen phosphate dihydrate* and 11.2 g of *sodium edetate* in 3 litres of *water* and mix. Adjust to pH 7.5 with a 40 g per litre solution of *sodium hydroxide*.

Test solution Dissolve 1 mg of the preparation under examination in 0.2 ml of solution (a) and incubate in a water-bath at 37 ± 1° for 2 hours. Add 20 µl of freshly prepared solution (b), mix and incubate at 37 ± 1° for a further 2 hours, protected from light. Add 10 µl of *2-mercaptoethanol* and mix. Dialyze against 1000 ml of solution (c). Add 200 µl of solution (c) and mix. Determine the protein content of the solution.

Reference solution (a). Dissolve 1 mg of the *follitropin IPRS* in 0.2 ml of solution (a) and incubate in a water-bath at 37 ± 1° for 2 hours. Add 20 µl of freshly prepared solution (b), mix and incubate at 37 ± 1° for a further 2 hours, protected from light.

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Add 10 µl of *2-mercaptoethanol* and mix. Dialyze against 1000 ml of solution (c). Add 200 µl of solution (c) and mix. Determine the protein content of the solution.

Reference solution (b). Dissolve 1 mg of fetuin in 0.2 ml of solution (a) and incubate in a water-bath at 37 ± 1° for 2 hours. Add 20 µl of freshly prepared solution (b), mix and incubate at 37 ± 1° for a further 2 hours, protected from light. Add 10 µl of *2-mercaptoethanol* and mix. Dialyze against 1000 ml of solution (c). Add 200 µl of solution (c) and mix. Determine the protein content of the solution.

Selective release of the glycans

Test solution. Dilute the test solution obtained in the previous step with solution (c) to obtain a concentration of 1.1 g per litre. Add 1 U of *peptide N-glycosidase F* to 500 µg of the solution, mix and incubate at 37 ± 1° for 24 hours. Place the solution in ice. Precipitate the protein and salts with 3 volumes of ice-cold *anhydrous ethanol* and allow to stand in ice for 10 minutes. Centrifuge at 16000 *g* for about 5 minutes and transfer the supernatant to a separate tube. Add 3 µl of a 1 µg per µl solution of *maltotriose*, then freeze-dry. Dissolve in 100 µl of *water*.

Reference solution (a). Dilute the reference solution obtained in the previous step with solution (c) to obtain a concentration of 1.1 g per litre. Add 1 U of *peptide N-glycosidase F* to 500 µg of the solution, mix and incubate at 37 ± 1° for 24 hours. Place the solution in ice. Precipitate the protein and salts with 3 volumes of ice-cold *anhydrous ethanol* and allow to stand in ice for 10 minutes. Centrifuge at 16000 *g* for about 5 minutes and transfer the supernatant to a separate tube. Add 3 µl of a 1 µg per µl solution of *maltotriose*, then freeze-dry. Dissolve in 100 µl of *water*.

Reference solution (b). Dilute the fetuin solution obtained in the previous step with solution (c) to obtain a concentration of 1.1 g per litre. Add 1 U of *peptide N-glycosidase F* to 500 µg of the solution, mix and incubate at 37 ± 1° for 24 hours. Place the solution in ice. Precipitate the protein and salts with 3 volumes of ice-cold *anhydrous ethanol* and allow to stand in ice for 10 minutes. Centrifuge at 16000 *g* for about 5 minutes and transfer the supernatant to a separate tube. Add 3 µl of a 1 µg per µl solution of *maltotriose*, then freeze-dry. Dissolve in 100 µl of *water*.

Determine the glycan content by chromatographic separation using liquid chromatography (2.4.14).

Chromatographic system

- a stainless steel pre-column 5 cm x 4.0 mm, packed with strongly basic anion-exchange resin,
- a stainless steel column 25 cm x 4.0 mm, packed with strongly basic anion-exchange resin,
- mobile phase: A. 20 g per litre solution of *sodium hydroxide*, maintain under helium,
  B. *water*, maintain under helium,
  C. dissolve 41 g of anhydrous *sodium acetate* in 800 ml of *water*, dilute to 1000 ml with the same solvent, maintain under helium,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- detector: pulsed amperometric,
- injection volume: 45 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)	Mobile phase C (per cent v/v)
0	20	80	0
0.2	20	80	0
94.0	20	34	46
97.0	20	34	46
97.1	20	80	0
115.0	20	80	0

Inject reference solution (b). The test is not valid unless the chromatogram obtained with the reference solution (b) is qualitatively similar to the chromatogram for fetuin supplied with the *follitropin IPRS*

Inject reference solution (a) and test solution. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution (a). identify the peaks due to neutral, mono-, di-, tri- and tetra-sialylated forms in the chromatogram obtained with reference solution (b); determine the area of each peak and express it as a percentage of the total; calculate the Z number using the following expression:

$$= (A_0 \times 0) + (A_1 \times 1) + (A_2 \times 2) + (A_3 \times 3) + (A_4 \times 4)$$

where, A_0 = peak area percentage due to the neutral form;
A_1 = peak area percentage due to the mono-sialylated form;
A_2 = peak area percentage due to the di-sialylated form;
A_3 = peak area percentage due to the tri-sialylated form;
A_4 = peak area percentage due to the tetra-sialylated form.

The Z number obtained for the reference solution (b) is not less than 290 and not more than 325 and for the test solution is not less than 178 and not more than 274.

Tests

Follitropin oligomers. Determine by Size-exclusion chromatography (2.4.16) using the normalisation procedure.

Solution (a). Dissolve 118 mg of *sodium dihydrogen phosphate*, 1.65 g of *disodium hydrogen phosphate dihydrate*;

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and 30.0 g of *sucrose* in 40 ml of *water* and dilute to 100 ml with the same solvent.

Solution (b). Dissolve 1 mg of *bovine albumin* in 30 ml of solution (a).

Test solution. Dissolve the preparation under examination in solution (a) to obtain a concentration of 0.25 mg per ml.

Reference solution (a). Dissolve *follitropin IPRS* in solution (a) to obtain a concentration of 0.25 mg per ml.

Reference solution (b). Dissolve the contents of a vial of *follitropin IPRS* in 200 µl of solution (a) and mix with the same volume of solution (b). If necessary, dilute further with solution (a) to obtain a concentration of 0.25 mg per ml.

Chromatographic system
- a stainless steel column 30 cm x 7.8 mm packed with hydrophilic silica gel of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10000 to 500000 (5µm),
- mobile phase. a buffer solution prepared by dissolving 14.2 g of *anhydrous sodium sulphate* in 1000 ml of *0.1 M phosphate buffer solution pH 6.7*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 100 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to bovine albumin and follitropin is not less than 1.2.

Inject reference solution (a) and the test solution. The chromatogram obtained with the test solution should correspond to the chromatogram obtained with reference solution (a). The sum of the peaks with the retention time less than that of the principal peak is not more than 0.5 per cent of the total peaks area.

Free subunits. Determine by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12) under non-reducing conditions.

Gel dimensions. 1.5 mm thick.

Resolving gel. 12 per cent acrylamide.

Sample buffer. Concentrated SDS-PAGE sample buffer.

Test solution. Dissolve the preparation under examination in water to obtain a concentration of 2 µg per µl. To 55 µl of the solution add 55 µl of the sample buffer. Allow to stand for 4 hours at room temperature.

Reference solution (a). Dissolve the contents of a vial of *follitropin IPRS* in *water* to obtain a concentration of 2 µg per µl. To 25 µl of the solution add 25 µl of the sample buffer. To 40 µl of this solution add 180 µl of the sample buffer and 180 µl of *water*. Allow to stand for 4 hours at room temperature, then boil for 5 minutes.

Reference solution (b). A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 14.4-94 kDa.

Application:

Well	Solution(s)	Volume (µl)
1	Reference solution (a)	40
2	Reference solution (a)	30
3	Reference solution (a)	20
4	Reference solution (a)	15
5	Reference solution (a)	10
6	Reference solution (a)	5
7	Test solution	50
8	Test solution + Reference solution (a)	50 + 25
9	Reference solution (b)	10

Detection. By Coomassie staining.

The test is not valid unless (i) reference solution (b) meets the validation criteria (2.4.12); (ii) the bands corresponding to follitropin heterodimer and subunits obtained with the electropherogram of test solution and reference solution (a) are clearly separated; (iii) reference solution (a) shows no bands corresponding to follitropin heterodimer.

In the electropherogram obtained, the content of *free subunits* is not more than 3 per cent.

Oxidised follitropin. Determine the oxidised follitropin content by chromatographic separation using Liquid chromatography (2.4.14).

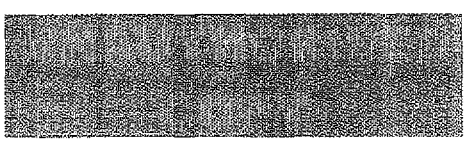
Solution (a). Dissolve 3.3 mg of *2,4-dichlorobenzoic acid* in 10.0 ml of *ethanol (96 per cent)*.

Test solution. Dilute the preparation under examination in *water* to obtain the concentration of 300 µg per ml.

Reference solution (a). Dilute the contents of *follitropin IPRS* in *water* to obtain a concentration of 300 µg per ml

Reference solution (b). Dilute 0.1 ml of strong *hydrogen peroxide solution* to 30 ml with *water*. Dissolve the contents of a vial of *follitropin IPRS* in this solution to obtain a concentration of 300 µg per ml. Incubate for 30 to 45 minutes. Add 10 µl of solution (a) and inject immediately.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with butylsilyl silica gel (5 µm),
- column temperature: 30°,
- mobile phase: A. *0.2 M phosphate buffer* solution adjusted to pH 2.5,



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B. 40 volumes of *water* and 60 volumes of *acetronitrile*,

C. *water*;

- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 210 nm,
- injection volume: 25 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)	Mobile phase C (per cent v/v)
0	50	25	25
8.4	50	39	11
8.5	50	45	5
15	50	45	5
15.1	50	25	25
25	50	25	25

Inject reference solution (b). The test is not valid unless (i) the peaks due to the oxidised follitropin α- and β-subunits are separated from the peaks due to the non-oxidised follitropin subunits and from the peak due to 2,4-dichlorobenzoic acid; (ii) the chromatogram obtained is similar to the chromatogram supplied with *follitropin IPRS*.

Inject test solution. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution (b).

Calculate the percentage of oxidation of the follitropin subunits using the following expression:

$$\frac{(A_2 + A_4) \times 100}{A_1 + A_2 + A_3 + A_4}$$

where, A_1 = area of the peak due to the follitropin α-subunit;

A_2 = area of the peaks due to the oxidised follitropin α-subunit;

A_3 = area of the peak due to the follitropin β-subunit;

A_4 = area of the peak due to the oxidised follitropin β-subunit.

The total oxidized forms are not more than 6 per cent of follitropin.

Bacterial Endotoxins (2.2.3). Not more than 0.1 IU per International Unit of follitropin activity

Assay

A. *Protein.* Determine by Size exclusion chromatography (2.4.16).

Solution (a). Dissolve 100 mg of *poloxamer 188* in 900 ml of *water* and dilute to 1000 ml with the same solvent.

Test solution. Dilute the preparation under examination in solution (a) to obtain a concentration of about 0.03 mg per ml.

Reference solution. Dilute the contents of a vial of *follitropin IPRS* in solution (a) to obtain a concentration of 0.03 mg per ml.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm packed with hydrophilic silica gel of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10000 to 500000 (5 µm),
- mobile phase: Mix 6.74 ml of *orthophosphoric acid*, 14.2 g of *anhydrous sodium sulphate* and 900 ml of *water*, pH adjusted to 6.7 with a 50 per cent w/v solution of *sodium hydroxide* and dilute to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 100 µl.

Inject the reference solution. The test is not valid unless the number of theoretical plates calculated for the peak of follitropin is not less than 1300.

Calculate the content of follitropin taking into account the assigned content of *follitropin IPRS*.

B. *Potency.* The follicle-stimulating activity of follitropin is estimated by comparing under given conditions its effect in enlarging the ovaries of immature rats treated with chorionic gonadotrophin with the same effect of the International Standard preparation of human recombinant follicle-stimulating hormone or of a reference preparation calibrated in International Units. The International Unit of FSH is the activity contained in stated amounts of the International Standard of human recombinant follicle-stimulating hormone. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Use immature female rats of the same strain, 19-28 days old, differing in age by not more than 3 days and having masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 6 equal groups of at least 5 rats. If sets of 6 litter mates are available, assign 1 litter mate from each set to each group and mark according to litter.

Choose three doses of the reference preparation and three doses of the preparation under examination such that the smallest dose produces a positive response in some of the rats and the largest dose does not produce a maximal response in all of the rats. Use doses in geometric progression and as an initial approximation total doses of 1.5 IU, 3.0 IU and 6.0 IU may be tried, although the dose will depend on the sensitivity of the rats used, which may vary widely.

Dissolve separately the total quantities of the preparation under examination and of the reference preparation

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corresponding to the daily doses to be used in sufficient *phosphate-albumin buffered saline pH 7.2* such that the daily dose is administered in a volume of about 0.5 ml. The buffer solution shall contain in the daily dose not less than 14 IU of chorionic gonadotrophin to ensure complete luteinisation. Add a suitable antimicrobial preservative such as 4 g per litre of phenol or 0.02 g per litre of thiomersal. Store the solutions at 5 ± 3°.

Inject subcutaneously into each rat the daily dose allocated to its group. Repeat the injection of each dose 24 hours and 48 hours after the first injection. About 24 hours after the last injection, euthanise the rats and remove the ovaries from each rat. Remove any extraneous fluid and tissue from the ovaries and weigh the 2 combined ovaries of each rat immediately. Calculate the results by the usual statistical methods using the mass of the 2 combined ovaries as the response. (The precision of the assay may be improved by a suitable correction of the organ mass with reference to the mass of the rat from which it was taken; an analysis of covariance may be used.)

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits (P = 0.95) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

Storage. Store in an airtight container, at temperature not exceeding -20°.

Labeling. The label states (1) Follicle Stimulating Hormone content in mg per ml; (2) the activity in International Unit per milliliter; (3) the name and the concentration of any other excipient.

Follicle Stimulating Hormone Injection

Follitropin Injection

Follicle Stimulating Hormone Injection is a heterodimeric glycoprotein having the structure of follicle stimulating hormone. It consists of 2 subunits: a 92 amino acid α-chain common to other glycoprotein hormones and a specific 111 amino acid β-chain.

Follicle stimulating hormone injection contains not less than 80 per cent and not more than 125 per cent of the stated potency

Usual strengths. 75 IU per 0.15 ml, 50 IU per 0.5 ml, 100 IU per 0.5 ml, 150 IU per 0.3 ml, 225 IU per 0.45 ml, 300 IU per 0.36 ml, 300 IU per 0.6 ml, 600 IU per 0.72 ml, 900 IU per 1.08 ml, 450 IU per 0.75 ml (33 mcg per 0.75 ml); 1050 IU per 1.75 ml (77 mcg per 1.75 ml) injection

Description. Clear and colorless solution essentially free of particulates.

Identification

A. It complies with the biological activity as described under Assay.

B. Determine by Isoelectric focusing (2.4.33).

Test solution. Desalt and concentrate the preparation under examination using a suitably validated procedure. Redissolve the recovered material in water to obtain the concentration of 5 mg per ml.

Reference solution. Desalt and concentrate the reference solution using a suitably validated procedure. Redissolve the recovered material in water to obtain the concentration of 5 mg per ml.

Focusing:

- pH gradient: a combination of ampholytes and electrode buffers giving a functional separation in the isoelectric point pI range of 3.5-5.5 is selected as defined by the system suitability criteria; where pre-cast gels are employed, proprietary electrode solutions may be used in conjunctions; otherwise suitable dilute mineral or organic acids and bases are employed at pH levels respectively lower and higher than the functional range of ampholytes,
- catholyte: 2 per cent w/v solution of glycine,
- anolyte: a mixture containing 0.34 per cent w/v solution of aspartic acid and 0.36 per cent w/v solution of glutamic acid adjusted to pH 2.8-3.8,
- application: 10 µl.

Detection. Proceed as described in Isoelectric Focusing (2.4.33).

In the electropherogram obtained with the reference solution, the number of bands seen in the pI region 3.5-5.5 corresponds to that shown in the electropherogram supplied with follitropin *IPRS*; the distribution of bands in the pI region 3.5-5.5 is qualitatively similar to that shown in the electropherogram supplied with *follitropin IPRS*.

The principal band in the electropherogram obtained with the test solution is similar in position to the principal band in the electropherogram obtained with the reference solution.

C. Examine the chromatograms in the test for follitropin oligomers.

In the chromatogram, the principal peak obtained with the test solution corresponds to the principal peak obtained with the reference solution.

D. Immunoblotting. Determine by electrophoresis (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) (SDS-PAGE) followed by immunoblotting (2.4.12).

Transfer buffer. Mix 0.3 per cent tris, 1.4 per cent glycine and 20 per cent v/v solution of *methanol*. Perform a SDS-PAGE as

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described in test for free subunits, except that the gel after electrophoresis is not stained, instead placed in a tray containing transfer buffer. The tray is placed on a shaker for 5-10 minutes and then assembled on an electro blotting cassette.

Assembly of transfer cassette. Soak two pieces of sponge sheets, 6 sheets of Whatman no. 1 paper and a nitro cellulose membrane in transfer buffer and place in a cassette with the nitro cellulose membrane covering the gel; place equal numbers of Whatman sheets above and below the gel-membrane setup. Then place this whole set up between two sheets of sponge and lower the whole cassette into the transblot apparatus containing the transfer buffer with the nitro cellulose membrane side facing the anode.

Transfer and development of protein bands. Transfer is done at 100 V for 1 hour at room temperature. Remove the membrane and stain briefly with 0.1 per cent Ponceau S (made in 5 per cent acetic acid) and destain with water, mark the protein bands with a pencil. Block the membrane for 1 hour with 5 per cent non-fat dried milk in *tris buffered saline (TBS)* at room temperature on a shaker. Remove the membrane blot and rinse with TBST. Wash the blot with TBST 3 times, for 5 minutes each, on a shaker.

Dilute primary antibody solution i.e. anti FSH antibody (from rabbit) to 1:1000 in TBST and incubate the blot with this solution for 1 hour on a shaker at room temperature. Remove the blot and rinse with 3 washes of TBST of 5 minutes each, on a shaker.

Then incubate the blot with the second antibody (i.e. goat anti rabbit, ALP conjugated) at a dilution of 1:10,000 in TBST, for 1 hour at room temperature on a shaker. Wash the blot 3 times with TBST, 5 minutes each, on a shaker.

Then rinse the blot once with water and pour the 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT) substrate solution on to the blot. After the development of bands remove the solution and wash the blot with water and air-dry.

Preparation of substrate solution. Use solution BCIP/NBT dissolved in 10 ml water as a substrate solution.

In the bands obtained with the test solution, the principal band corresponds to the band obtained with the reference and no significant additional bands with higher or lower molecular weights are present.

Tests

Follitropin oligomers. Determine by Size-exclusion chromatography (2.4.16) using the normalisation procedure.

Solution (a). Dissolve 118 mg of *sodium dihydrogen phosphate*, 1.65 g of *disodium hydrogen phosphate dihydrate*, and 30.0 g of *sucrose* in 40 ml of *water* and dilute to 100 ml with the same solvent.

Solution (b). Dissolve 1 mg of bovine albumin in 30 ml of solution (a).

Test solution. Use the Drug product as such.

Reference solution (a). Dissolve *follitropin IPRS* in solution (a) to obtain a concentration of 0.037 mg per ml.

Reference solution (b). Dissolve the *follitropin IPRS* in equal volume of solution (a) and solution (b). If necessary, dilute further with solution (a) to obtain a concentration of 0.037 mg per ml.

Chromatographic system
- a stainless steel column 30 cm x 7.8 mm packed with hydrophilic silica gel of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10000 to 500000 (5 µm),
- mobile phase: a buffer solution prepared by dissolving 14.2 g of *anhydrous sodium sulphate* in 1000 ml of *0.1 M phosphate buffer solution pH 6.7*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 100 µl.

Inject reference solution (b). The test is not valid unless the resolution between the peaks due to bovine albumin and follitropin is not less than 1.2.

Inject reference solution (a) and the test solution. The chromatogram obtained with the test solution should correspond to the chromatogram obtained with reference solution (a). The sum of the peaks with the retention time less than that of the principal peak is not more than 0.5 per cent of the total peaks area.

Free subunits. Determine by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12) under non-reducing conditions.

Gel dimensions. 1.5 mm thick.

Resolving gel. 12 per cent acrylamide.

Sample buffer. Concentrated SDS-PAGE sample buffer.

Test solution. Dissolve the preparation under examination in water to obtain a concentration of 2 µg per µl. To 55 µl of the solution add 55 µl of the sample buffer. Allow to stand for 4 hours at room temperature.

Reference solution (a). Dissolve the contents of a vial of *follitropin IPRS* in *water* to obtain a concentration of 2 µg per µl. To 25 µl of the solution add 25 µl of the sample buffer. To 40 µl of this solution add 180 µl of the sample buffer and 180 µl of *water*. Allow to stand for 4 hours at room temperature, then boil for 5 minutes.

Reference solution (b). A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 14.4-94 kDa.

Application:

Well	Solution(s)	Volume (µl)
1	Reference solution (a)	40
2	Reference solution (a)	30
3	Reference solution (a)	20
4	Reference solution (a)	15
5	Reference solution (a)	10
6	Reference solution (a)	5
7	Test solution	50
8	Test solution + Reference solution (a)	50+25
9	Reference solution (b)	10

Detection. By Coomassie staining.

The test is not valid unless (i) reference solution (b) meets the validation criteria (2.4.12); (ii) the bands corresponding to follitropin heterodimer and subunits obtained with the electropherogram of test solution and reference solution (a) are clearly separated; (iii) reference solution (a) shows no bands corresponding to follitropin heterodimer; (iv) the recovery is not less than 75 per cent and not more than 125 per cent.

In the electropherogram obtained, the content of *free subunits* is not more than 3 per cent.

Oxidised follitropin. Determine the oxidised follitropin content by chromatographic separation using Liquid chromatography (2.4.14).

Solution (a). Dissolve about 3.3 mg of *2,4-dichlorobenzoic acid* in 10.0 ml of *ethanol (96 per cent)*.

Test solution. Dilute the preparation under examination in *water* to obtain the concentration of 300 µg per ml.

Reference solution (a). Dilute the contents of *follitropin IPRS* in *water* to obtain a concentration of 300 µg per ml

Reference solution (b). Dilute 0.1 ml of *strong hydrogen peroxide solution* to 30 ml with *water*. Dissolve the contents of a vial of *follitropin IPRS* in this solution to obtain a concentration of 300 µg per ml. Incubate for 30 to 45 minutes. Add 10 µl of solution (a) and inject immediately.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm packed with butylsilyl silica gel (5 µm),
- column temperature: 30°,
- mobile phase: A. *0.2 M phosphate buffer solution* adjusted to pH 2.5,

B. 40 volumes of *water* and 60 volumes of *acetronitrile*,

C. *water*,
- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 210 nm,
- injection volume: 25 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)	Mobile phase C (per cent v/v)
0	50	25	25
8.4	50	39	11
8.5	50	45	5
15	50	45	5
15.1	50	25	25
25	50	25	25

Inject reference solution (b). The test is not valid unless (i) the peaks due to the oxidised follitropin α- and β-subunits are separated from the peaks due to the non-oxidised follitropin subunits and from the peak due to 2,4-dichlorobenzoic acid; (ii) the chromatogram obtained is similar to the chromatogram supplied with *follitropin IPRS*.

Inject reference solution (b) and test solution. The chromatogram obtained with the test solution corresponds to the chromatogram obtained with the reference solution (b).

Calculate the percentage of oxidation of the follitropin subunits using the following expression:

$$\frac{(A_2 + A_4) \times 100}{A_1 + A_2 + A_3 + A_4}$$

where, A_1 = area of the peak due to the follitropin α-subunit;

A_2 = area of the peaks due to the oxidised follitropin α-subunit;

A_3 = area of the peak due to the follitropin β-subunit;

A_4 = area of the peak due to the oxidised follitropin β-subunit.

The total oxidized forms are not more than 6 per cent.

Bacterial Endotoxins (2.2.3). Not more than 0.1 IU per international unit of follitropin activity.

Other tests. Complies with the tests stated under Parenteral Preparations (Injections).

Assay

A. *Protein.* Determine by Size exclusion chromatography (2.4.16).

Solution (a). Dissolve 100 mg of *poloxamer 188* in 900 ml of *water* and dilute to 1000 ml with the same solvent.

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Test solution. Dilute the preparation under examination on solution (a) to obtain a concentration of about 0.03 mg per ml.

Reference solution. Dilute the contents of a vial of *follitropin IPRS* in solution (a) to obtain a concentration of 0.03 mg per ml.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm packed with hydrophilic silica gel of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10000 to 500000 (5µm),
- mobile phase: Mix 6.74 ml of *orthophosphoric acid*, 14.2 g of *anhydrous sodium sulphate* and 900 ml of *water*, adjusted to pH 6.7 with a 50 per cent w/v solution of *sodium hydroxide* and dilute to 1000 ml with *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 100 µl.

Inject the reference solution. The test is not valid unless the number of theoretical plates calculated for the peak of follitropin is not less than 1300.

Calculate the content of follitropin using the assigned content of *follitropin IPRS*.

B. *Potency*. The follicle-stimulating activity of follitropin is estimated by comparing under given conditions its effect in enlarging the ovaries of immature rats treated with chorionic gonadotrophin with the same effect of the International Standard preparation of human recombinant follicle-stimulating hormone or of a reference preparation calibrated in International Units. The International Unit of FSH is the activity contained in stated amounts of the International Standard of human recombinant follicle-stimulating hormone. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Use immature female rats of the same strain, 19-28 days old, differing in age by not more than 3 days and having masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 6 equal groups of at least 5 rats. If sets of 6 litter mates are available, assign 1 litter mate from each set to each group and mark according to litter.

Choose 3 doses of the reference preparation and 3 doses of the preparation under examination such that the smallest dose produces a positive response in some of the rats and the largest dose does not produce a maximal response in all of the rats. Use doses in geometric progression and as an initial approximation total doses of 1.5 IU, 3.0 IU and 6.0 IU may be used, although the dose will depend on the sensitivity of the rats used, which may vary widely.

Dissolve separately the total quantities of the preparation under examination and of the reference preparation corresponding to the daily doses to be used in sufficient *phosphate-albumin buffered saline pH 7.2* such that the daily dose is administered in a volume of about 0.5 ml. The buffer solution shall contain in the daily dose not less than 14 IU of chorionic gonadotrophin to ensure complete luteinisation. Add a suitable antimicrobial preservative such as 4 g per litre of phenol or 0.02 g per litre of thiomersal. Store the solutions at 5 ± 3°.

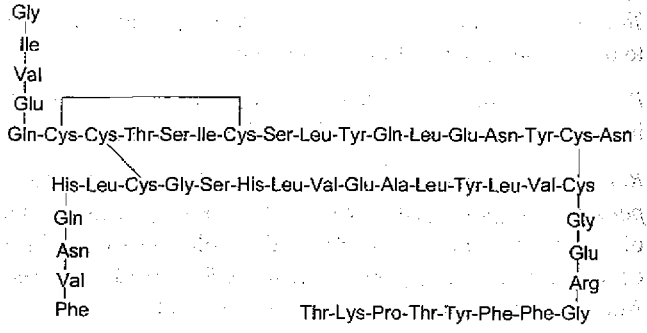
Inject subcutaneously into each rat the daily dose allocated to its group. Repeat the injection of each dose 24 hours and 48 hours after the 1st injection. About 24 hours after the last injection, euthanise the rats and remove the ovaries from each rat. Remove any extraneous fluid and tissue from the ovaries and weigh the 2 combined ovaries of each rat immediately. Calculate the results by the usual statistical methods using the mass of the 2 combined ovaries as the response. (The precision of the assay may be improved by a suitable correction of the organ mass with reference to the mass of the rat from which it was taken; an analysis of covariance may be used).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits (P = 0.95) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

Storage. In an airtight glass vial, at temperature in the range of 2-8°.

Labeling. The label states (1) Follicle Stimulating Hormone content in mg per ml or PFS; (2) the activity in International Unit per milliliter; (3) the name and the concentration of any other excipient: (4) storage temperature.

Human Insulin



$C_{257}H_{383}N_{65}O_{77}S_6$ Mol. Wt. 5808.0

Human insulin is a 2-chain peptide having the structure of the antidiabetic hormone produced by the human pancreas. It is produced by a method based on recombinant DNA (rDNA) technology.

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Human insulin contains not less than 27.5 IU per mg of human insulin, $C_{257}H_{383}N_{65}O_{77}S_6$, calculated on the dried basis.

Description. A white or almost white powder.

Identification

A. In the Assay, the principal peak due to insulin in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with appropriate reference solution.

B. Determine by peptide mapping (2.3.47).

Test solution. Prepare a 0.2 per cent w/v solution of the substance under examination in *0.01 M hydrochloric acid* and transfer 500 µl of this solution to a stoppered clean tube. Add 2.0 ml of *HEPES buffer solution pH 7.5* and 400 µl of a 0.1 per cent w/v solution of *Staphylococcus aureus strain V8 protease*. Close the tube and incubate at 25° for 6 hours. Stop the reaction by adding 2.9 ml of *sulphate buffer solution pH 2.0*.

Reference solution. Prepare in the same manner as for the test solution but using *human insulin IPRS* in place of the substance under examination.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilane silica gel (3 µm) with a pore size of 8 nm,
- column temperature: 40°,
- mobile phase: A. a mixture of 100 ml of *acetonitrile*, 200 ml of *sulphate buffer solution pH 2.0* and 700 ml of *water*,

  B. a mixture of 200 ml of *sulphate buffer solution pH 2.0*, 400 ml of *acetonitrile* and 400 ml of *water*,
- a gradient programme using the conditions given below
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
60	30	70
65	0	100
70	0	100
71	90	10
80	90	10

At initial conditions equilibrate the column for at least 15 minutes. Carry out a blank run using the above-mentioned gradient.

Inject the reference solution. The test is not valid unless the tailing factor is not more than 1.5 for the chromatograms obtained with the reference solution for the peaks due to fragments II and III and the resolution factor between the peaks due to fragments II and III is not less than 3.4.

Inject the reference solution and the test solution. The chromatograms obtained with the test and reference solutions are qualitatively similar. In the chromatogram obtained with the reference solution identify the peaks due digest fragments I, II and III.

The profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

Tests

Light absorption (2.4.7). Absorbance of a 0.05 per cent w/v solution in *0.01 M hydrochloric acid* at the maximum at about 276 nm, 0.48 to 0.56.

Total zinc. Not more than 1.0 per cent, calculated on the dried basis, determined by the following method. To 5 ml of a 0.5 per cent w/v solution in *0.01 M hydrochloric acid* add 10 ml of *alkaline borate buffer pH 9.0*, 3 ml of *zincon solution* and sufficient *water* to produce 50 ml. Allow to stand for 1 hour and measure the absorbance of the resulting solution at about 620 nm, using as the blank a solution prepared by treating 5 ml of *water* instead of the substance being examined in a similar manner. Calculate the content of zinc from the absorbance obtained by repeating the procedure using a suitable aliquot of a mixture of 4 volumes of *zinc sulphate solution* and 6 volumes of *water*.

Other tests. Comply with the tests for Impurities with molecular masses greater than that of insulin, Related proteins and Total zinc stated under Insulin Preparations.

Sulphated ash (2.3.18). Not more than 2.5 per cent, calculated on the dried basis, determined on 0.2 g.

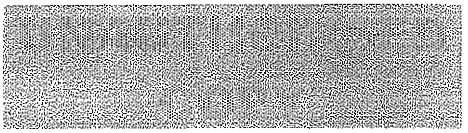
Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 0.2 g by drying in an oven at 100° to 105° for 24 hours.

Assay. Determine as described under Assay of Insulins (2.3.46).

Test solution. Dissolve a suitable quantity of the substance under examination in *0.01 M hydrochloric acid* to obtain a concentration of 4.0 mg per ml.

Human Insulin intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 10 Endotoxin Units per mg.



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Storage. Store protected from light, at a temperature not exceeding -18° until released by the manufacturer. When thawed, it should be stored in a refrigerator (2° to 8°) and used for the manufacture of preparations within a short period of time.

Labelling. The label states (1) whether the material is produced by enzymatic modification of porcine insulin or by rDNA technology; (2) where applicable, that the material is free from bacterial endotoxins; (3) the storage conditions.

NOTE— 0.0347 mg of human insulin is equivalent to 1 Unit of insulin.

Insulin Injection

Neutral Insulin; Neutral Insulin Injection; Soluble Insulin; Plain Insulin

Insulin Injection is a neutral, sterile solution of bovine, porcine or human insulin.

Insulin Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of IU of Insulin.

Usual strengths. 40 IU per ml; 80 IU per ml; 100 IU per ml.

Description. A colourless liquid, free from turbidity and foreign matter; during storage, traces of a very fine sediment may be deposited.

Identification

In the chromatograms obtained in the Assay, the position of the peak due to insulin obtained with the test solution corresponds to that of the principal peak obtained with the appropriate reference solution.

Tests

pH (2.4.24). 6.8 to 7.8.

Total zinc. Not more than 40 µg per 100 IU of insulin, determined by either of the methods stated under Insulin Preparations.

Test solution. Dilute a volume of the gently shaken preparation under examination containing 200 IU to 25.0 ml with *water*. Dilute if necessary to a suitable concentration with *water*.

Bacterial endotoxins (2.2.3). Not more than 80.0 Endotoxin Units for each 100 insulin unit.

Other tests. Comply with the tests stated under Insulin Preparations.

Assay. Determine as described under Assay of Insulins (2.3.46).

Test solution. Add 4 µl of *6 M hydrochloric acid* per millilitre of the preparation under examination to obtain a clear solution.

For a preparation containing more than 100 IU per ml, an additional dilution with *0.01 M hydrochloric acid* is necessary to avoid overloading the column. Dilute a suitable volume of the injection with *0.01 M hydrochloric acid* to obtain a concentration of 40 IU per ml.

Storage. Store in multiple dose containers in a refrigerator (2° to 8°). It should not be allowed to freeze.

Labelling. The label states (1) the strength in terms of the number of IU per ml; (2) the animal source or sources of the insulin; (3) that the preparation should not be allowed to freeze; (4) the storage conditions.

Insulin Aspart



$C_{256}H_{381}N_{65}O_{79}S_6$ Mol.Wt. 5,826

Insulin Aspart is 28^B–L-Aspartate insulin (human). It is a 2-chain peptide containing 51 amino acids. The A-chain is composed of 21 amino acids and the B-chain is composed of 30 amino acids. It is identical in primary structure to human insulin, except that it has aspartic acid instead of proline at position 28 of the B-chain. As in human insulin, insulin aspart contains 2 interchain disulphide bonds and 1 intrachain disulphide bond.

Insulin aspart is produced by a method based on recombinant DNA (rDNA) technology under conditions designed to minimise the degree of microbial contamination of the product.

Insulin Aspart contains not less than 90.0 per cent and not more than 104.0 per cent of $C_{256}H_{381}N_{65}O_{79}S_6$ plus A21Asp insulin aspart, B3Asp insulin aspart, B3isoAsp insulin aspart and B28isoAsp insulin aspart, calculated on the dried basis.

By convention, for the purpose of labeling insulin aspart preparations, 0.0350 mg of insulin aspart is equivalent to 1 unit and 6 nmol.

Category. Antidiabetic.

Description. A white or almost white powder.

Host cell DNA. Not more than 10 ng per dose.

Host-cell-derived proteins. Not more than 100 ppm.

Single-chain precursor. Not more than 10 ppm.

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Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

B. Determine by peptide mapping (2.3.47).

Test solution. Dissolve 200 mg of substance under examination in 100 ml of *0.01 M hydrochloric acid*. Transfer 25 µl of this solution to a clean tube. Add 100 µl of *HEPES buffer solution pH 7.5* and 20 µl of a 1 mg per ml solution of *Staphylococcus aureus strain V8 protease*. Cap the tube and incubate at 25° for 6 hours. Stop the reaction by adding 145 µl of sulphate buffer solution pH 2.0.

Reference solution. Prepare same as that of test solution using *insulin aspart IPRS* instead of the substance under examination.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecylsilyl silica gel (3 µm) with a pore size of 8 nm

or

- a stainless steel column 25 cm x 4.0 mm, packed with octylsilyl silica gel for chromatography,
- column temperature: 40°,
- mobile phase: A. a mixture of 100 volumes of *acetonitrile*, 200 volumes of *sulphate buffer solution* pH 2.0 and 700 volumes of *water*,

B. a mixture of 200 volumes of *sulphate buffer solution* pH 2.0, 400 volumes of *acetonitrile* and 400 volumes of *water*,

- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
60	30	70
65	0	100
70	0	100

Equilibrate the column at initial conditions for at least 15 minutes. Carry out a blank run using the above mentioned gradient.

Inject the reference solution and the test solution. The chromatogram obtained with the test solution and the reference solution, are qualitatively similar to the chromatogram of insulin aspart digest supplied with *insulin aspart IPRS*. In the chromatogram obtained with reference solution, identify the peaks due to digest fragments I, II and III. The test is not valid unless the tailing factor corresponds to fragment II and III is not more than 1.5. The resolution between the peaks due to fragment II and III is not less than 8.0 in the chromatogram obtained with reference solution.

The profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

NOTE— The retention times of fragments I, II and IV are the same as for human insulin. The retention time of fragment III differs from human insulin due to substitution of proline by aspartic acid.

Tests

Impurities with molecular masses greater than that of insulin aspart. Determine by size-exclusion chromatography (2.4.16).

Test solution. Dissolve 0.4 mg of the substance under examination in 100 ml of *0.01 M hydrochloric acid*. Maintain the solution at 2° to 8° and use within 48 hours.

Reference solution. Use a solution of insulin (about 4 mg per ml), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of *6 M hydrochloric acid*, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in *0.01 M hydrochloric acid*, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days. Maintain the solution at 2° to 8° and use within 7 days.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm, packed with hydrophilic silica gel (5-10 µm) with a pore size of 12-12.5 nm,
- mobile phase: a mixture of 15 volumes of *glacial acetic acid*, 20 volumes of *acetonitrile* and 65 volumes of a 0.1 per cent w/v solution of *arginine*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 276 nm,
- injection volume: 100 µl,
- Run time: about 35 minutes.

Equilibrate the column until repeatable results are obtained for 2 subsequent injections. Inject atleast 3 injections of the reference solution.

Inject the reference solution. The test is not valid unless the peak-to-valley ratio, where H_p is the height above the baseline of the peak due to the dimer and H_v is the height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer, is not less than 2.0.

Inject the test solution. Run the chromatogram for atleast 35 minutes. The retention times for Insulin aspart polymers, insulin aspart dimer, insulin aspart monomer and salts are about 13-17 minutes, about 17.5 minutes, about 20 minutes and about 22

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minutes respectively. The sum of the areas of all the peaks with a retention time less than that of monomer is not more than 0.5 per cent of the total area of all the peaks. Ignore any peak with a retention time greater than that of insulin aspart monomer.

Related proteins. Determine by liquid chromatography (2.4.14), as described in the Assay.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, area of the peak due to B28isoAsp insulin aspart is not more than 1.0 per cent. The sum of the areas of the peaks due to A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart is not more than 2.0 per cent and the sum of the areas of peaks due to all other impurities is not more than 1.5 per cent.

Sulphated ash (2.3.18). Not more than 6.0 per cent, determined on 0.2 g dried substance.

Bacterial endotoxins (2.2.3). Less than 10 Endotoxin Units per mg.

Loss on drying (2.4.19). Not more than 10.0 per cent, determined on 0.2 g by drying in an oven at 105° for 24 hours.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve a suitable quantity of the substance under examination *0.01M hydrochloric acid* to obtain a concentration of 4.0 mg per ml. Maintain the solution at 2° to 8° and use within 24 hours.

Reference solution (a). Dissolve the contents of a vial of *insulin aspart IPRS* in *0.01M hydrochloric acid* to obtain a concentration of 4.0 mg per ml. Maintain the solution at 2° to 8° and use within 48 hours.

Reference solution (b). A solution containing not less than 1 per cent w/v of B3Asp insulin aspart and A21Asp insulin aspart. This may be achieved by storing reference solution at room temperature for about 1 to 3 days. Maintain the solution at 2 to 8°and use within 72 hours.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilyl silica gel (5 µm),
- column temperature: 40°,
- mobile phase: A. dissolve 28.4 g of *anhydrous sodium sulphate* in *water*; add 2.7 ml of *orthophosphoric acid* and dilute to 1000 ml with *water*, adjusted to pH 3.6, if necessary, with strong *sodium hydroxide solution*; mix 9 volumes of the solution with 1 volume of *acetonitrile*, B. a mixture of equal volumes of *water* and *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	58	42
35	58	42
40	20	80
45	20	80
46	58	42
60	58	42

Adjust the mobile phase composition and the duration of the isocratic elution to obtain a retention time of about 22 minutes for insulin aspart and to ensure that B3isoAsp insulin aspart is eluted before the gradient starts.

Inject reference solution (a) and reference solution (b). The test is not valid unless the tailing factor is not more than 1.5 and the relative retention times with reference to insulin aspart (retention time is about 20-24 minutes), for B28isoAsp insulin aspart, B3Asp insulin aspart plus A21Asp insulin aspart (generally coeluted) and B3isoAsp insulin aspart are about 0.9, 1.3 and 1.5, respectively. The test is not valid unless resolution between the peak due to insulin aspart and the peak due to A21Asp insulin aspart and to B3Asp insulin aspart is not less than 2.0 in the chromatogram obtained with reference solution (b).

Inject reference solution (a) and the test solution.

Calculate the content of $C_{256}H_{381}N_{65}O_{79}S_6$, plus B28isoAsp insulin aspart, A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart using the areas of the corresponding peaks in the chromatograms obtained with the test solution and reference solution.

Storage. Store protected from light in an air tight container at a temperature not exceeding -18° until released by the manufacturer. When thawed, insulin aspart is stored at 2° to 8° and used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, insulin aspart must be at room temperature before opening the container.

Labelling. The label states (1) the material is produced by microbial synthesis via recombinant DNA technology; (2) that the material has endotoxin within the specified limits; (3) the storage conditions.

Insulin Aspart Injection

Insulin Aspart Injection is a sterile, neutral, aqueous solution of Insulin Aspart.

Insulin Aspart Injection contains not less than 90 per cent and not more than 110 per cent of the stated amount of Insulin aspart.

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Usual strength. 100 IU per ml.

Description. A colourless liquid, free from turbidity and foreign matter; during storage traces of very fine sediment may be deposited.

Identification

In the Assay, principal peak in the chromatogram of the test solution corresponds to that in the chromatogram of the reference solution.

Tests

pH (2.4.24). 6.9 to 7.8.

Related proteins. Determine by liquid chromatography (2.4.14) as described under assay by using the normalization procedure.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, area of the peak due to B28isoAsp insulin aspart is not more than 2.5 per cent. The sum of the areas of the peaks due to A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart is not more than 5.0 per cent and the sum of the areas of peaks due to all other impurities is not more than 3.5 per cent.

Total Zinc. Determine by Atomic Absorption Spectrometry (2.4.2). Not more than 40 µg per 100 units of Insulin Aspart.

Test solution. Dilute a volume containing 100 units of insulin aspart to 25.0 ml with *0.01 M hydrochloric acid.* Dilute, if necessary, to a suitable concentration of zinc (for example 0.2 µg to 1.0 µg of Zn per ml) with *0.01 M hydrochloric acid.*

Reference solutions. Solutions containing a suitable range of concentrations, for example 0.20 µg, 0.40 µg, 0.60 µg, 0.80 µg and 1.00 µg of Zn per ml, freshly prepared by diluting *zinc standard solution* (5 mg per ml Zn) with *0.01 M hydrochloric acid.*

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as source of radiation and an air-acetylene flame of suitable composition (for example 11 liters of air and 2 liters of acetylene per minute).

Bacterial endotoxins (2.2.3). Not more than 80 Endotoxin Units per 100 units of Insulin Aspart.

Impurities with molecular masses greater than that of Insulin Aspart. Determine by size exclusion chromatography (2.4.16) using the normalization procedure.

Test solution. A 4 µl per ml solution of preparation under examination in *6 M hydrochloric acid.* When sample is a suspension, agitate the material prior to sampling in order to obtain a homogenous sample. If a suspension does not turn clear within 5 minutes of the initial addition of hydrochloric acid, add small aliquots of the acid (less than 4 µl per ml) until a solution is obtained.

Reference solution. Use a solution of insulin (about 4 mg per ml), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of *6 M hydrochloric acid*, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in *0.01 M hydrochloric acid*, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days. Maintain the solution at 2° to 8° and use within 7 days.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm, packed with hydrophilic silica gel (5-10 µm) with a pore size of 12 to 12.5 nm,
- mobile phase: a mixture of 15 volumes of *glacial acetic acid*, 20 volumes of *acetonitrile* and 65 volumes of a 0.1 per cent w/v solution of *arginine*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 276 nm,
- injection volume: 100 µl,
- run time: about 35 minutes.

Equilibrate the column until repeatable results are obtained for 2 subsequent injections. Inject atleast 3 injections of the reference solution.

Inject the reference solution and the test solution. The sum of the areas of all the peaks with a retention time less than that of the principal peak is not more than 2.0 per cent of the total areas of the peaks. Disregard any peak with a retention time greater than that of the peak due to insulin aspart monomer.

Other tests. Comply with the tests stated under Insulin Preparations and Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Acidify each ml of the preparation under examination with 4 µl of *6 M hydrochloric acid.* Dilute, if necessary, a portion of the acidified solution with *0.01 M hydrochloric acid* to obtain a solution containing about 100 insulin aspart units per ml, maintain the solution at 2° to 8°.

Reference solution (a). Dissolve the contents of a vial of *insulin aspart IPRS* in *0.01 M hydrochloric acid* to obtain a known concentration of 100 insulin aspart units per ml. Add 4 µl of *6 M hydrochloric acid* per ml and mix. Maintain the solution at 2° to 8°.

Reference solution (b). A solution containing not less than 1 per cent w/v of B3Asp insulin aspart and A21Asp insulin aspart. This may be achieved by storing reference solution at room temperature for about 1 to 3 days. Maintain the solution at 2° to 8°and use within 72 hours.

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Chromatographic system

- a stainless steel column, 25 cm x 4.0mm, packed with octadecyl silyl silica gel (5 µm),
- column temperature: 35°,
- mobile phase: A. dissolve 14.0 g of *anhydrous sodium sulphate* in approximately 900 ml of *water*; add 1.3 ml of *orthophosphoric acid* and adjusted to pH 3.4 with *sodium hydroxide*. Dilute to 1000 ml with *water*. Mix 900 ml of this solution with 100 ml of *acetonitrile*,

  B. a mixture of equal volumes of *water* and *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	58	42
35	58	42
40	20	80
45	20	80
46	58	42
60	58	42

Adjust the mobile phase composition and the duration of the isocratic elution to obtain a retention time of about 22 minutes for insulin aspart and to ensure that B3isoAsp insulin aspart is eluted before the gradient starts.

Inject reference solutions (a) and (b). The test is not valid unless the tailing factor of the principal peak is not more than 1.8 and the relative standard deviation for replicate injections is not more than 1.4 per cent in the chromatogram obtained with reference solution (a). The resolution between the peaks due insulin aspart, B3Asp insulin aspart and A21Asp insulin aspart is not less than 1.6 in the chromatogram obtained with reference solution (b).

The relative retention times with reference to insulin aspart (retention time, 20 to 26 minutes) are: B28isoAsp insulin aspart = approximately 0.9; B3Asp insulin aspart and A21Asp insulin aspart (generally coeluted) = approximately 1.3; B3isoAsp insulin aspart = approximately 1.5.

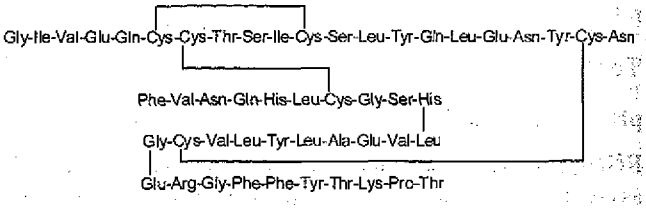
Inject reference solution (a) and the test solution.

Calculate the content of insulin aspart together with B28isoAsp insulin aspart, A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart using the areas of the corresponding peaks in the chromatograms obtained with test solution and reference solution and the declared content of insulin aspart together with B28isoAsp insulin aspart, A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart in *insulin aspart IPRS*.

Storage. Store protected from light at 2° to 8° and avoid freezing.

Labelling. The label states that (1) the strength in terms of insulin aspart units per ml; (2) the material is produced by microbial synthesis via recombinant DNA technology; (3) the storage conditions.

Insulin Lispro



$C_{257}H_{383}N_{65}O_{77}S_6$ Mol. Wt. 5808

Insulin Lispro is 28^B-L-Lysine-29^B-L-proline insulin (human).

Insulin Lispro is a 2-chain peptide containing 51 amino acids. The A- chain is composed of 21 amino acids and the B- chain is composed of 30 amino acids. It is identical in primary structure to human insulin, only differing in amino acid sequence at positions 28 and 29 of the B- chain. Human insulin is Pro (B28), Lys (B29), whereas insulin Lispro is Lys (B28), Pro (B29). As in human insulin, insulin Lispro contains 2 interchain disulfide bonds and 1 intrachain disulfide bond. Insulin Lispro is produced by a method based on recombinant DNA (r-DNA) technology.

Insulin Lispro contains not less than 27.0 Insulin Lispro units per mg calculated on the dried basis. It contains not less than 94.0 per cent and not more than 104.0 per cent of insulin Lispro $C_{257}H_{383}N_{65}O_{77}S_6$, calculated on dried basis.

0.0347 mg insulin lispro is equivalent to 1 unit of insulin lispro.

Category. Antidiabetic.

Description. A white or almost white powder.

Host cell DNA. Not more than 10 ng per dose.

Host cell-derived protein. Not more than 100 ppm.

Single-chain precursor. Not more than 10 ppm.

Identification

A. In the Assay, principal peak in the chromatogram of the test solution corresponds to that in the chromatogram of the reference solution.

B. Determine by peptide mapping (2.3.47).

Test solution. Dissolve 500 µl of 2.0 mg per ml solution of the substance under examination in *0.01 M hydrochloric acid* with 2 ml of *HEPES buffer solution pH 7.5* and 400 µl of a 1 mg per ml solution of *Staphylococcus aureus strain V8 protease*.

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Cap the tube and incubate at 25° for 6 h. Stop the reaction by adding 2.9 ml of *sulphate buffer solution pH 2.0*.

Reference solution. Prepare as that of test solution, but using *insulin lispro IPRS* instead of the substance under examination.

Chromatographic system

- a stainless steel column 10 cm x 4.6 mm, packed with octadecyl silane bonded to porous silica or ceramic micro-particles (1.5 to 10 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 100 volumes of *acetonitrile*, 200 volumes of *sulphate buffer solution* pH 2.0 and 700 volumes of *water*,

  B. a mixture of 200 volumes of *sulphate buffer solution pH 2.0*, 400 volumes of *water* and 400 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
3	95	5
30	41	59
35	20	80
40	95	5
50	95	5

Equilibrate the column with initial conditions for at least 15 minutes and carry out a blank run using the above-mentioned gradient.

Inject the reference solution. In the chromatogram identify the peaks due to digest fragments I, II, III and IV. The test is not valid unless the tailing factor is not more than 1.5, for the peaks due to fragments II and III and the resolution not less than 3.4, between the peaks due to fragments II and III.

Inject the reference solution and the test solution. The chromatogram obtained with the test solution and the reference solution are qualitatively similar to the chromatogram of insulin lispro digest supplied with *insulin lispro IPRS*. The chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

NOTE—Fragment I elutes at the same time in insulin derived from pork and Insulin Human; Fragment II elutes at the same time in all insulins; and Fragment III elutes at the same time in insulin derived from beef and pork.

Fragment I consists of amino acids A5 to A17 and B1 to B13. Fragment II consists of amino acids A18 to A21 and B14 to B21. Fragment III consists of amino acids B22 to B30. Fragment IV consists of amino acids A1 to A4. A refers to the A chain of Insulin Human, and B refers to the B- chain of Insulin Human.

Prior to release, the following tests are carried out on each batch of the final bulk product, unless exemption has been granted by the competent authority.

Bacterial endotoxins (2.2.3). Less than 10 Endotoxin Units per mg insulin lispro by Kinetic chromogenic method.

Loss on drying (2.4.19). Not more than 10.0 per cent of its weight, determined on 300 mg by drying in an oven at 105° for 16 hours.

Sulphated ash (2.3.18). Not more than 2.5 percent, determined on 0.3 g (dried substance).

Zinc. Determine by Atomic absorption spectrometry (2.4.2) as per insulin preparations. Not more than 1.0 per cent (dried substance).

Impurities with molecular masses greater than that of Insulin Lispro. Determine by Size Exclusion Chromatography (2.4.16). Not more than 0.25 per cent.

Proceed as directed in the test for Impurities with molecular masses greater than that of Insulin preparations except that Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 5 days.

Related proteins. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve 3.5 mg of the substance under examination in 1.0 ml of *0.01 M hydrochloric acid*. Maintain the solution at 2° to 8° and use with in 56 hours.

Reference solution. Dissolve 3.5 mg of the preparation under examination in *0.01 M hydrochloric acid*. Allow it to stand at room temperature to obtain 0.8 per cent and 11 per cent w/v of A 21 desamido insulin lispro.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecyl silane bonded to porous silica or ceramic micro-particles (1.5 to 10 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 82 volumes of a 2.84 per cent w/v solution of *anhydrous sodium sulphate* adjusted to pH 2.3 with *orthophosphoric acid* and 18 volumes of *acetonitrile*,

  B. a mixture of equal volumes 2.84 per cent w/v solution of *anhydrous sodium sulphate* adjusted to pH 2.3 with *orthophosphoric acid* and *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	81	19
60	81	19
83	51	49
84	81	19
94	81	19

Adjust the mobile phase composition to obtain a retention time of about 41 minutes for insulin Lispro; A21 desamido insulin Lispro elutes near the start of the gradient elution.

Inject the reference solution. The test is not valid unless tailing factor is not more than 2.0 and the resolution between the peaks due to the insulin lispro peak and the A21 desamido insulin lispro is not less than 2.5.

Inject the reference solution and test solution. In the chromatogram obtained with the test solution, the area of the peak due to A21 desamido insulin lispro is not more than 1.0 per cent. The area of any secondary peak is not more than 0.5 per cent and the sum of all the secondary peaks other than A21 desamido insulin Lispro peak is not more than 2.0 per cent.

Assay. Determine by Liquid Chromatography (2.4.14).

Test solution. Dissolve about 80 mg of substance under examination in 100 ml of *0.01 M hydrochloric acid.*

Reference solution (a). A 0.07 per cent w/v solution of *insulin Lispro IPRS* in *0.01 M hydrochloric acid.*

Reference solution (b). Dissolve 1 mg of the preparation under examination in *0.01 M hydrochloric acid.* Allow it to stand at room temperature to obtain 0.8 per cent and 11 per cent w/v of A 21 desamido insulin.

Chromatographic system

- a stainless steel column, 10 cm x 4.6 mm, packed with octadecyl silane bonded to porous silica or ceramic micro-particles (1.5 to 10 µm),
- column temperature: 40°,
- mobile phase: a mixture of 745 volumes of 2.84 per cent w/v solution of *anhydrous sodium sulfate* adjusted to pH 2.3 with *orthophosphoric acid* and 255 volumes of *acetonitrile*,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Inject reference solutions (a) and (b). The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.1 per cent in the chromatogram obtained with reference solution (a). The resolution between the peaks due to insulin Lispro and A21 desamido insulin Lispro is not less than 3.0 in the chromatogram obtained with reference solution (b).

Inject reference solutions (a) and the test solution.

Calculate the content of insulin Lispro $C_{257}H_{383}N_{65}O_{77}S_6$ on the as- is basis and the declared content of insulin Lispro in *insulin Lispro IPRS* using the formula:

$$(C_s/C_u)(r_u/r_s)$$

Where C_s is the concentration (in insulin lispro units/ml) of *insulin Lispro IPRS* in reference solution; C_u the concentration (in insulin Lispro mg/ml) of insulin Lispro in test solution; and r_u and r_s are the insulin Lispro peak areas obtained from the test solution and reference solution respectively. From the value obtained in the test for Loss on drying, calculate the potency on the dried basis.

Storage. Store protected from light and moisture , at or below -18° until required for manufacture. When thawed, insulin lispro is stored and weighed under conditions defined by the manufacturer to maintain the quality attributes of the drug substance and is used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, insulin lispro must be at room temperature before opening the container.

Labelling. The label states the storage conditions.

Insulin Lispro Injection

Insulin Lispro Injection is sterile, isotonic, aqueous solution of insulin lispro.

Insulin Lispro Injection contains not less than 95 per cent and not more than 105 per cent of the stated amount of Insulin Lispro.

Usual strength. 40 IU per ml, 100 IU per ml.

Description. A clear colourless solution, free from turbidity and foreign matter.

Identification

In the Assay, principal peak in the chromatogram of the test solution corresponds to that in the chromatogram of the reference solution.

Tests

pH (2.4.24). 7.0 to 7.8

Related proteins. Determine by liquid chromatography (2.4.14) proceed as directed in the test for related proteins under Insulin Lispro.

Test solution. Dissolve 3.5 mg of the substance under examination in 1.0 ml of *0.01 M hydrochloric acid.* Acidify each ml of injection with 3µl of *9.6 M hydrochloric acid.*

Reference solution. Dissolve 3.5 mg of the preparation under examination in *0.01 M hydrochloric acid.* Allow it to stand at room temperature to obtain 0.8 per cent and 11 per cent w/v of A 21 desamido insulin.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecyl silane bonded to porous silica or ceramic micro-particles (1.5 to 10 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 82 volumes of a 2.84 per cent w/v solution of *anhydrous sodium sulfate* adjusted to pH 2.3 with phosphoric acid and 18 volumes of acetonitrile,

  B. a mixture of equal volumes 2.84 per cent w/v solution of *anhydrous sodium sulfate* adjusted to pH 2.3 with phosphoric acid and acetonitrile,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	81	19
60	81	19
83	51	49
84	81	19
94	81	19

Adjust the mobile phase composition to obtain a retention time of about 41 minutes for insulin Lispro; A21 desamido insulin Lispro elutes near the start of the gradient elution.

Inject the reference solution. The test is not valid unless tailing factor is not more than 2.0 and the resolution between the peaks due to the insulin lispro peak and the A21 desamido insulin lispro is not less than 2.5.

Inject the reference solution and test solution. In the chromatogram obtained with the test solution, the area of the peak due to A21 desamido insulin lispro is not more than 1.5 per cent. The sum of all the secondary peaks other than A21 desamido insulin Lispro peak is not more than 4.0 per cent.

Total Zinc. Not more than 40 µg per 100 units of Insulin Lispro units.

Bacterial endotoxins (2.2.3, method D). Not more than 80 Endotoxin Units per 100 units of insulin Lispro.

Impurities with molecular masses greater than that of Insulin Lispro. Determine by size exclusion chromatography (2.4.16). Not more than 1.5 per cent.

Proceed as directed for procedure in the test for Impurities with molecular masses greater than that of Insulin under insulin preparations except that Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 5 days.

Other tests. Comply with the tests under Parenteral Preparations.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 80 mg of substance under examination in 100 ml of *0.01 M hydrochloric acid.* Acidify each ml of injection with 3 µl of *9.6 M hydrochloric acid.* Dilute the acidified solution with *0.01 M hydrochloric acid* to obtain a solution containing about 20 insulin Lispro units per ml.

Reference solution (a). A 0.07 per cent w/v solution of *insulin Lispro IPRS* in *0.01 M hydrochloric acid.*

Reference solution (b). Dissolve 1 mg of the preparation under examination in *0.01 M hydrochloric acid.* Allow it to stand at room temperature to obtain 0.8 per cent and 11 per cent w/v of A 21 desamido insulin.

Chromatographic system

- a stainless steel column, 10 cm x 4.6 mm, packed with octadecyl silane bonded to porous silica or ceramic micro-particles (1.5 to 10 µm),
- column temperature: 40°,
- mobile phase: a mixture of 745 volumes of 2.84 per cent w/v solution of *anhydrous sodium sulfate* adjusted to pH 2.3 with orthophosphoric acid and 255 volumes of acetonitrile,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Inject the reference solution (a) and (b). The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for 3 replicate injections is not more than 1.1 per cent in the chromatogram obtained with reference solution (a). The resolution between the peaks due to insulin Lispro and A21 desamido insulin Lispro is not less than 3.0 in the chromatogram obtained with reference solution (b).

Inject the reference solution (a) and the test solution.

Calculate the potency, in insulin Lispro units, in each ml of injection by the formula

$$CD\,(r_u/r_s)$$

where C is the concentration, in insulin Lispro units per ml, of insulin lispro in the reference solution.

D is the dilution factor used to prepare the test solution and r_u and r_s are the insulin lispro peak areas in the chromatogram obtained with the test solution and the reference solution, respectively.

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Storage. Store protected from light at 2° to 8° and avoid freezing.

Labelling. The label states that (1) the strength in terms of insulin lispro units per ml; (2) the material is produced by microbial synthesis via recombinant DNA technology; (3) the storage conditions.

Biphasic Insulin Aspart Injection

Biphasic Insulin Aspart Injection is a sterile buffered suspension of insulin aspart complexed with protamine crystallized in a solution of insulin aspart.

It is prepared by the procedure described under Insulin Preparations. It is a mixture of insulin aspart and protamine crystallized insulin aspart in defined ratios.

Biphasic Insulin Aspart Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of Insulin Aspart.

Usual strength. 100 IU per ml (30:70); 100 IU per ml (50:50); 100 IU per ml (70:30).

Description. A white suspension.

Identification

In the Assay, principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 6.9 to 7.8.

Related proteins. Determine by liquid chromatography (2.4.14) as described under Assay.

Inject reference solution (a) and the test solution. In the chromatogram obtained with the test solution, area of the peak due to and reference solution. B28isoAsp insulin aspart is not more than 2.5 per cent. The sum of the areas of the peaks due to A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart is not more than 5.0 per cent and the sum of the areas of peaks due to all other impurities is not more than 3.5 per cent, calculated by area normalization procedure.

Total Zinc. Determine by Atomic Absorption Spectrometry (2.4.2). Not more than 40 µg per 100 units of Insulin Aspart.

Test solution. Dilute a volume containing 100 units of insulin aspart to 25.0 ml with *0.01M hydrochloric acid.* Dilute, if necessary, to a suitable concentration of zinc (for example 0.2 µg to 1.0 µg of Zn per ml) with *0.01M hydrochloric acid.*

Reference solutions. Solutions containing a suitable range of concentrations, for example 0.20 µg, 0.40 µg, 0.60 µg, 0.80 µg and 1.00 µg of Zn per ml, freshly prepared by diluting *zinc standard solution* (5 mg per ml Zn) with *0.01M hydrochloric acid.*

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as source of radiation and an air-acetylene flame of suitable composition (for example 11 liters of air and 2 liters of acetylene per minute).

Per cent Insulin aspart in solution in Biphasic Insulin aspart samples. Determine by using method A or B.

Biphasic Insulin Aspart Injection contains not less than 24 per cent and not more than 36 per cent for 30/70 Biphasic Insulin Aspart Injection, not less than 42 per cent and not more than 58 per cent for 50/50 Biphasic Insulin Aspart Injection and not less than 62 per cent and not more than 78 per cent for 70/30 Biphasic Insulin Aspart Injection.

A. Determine insulin aspart in solution (ins.asp.sol.) and total insulin aspart (ins.asp.tot.) by GPC-HPLC.

Tris/EDTA buffer (approx. pH 8.6). Dissolve 6.05g of Tris and 4.50g of EDTA in 80ml of water in a 100ml volumetric flask. Add 1.80 ml *4M hydrochloric acid* and add water to volume. Do not adjust pH.

Insulin aspart in solution (ins.asp.sol.). Transfer whole content of the preparation under examination into a centrifuge tube and add 25µl of Tris/EDTA-buffer per 1.5 ml of preparation under examination and mix for 5 seconds by means of a tube shaker. The tube (plugged) is placed at 25° for 1 hour. Centrifuge for 10 minutes at 3000 rpm at 21 to 25°. Decant the clear supernatant. Centrifuge this supernatant for 10 minutes at 3000 rpm at 21° to 25°. Decant the clear supernatant. Add 4µl of *6 M hydrochloric acid* per ml of the clear supernatant and mix thoroughly.

Total insulin aspart (ins.asp.tot.). Mix 4µl of *6M hydrochloric acid* per ml of preparation under examination and mix thoroughly.

Chromatographic system

- a stainless steel column 30 cm × 7.8 mm, packed with hydrophilic silica gel (5-10 µm) with a pore size of 12-12.5 nm,
- Mobile phase: *2.5 M acetic acid,*
- flow rate: 2 ml per minute,
- spectrophotometer set at 280 nm,
- injection volume: 50 µl,
- run time: 10 minutes.

The number of theoretical plates, N (5 sigma) calculated from the insulin aspart monomer peak in the chromatogram obtained with the total insulin aspart solution is not less than 1000.

$$N = 25\left(\frac{t_R}{W}\right)$$

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Where, t_R = the retention time (approx. 5 minutes)
W = the peak width at 4.4 per cent of peak height.

The relative content of insulin aspart in solution is calculated by means of the insulin aspart monomer peak areas in the chromatograms obtained with the solution 'insulin aspart in solution', $A_{ins.asp.sol.}$, and with the solution 'total insulin aspart', $A_{ins.asp.tot}$:

$$\text{per cent insulin aspart in solution} = \frac{A_{ins\ asp.\ sol.} \times 1525 \times 100 \text{ per cent}}{A_{ins\ asp.\ tot.} \times 1500}$$

B. Determine insulin aspart in solution (ins.asp.sol.) and total insulin aspart (ins.asp.tot.) by GPC-HPLC.

Tris/EDTA buffer (approx. pH 8.6). Dissolve 6.05g of Tris and 4.50g of EDTA in 80 ml of *water* in a 100-ml volumetric flask. Add 1.80 ml *4 M hydrochloric acid* and add *water* to volume. Do not adjust pH.

Insulin aspart in solution (ins.asp.sol.). Transfer whole content of the preparation under examination into a centrifuge tube and add 25µl of Tris/EDTA-buffer per 1.5 ml of preparation under examination and mix for 5 seconds by means of a tube shaker. The tube (plugged) is placed at 25° for 1 hour. Centrifuge for 10 minutes at 3000 rpm at 21° to 25°. Decant the clear supernatant. Centrifuge this supernatant for 10 minutes at 3000 rpm at 21° to 25°. Decant the clear supernatant. Add 4µl of *6 M hydrochloric acid* per ml of the clear supernatant and mix thoroughly.

Total insulin aspart (ins.asp.tot.). Mix 4 µl of *6M hydrochloric acid* per ml of preparation under examination and mix thoroughly.

Reference solution. Use a solution of insulin (about 4 mg per ml), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of *6 M hydrochloric acid*, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in *0.01M hydrochloric acid* may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days. Maintain the solution at 2° to 8° and use within 7 days.

Chromatographic system
- a stainless steel column 30 cm × 7.8 mm, packed with hydrophilic silica gel (5-10 µm) with a pore size of 12-12.5 nm,
- mobile phase: mix 15 volumes of *glacial acetic acid*, 20 volumes of *acetonitrile* and 65 volumes of a 0.1 per cent w/v solution of *arginine*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 276 nm,
- injection volume: 100 µl,
- run time: 35 minutes.

Equilibrate the column with at least 3 injections of the reference solution; the column is equilibrated when repeatable results are obtained from 2 subsequent injections.

Inject reference solution. The test is not valid unless the peak-to-valley ratio is not more than 2.0, where H_p = height above the baseline of the peak due to the dimer and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

The relative content of insulin aspart in solution is calculated by means of the insulin aspart monomer peak areas in the chromatograms obtained with the solution 'insulin aspart in solution', $A_{ins.asp.sol.}$, and with the solution 'total insulin aspart', $A_{ins.asp.tot}$:

$$\text{per cent insulin aspart in solution} = \frac{A_{ins\ asp.\ sol.} \times 1525 \times 100 \text{ per cent}}{A_{ins\ asp.\ tot.} \times 1500}$$

Bacterial endotoxins (2.2.3). Not more than 80 Endotoxin Units per 100 units of Insulin Aspart.

Impurities with molecular masses greater than that of Insulin Aspart. Determine by size exclusion chromatography (2.4.16) using the normalization procedure.

Test solution. A 4 µl per ml solution of preparation under examination in *6 M hydrochloric acid*. When sample is a suspension, agitate the material prior to sampling in order to obtain a homogenous sample. If a suspension does not turn clear within 5 minutes. of the initial addition of *hydrochloric acid*, add small aliquots of the acid (less than 4 µl per ml) until a solution is obtained.

Reference solution. Use a solution of insulin (about 4 mg per ml), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of *6 M hydrochloric acid*, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in *0.01 M hydrochloric acid*, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days. Maintain the solution at 2° to 8° and use within 7 days.

Chromatographic system
- a stainless steel column 30 cm x 7.8 mm, packed with hydrophilic silica gel (5-10 µm) with a pore size of 12-12.5 nm,
- mobile phase: a mixture of 15 volumes of *glacial acetic acid*, 20 volumes of *acetonitrile* and 65 volumes of a 0.1 per cent w/v solution of *arginine*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 276 nm,
- injection volume: 100 µl,
- run time: about 35 minutes.

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Equilibrate the column until repeatable results are obtained for 2 subsequent injections. Inject atleast 3 injections of the reference solution.

Inject the reference solution and the test solution. The sum of the areas of all the peaks with a retention time less than that of the principal peak is not more than 3.0 per cent (protamine containing preparations) of the total areas of the peaks. Disregard any peak with a retention time more than that of the peak due to insulin aspart monomer.

Other tests. Comply with the tests stated under Insulin Preparations and Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14)

Test solution. Acidify each ml of the preparation under examination with 4 µl of *6 M hydrochloric acid*. Dilute, if necessary, a portion of the acidified solution with *0.01 M hydrochloric acid* to obtain a solution containing about 100 insulin aspart units per ml, maintain the solution at 2° to 8°.

Reference solution (a). Dissolve the contents of a vial of *insulin aspart IPRS* in 0.01 M hydrochloric acid to obtain a known concentration of 100 insulin aspart units per ml. Add 4 µl of *6 M hydrochloric acid* per ml and mix. Maintain the solution at 2° to 8°.

Reference solution (b). A solution containing not less than 1 per cent w/v of B3Asp insulin aspart and A21Asp insulin aspart. This may be achieved by storing reference solution at room temperature for about 1to 3 days. Maintain the solution at 2° to 8°and use within 72 hours.

Chromatographic system

- A stainless steel column, 25 cm x 4.0 mm, packed with octadecylsilyl silica gel (5 µm),
- column temperature: 35°,
- mobile phase: A. dissolve 70.0 g of *anhydrous sodium sulphate* in approximately 4500 ml of *water*; add 6.5 ml of *phosphoric acid* and adjust to pH 3.4 with *sodium hydroxide*. Dilute to 5000 ml with *water*. Mix 900 ml of this solution with 100 ml of *acetonitrile*,

  B. a mixture of equal volumes of *water* and *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 10 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	58	42
35	58	42
40	20	80
45	20	80
46	58	42
60	58	42

Adjust the mobile phase composition and the duration of the isocratic elution to obtain a retention time of about 22 minutes for insulin aspart and to ensure that B3isoAsp insulin aspart is eluted before the gradient starts.

Inject reference solutions (a) and (b). The test is not valid unless the tailing factor of the principal peak is not more than 1.8 and the relative standard deviation for replicate injections is not more than 1.4 per cent in the chromatogram obtained with reference solution (a). The resolution between the peaks due insulin aspart, B3Asp insulin aspart and A21Asp insulin aspart is not less than1.6 in the chromatogram obtained with reference solution (b).

The relative retention times with reference to insulin aspart (retention time, 20 to 26 minutes) are: B28isoAsp insulin aspart about 0.9; B3Asp insulin aspart and A21Asp insulin aspart (generally co-eluted) about 1.3; B3isoAsp insulin aspart about 1.5.

Inject reference solution (a) and the test solution.

Calculate the content of insulin aspart together with B28isoAsp insulin aspart, A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart using the areas of the corresponding peaks in the chromatograms obtained with test solution and reference solution and the declared content of insulin aspart together with B28isoAsp insulin aspart, A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart in *insulin aspart IPRS*.

Storage. Store protected from light at 2° to 8° and avoid freezing.

Labelling. The label states that (1) the strength in terms of insulin aspart units per ml; (2) the material is produced by microbial synthesis via recombinant DNA technology; (3) the storage conditions

Biphasic Insulin Lispro Injection

Insulin Lispro Biphasic Injection

Biphasic Insulin Lispro Injection is a sterile buffered suspension of insulin lispro complexed with protamine crystallized in a solution of insulin lispro.

It is prepared by the procedure described under Insulin Preparations. It is a mixture of insulin lispro and protamine crystallized insulin lispro in defined ratios.

Biphasic Insulin Lispro Injection contains not less than 95.0 per cent and not more than 105.0 per cent of the stated amount of Insulin Lispro.

Usual strength. 100 IU per ml (25:75); 100 IU per ml (50:50).

Description. A white suspension.

Identification

In the Assay, principal peak in the chromatogram obtained with the test solution corresponds to that in the chromatogram obtained with the reference solution.

Tests

pH (2.4.24). 7.0 to 7.8.

Soluble insulin lispro content. Determine by liquid chromatography (2.4.14) using mobile phase, reference solution (a), reference solution (b) and chromatographic system as directed in the Assay.

0.1 M Tris buffer solution. Dissolve 3.54 ± 0.01 g of *Tris(hydroxymethyl)-aminomethane hydrochloride* and 3.34 ± 0.01 g of *Tris(hydroxymethyl)-aminomethane* in 500 ml of *water*. The pH of the 0.1 M Tris buffer solution must be between 8.15 and 8.35.

Biphasic Insulin Lispro Injection contains soluble insulin lispro not less than L-10 and not more than L+5, where L is the concentration of soluble insulin lispro stated on the label. The concentration of soluble insulin lispro is in the range of 15.0 to 30.0 IU per ml for 25/75 Biphasic Insulin Lispro Injection. The concentration of soluble insulin lispro is in the range of 40.0 to 55.0 IU per ml for 50/50 Biphasic Insulin Lispro Injection.

Test solution. Re-suspend the insulin lispro vial or cartridge according to the manufacturer's directions. Dilute a suitable volume of well-mixed suspension with *0.1 M Tris buffer* in a ratio of 1:1. Immerse the container in a water bath at 25° ± 2° for 30 ± 2 minutes. Immediately pass this solution through a 0.2-µm low protein binding filter. Transfer 2 parts of the filtrate to a suitable vessel, and add 1 part *0.2 N hydrochloric acid* and 2 parts *0.01 N hydrochloric acid* so that the final concentration is a five fold dilution of the resuspended sample.

Separately inject about 20 µl of the reference solution and Soluble insulin lispro test solution, record the chromatograms, and measure the peak responses for insulin lispro. Calculate the concentration of soluble insulin lispro, in Insulin Lispro Units per mL, by the formula:

$$CD\,(r_u/r_s)$$

in which C is the concentration, in insulin lispro Units per ml, of insulin lispro *IPRS* in the reference solution; D is the dilution factor used to prepare the Soluble insulin lispro test solution; and r_u and r_s are the insulin lispro peak areas obtained from the Soluble insulin lispro test solution and the reference solution, respectively.

Related proteins. Determine by liquid chromatography (2.4.14) proceed as directed in the test for related proteins under Insulin Lispro

Test solution. Dissolve 3.5 mg of the substance under examination in 1.0 ml of *0.01 M hydrochloric acid*. Acidify each ml of injection with 3µl of *9.6 M hydrochloric acid*.

Reference solution. Dissolve 3.5 mg of the preparation under examination in *0.01 M hydrochloric acid*. Allow it to stand at room temperature to obtain 0.8 per cent and 11 per cent w/v of A 21 desamido insulin.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecyl silane bonded to porous silica or ceramic micro-particles (1.5 to 10 µm),
- column temperature: 40°,
- mobile phase: A. a mixture of 82 volumes of a 2.84 per cent w/v solution of *anhydrous sodium sulphate* adjusted to pH 2.3 with phosphoric acid and 18 volumes of acetonitrile,

  B. a mixture of equal volumes 2.84 per cent w/v solution of *anhydrous sodium sulphate* adjusted to pH 2.3 with phosphoric acid and acetonitrile,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	81	19
60	81	19
83	51	49
84	81	19
94	81	19

Adjust the mobile phase composition to obtain a retention time of about 41 minutes for insulin Lispro; A21 desamido insulin Lispro elutes near the start of the gradient elution.

Inject the reference solution. The test is not valid unless tailing factor is not more than 2.0 and the resolution between the peaks due to the insulin lispro peak and the A21 desamido insulin lispro is not less than 2.5

Inject the reference solution and test solution. In the chromatogram obtained with the test solution, the area of the peak due to A21 desamido insulin lispro is not more than 1.5 per cent. The sum of area of all the secondary peaks other than A21 desamido insulin Lispro peak is not more than 4.0 per cent.

Total Zinc. Not more than 40 µg per 100 units of Insulin Lispro units.

Bacterial endotoxins (2.2.3, method D). It contains not more than 80 Endotoxin Units per 100 units of insulin Lispro.

Impurities with molecular masses greater than that of Insulin Lispro. Determine by size exclusion chromatography (2.4.16). Not more than 3.0 per cent.

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Proceed as directed for procedure in the test for Impurities with molecular masses greater than that of Insulin under insulin preparations except that Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 5 days.

Other tests. Comply with the tests stated under Insulin Preparations and Parenteral Preparations.

Assay. Determine by liquid chromatography (2.4.14).

Test solution. Dissolve about 80 mg of substance under examination in 100 ml of *0.01 M hydrochloric acid*. Acidify each ml of injection with 3 µl of *9.6 M hydrochloric acid*. Dilute the acidified solution with *0.01 M hydrochloric acid* to obtain a solution containing about 20 insulin Lispro units per ml.

Reference solution (a). A 0.07 per cent w/v solution of *insulin Lispro IPRS* in *0.01 M hydrochloric acid*.

Reference solution (b). Dissolve 1 mg of the preparation under examination in *0.01 M hydrochloric acid*. Allow it to stand at room temperature to obtain 0.8 per cent and 11 per cent w/v of A 21 desamido insulin.

Chromatographic system

- a stainless steel column, 10 cm x 4.6 mm, packed with octadecyl silane bonded to porous silica or ceramic micro-particles (1.5 to 10 µm),
- column temperature: 40°,
- mobile phase: a mixture of 745 volumes of 2.84 per cent w/v solution of *anhydrous sodium sulphate* adjusted to pH 2.3 with orthophosphoric acid and 255 volumes of acetonitrile,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Inject the reference solution (a) and (b). The test is not valid unless the tailing factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 1.1 per cent in the chromatogram obtained with reference solution (a). The resolution between the peaks due to insulin Lispro and A21 desamido insulin Lispro is not less than 3.0 in the chromatogram obtained with reference solution (b).

Inject the reference solution (a) and the test solution.

Calculate the potency, in insulin Lispro units, in each ml of injection by the formula:

$$CD\,(r_u/r_s)$$

where C is the concentration, in insulin Lispro units per ml, of insulin lispro in the reference solution;

D is the dilution factor used to prepare the test solution and r_u and r_s are the insulin lispro peak areas in the chromatogram obtained with the test solution and the reference solution, respectively.

Storage. Store protected from light at 2° to 8° and avoid freezing.

Labelling. The label states that (1) the strength in terms of insulin lispro units per ml; (2) the material is produced by microbial synthesis via recombinant DNA technology; (3) the storage conditions.

Biphasic Isophane Insulin Injection

Biphasic Isophane Insulin Injection is a sterile buffered suspension of either porcine or human insulin, complexed with protamine sulphate or any other suitable protamine, in a solution of insulin of the same species.

It is prepared by the procedure described under Insulin Preparations. It is produced by mixing, in defined ratios, soluble insulin injection and isophane insulin injection.

Biphasic Isophane Insulin Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated number of IU of Insulin.

Usual strengths. 40 IU per ml (30:70); 100 IU per ml (30: 70); 40 IU per ml (50: 50); 100 IU per ml (50:50).

Description. A white suspension which on standing deposits a white sediment and leaves a colourless or almost colourless supernatant liquid; the sediment is readily resuspended by gently shaking. When examined under a microscope, the particles are seen to be rod-shaped crystals, the majority with a maximum dimension greater than 1 µm but rarely exceeding 60 µm, free from large aggregates.

Identification

In the chromatograms obtained in the Assay the position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with the appropriate reference solution.

Tests

pH (2.4.24). 6.9 to 7.8.

Total zinc. Not more than 40 µg per 100 IU of insulin, determined by either of the methods stated under Insulin Preparations.

Other tests. Comply with the tests stated under Insulin Preparations, except Insulin in the supernatant.

Soluble insulin human content. The limit should be L+/- 5, where L is the per cent of soluble insulin human stated on the label.

Determine by liquid chromatography (2.4.14), using Method A or B.

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Method A

Soluble insulin test solution. Maintain the temperature at 25 ± 1° throughout the procedure. Transfer 5.0 ml of the injection to a centrifuge tube. Add 20 µl of *1M sodium hydroxide*, and adjust with *0.05 M hydrochloric acid* or *0.05 M sodium hydroxide* to a pH of 8.20 ± 0.02 if the total zinc concentration is approximately 20 µg per 100 IU of insulin or adjust to a pH of 8.35 ± 0.02 if the total zinc concentration is approximately 30 µg per 100 IU of insulin. Record the volume, in µl, of acid or base needed to adjust the pH. Mix, and allow to stand for 1hour. Centrifuge, transfer the supernatant to another centrifuge tube, and repeat the centrifugation. Transfer 2 ml of the supernatant to another tube, add 5 µl of *9.6 M hydrochloric acid*, and mix.

Total insulin test solution. Transfer 2 ml of Injection to a suitable vessel, add 5 µl of *9.6 M hydrochloric acid*, and allow the suspension to clarify. Dilute the resulting solution with *0.01 M hydrochloric acid* to the same theoretical concentration of insulin as the soluble insulin test solution (e.g., if the Injection is labeled to contain 20 per cent soluble insulin, the dilution factor is 100/20 = 5).

System suitability solution. A 0.15 per cent w/v solution of Insulin in *0.01 M hydrochloric acid*. Allow it to stand at room temperature for not less than 3 days to obtain a solution containing not less than 5 per cent of A-21 desamido insulin.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm),
- column temperature: 40°,
- mobile phase: a mixture of 74 volumes of buffer solution prepared by dissolving 28.4 g of *anhydrous sodium sulphate* in *water* and dilute to 1000 ml with same solvent, add 2.7 ml of *orthophosphoric acid*, adjusted to pH 2.3 with *ethanolamine* and 26 volumes of *acetonitrile*,
- flow rate: 1ml per minute,
- spectrophotometer se at 214 nm,
- injection volume: 20 µl.

Inject system suitability solution. The test is not valid unless the resolution between the peaks due to insulin and A-21 desamido insulin is not less than 2.0 and the relative standard deviation for replicate injections is not more than 1.6 per cent for the principal peak.

Inject the soluble insulin test solution and total insulin test solution.

Calculate the content of soluble insulin human as a per cent of the total insulin content of the injection by the formula:

$$(100/D)[(5020 + V_A)/5000](r_S / r_T)$$

in which D is the dilution factor for the total insulin test solution; V_A is the number of µl added to adjust the pH of the soluble insulin test solution; and r_S and r_T are the responses of the soluble insulin test solution and the total insulin test solution, respectively.

Method B

Use system suitability solution and chromatographic system as described in method A.

0.1 M tris buffer solution. Dissolve 3.54 g of *tris(hydroxymethyl)aminomethane hydrochloride* and 3.34 g of *tris(hydroxymethyl)aminomethane* in 500 ml of *water*. The pH of the solution must be between 8.15 and 8.35. If the pH is outside of this range, discard the solution and prepare fresh; do not adjust the pH.

Soluble insulin test solution. Dilute a suitable volume of Injection with 0.1 M tris buffer solution to obtain a solution containing about 6 Insulin Human Units of soluble insulin per ml (e.g., 2 ml of 70/30 Human Insulin Isophane Suspension and Human Insulin Injection containing 100 Insulin Human Units per ml would be diluted with 8 ml of 0.1M tris buffer solution to obtain a filtrate that contains 6 Insulin Human Units of soluble insulin per ml). Immerse the container in a water-bath at 25 ± 1° for 30 minutes. Immediately pass this solution through a 0.2 µm filter using a disposable syringe. Transfer 2 parts of the filtrate to a suitable vessel, and add 1 part *0.2M hydrochloric acid* (e.g., the dilution factor for the soluble insulin test solution that contains 30 per cent soluble insulin is 5 × 3/2 = 7.5).

Total insulin test solution. For each ml of injection, add 3.0 µl of *9.6 M hydrochloric acid*, mix, and allow the suspension to clarify. Dilute the resulting solution with *0.01 M hydrochloric acid* to 4 Insulin Human Units per ml (e.g., if the product is labeled to contain a total of 100 Insulin Human Units per ml, the dilution factor is 25).

Inject the soluble insulin test solution and the total insulin test solution.

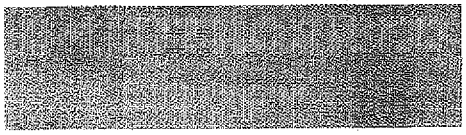
Calculate the content of soluble insulin human as a per cent of the total human insulin content of the injection by the formula:

$$(100D_S / D_T)(r_S / r_T)$$

in which D_S and D_T are the dilution factors for the soluble insulin test solution and total insulin test solution, respectively; and r_S and r_T are the human insulin peak responses obtained from the soluble insulin test solution and the total insulin test solution, respectively.

Assay. Determine as described under Assay of Insulins (2.3.46).

Test solution. To 10 ml of the preparation under examination add 40 µl of *5 M hydrochloric acid*, mix well, allow to stand for 1 hour to ensure solution of the sediment and dilute with *0.03 M hydrochloric acid* to obtain a solution containing 40 IU per ml.



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Labelling. The label states the ratio of soluble insulin injection to isophane insulin injection used in the manufacture of biphasic isophane insulin injection.

Isophane Insulin Injection

Isophane Insulin; Isophane Insulin (NPH)

Isophane Insulin injection is a sterile suspension of bovine, porcine or human insulin, complexed with protamine sulphate or another suitable protamine.

It is prepared by the procedure described under Insulin Preparations. The amount of protamine is based on the known isophane ratio and is not less than the equivalent of 0.3 mg and not more than the equivalent of 0.6 mg of protamine sulphate for each 100 IU of insulin in the insulin-protamine complex. It contains sodium phosphate as a buffering agent.

Isophane Insulin Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated number of IU of Insulin.

Usual strength. 40 IU per ml.

Description. A white suspension which on standing deposits a white sediment and leaves a colourless or almost colourless supernatant liquid; the sediment is readily resuspended by gently shaking. When examined under a microscope, the particles are seen to be rod-shaped crystals, the majority with a maximum dimension greater than 1 µm but rarely exceeding 60 µm, free from large aggregates.

Identification

In the chromatograms obtained in the Assay the position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with the appropriate reference solution.

Tests

pH (2.4.24). 6.9 to 7.5.

Total zinc. Not more than 40 µg per 100 IU of insulin, determined by either of the methods stated under Insulin Preparations.

Bacterial endotoxins (2.2.3). Not more than 80.0 Endotoxin Units per 100 insulin unit.

Other tests. Comply with the tests stated under Insulin Preparations.

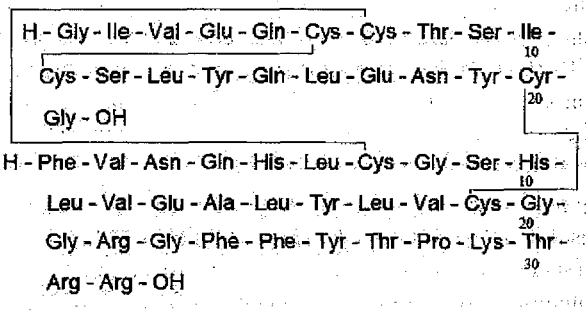
Assay. Determine as described under Assay of Insulins (2.3.46).

Test solution. To 10 ml of the preparation under examination add 40 µl of *5 M hydrochloric acid*, mix well, allow to stand for 1 hour to ensure solution of the sediment and dilute with *0.03 M hydrochloric acid* to obtain a solution containing 40 IU per ml.

Storage. Store in multiple dose containers in a refrigerator (2° to 8°). It should not be allowed to freeze.

Labelling. The label states (1) the strength in terms of the number of IU per ml; (2) the animal source or sources of the insulin; (3) that the preparation should not be allowed to freeze; (4) that the container should be gently shaken before a dose is withdrawn; (5) the storage conditions.

Insulin Glargine



$C_{267}H_{404}N_{72}O_{78}S_6$ Mol. Wt. 6063

21^A-Glycine-30^{B}a-L-arginine-30^{B}b-L-arginine-insulin (human).

Insulin glargine is a 2-chain peptide containing 53 amino acids. The A-chain is composed of 21 amino acids and the B-chain is composed of 32 amino acids. It is identical in primary structure to human insulin, only differing in amino acid sequence at position 21 in the A-chain and at the C-terminal end of the B-chain where it contains two additional amino acids. Human insulin is Asn (A21), whereas insulin glargine is Gly (A21), Arg (B31), Arg (B32). As in human insulin, insulin glargine contains two interchain disulphide bonds and one intrachain disulphide bond.

Insulin glargine contains not less than 94 per cent and not more than 105 per cent (anhydrous substance). By convention, for the purpose of labelling insulin glargine preparations, 0.0364 mg of insulin glargine is equivalent to 1 unit.

Insulin glargine is produced by a method based on recombinant DNA (rDNA) technology under conditions designed to minimise the degree of microbial contamination.

Category. Antidiabetic.

Single-chain precursor. Use a suitably sensitive method. It complies with the limits approved by National Regulatory Authority.

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Description. White or almost white, hygroscopic powder. Practically insoluble in *water* and in anhydrous *ethanol*, soluble in dilute minerals acids.

Host-cell-derived proteins. Not more than 100 ppm.

Host cell or vector-derived DNA. Not more than 10 ng per dose.

Identification

A. In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

B. Determine by peptide mapping (2.3.47).

Test solution. Dilute the substance under examination in *0.01M hydrochloric acid* to a concentration of 10.0 mg per ml of insulin glargine and transfer 5 µl of this solution to a clean tube. Add 1 ml of *1 M tris- hydrochloride buffer solution* pH 7.5 and 100 µl of a 20 units per ml solution of *Staphylococcus aureus strain V8 protease, type XVII-B* in *1 M tris hydrochloride buffer solution* pH 7.5. Mix and incubate at 45° for about 2 hours. Stop the reaction by adding 2 µl of *orthophosphoric acid*.

Reference solution. Prepare at the same time and in the same manner as that of test solution but use *insulin glargine IPRS* instead of the substance under examination.

Buffer solution. Dissolve 11.6 g *orthophosphoric acid* and 42.1 g of *sodium perchlorate* in 1600 ml of *water*, adjust pH to 2.3 with *triethylamine* and dilute to 2000 ml with *water*.

Chromatographic system
- a stainless steel column 12. 5 cm x 3.0 mm, packed with spherical end-capped octadecylsilyl silica gel (4 µm),
- column temperature: 35°,
- mobile phase: A. a mixture of 7 volumes of *acetonitrile* and 93 volumes of *buffer solution pH 2.3*,
  B. a mixture of 57 volumes *acetonitrile* and 43 volumes of *buffer solution pH 2.3*,
- flow rate: 0.6 ml per minute,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 214 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
30	20	80
35	20	80

Equilibrate the column with initial condition for at least 15 minutes.

Inject the reference solution and the test solution. The chromatograms obtained with the test solution and the reference solution are qualitatively similar to the chromatogram of insulin glargine digest supplied with *insulin glargine IPRS*. In the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II, and III, the symmetry factor should not be more than 1.5 for the peaks due to fragments II and III and resolution between them is not less than 3.4.

The retention times of fragments I and IV are the same as for human insulin, the retention times of fragment II and III differ from human insulin due to the difference in the sequence at position 21 of the A-chain and to the 2 additional amino acids of the B-chain.

Tests

Dimers and related substances of higher molecular mass. Determine by size-exclusion chromatography (2.4.16) using normalisation procedure.

Test solution. Dissolve 15.0 mg of the substance under examination in 1.5 ml of *0.01M hydrochloric acid* and dilute to 10.0 ml with *water*.

Reference solution (a). Dry about 200 mg of the substance under examination in an oven at 100° for 1.5 to 3 hours. Dissolve 15.0 mg of the dried substance in 1.5 ml of *0.01M hydrochloric acid* and dilute to 10.0 ml with *water*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *water*. Dilute 3.0 ml of this solution to 20.0 ml with *water*.

Chromatographic system
- two columns coupled in series, the coupling volume between the 2 columns being kept to a minimum;
- a stainless steel column 30 cm x 8 mm, packed with hydrophilic silica gel (5 µm) with a pore size of 15 nm, of a grade suitable for fractionation of global proteins in the relative molecular mass range of 2000 to 80000,
- mobile phase: a mixture of 200 volumes of *anhydrous acetic acid*, 300 volumes of *acetonitrile* and 500 volumes of *water*, adjust pH to 3.0 with *concentrated ammonia*.
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 276 nm,
- injection volume: 100 µl, if splitting of the principal peak is observed, the injection volume may be decreased.

Inject reference solution (a). The test is not valid unless symmetry factor is not more than 2.0 for the peak due to Insulin glargine; peak-to-valley ratio calculated between height above the baseline of the peak due to high molecular mass proteins and height above the baseline of the lowest point of the curve separating this peak from the peak due to insulin glargine is not less than 2 per cent. Inject reference solution (b). The test is not valid unless signal-to-noise ratio is not less than 10 for the principal peak.

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Inject the test solution. The total of impurities with a retention time less than that of Insulin glargine is not more than 0.3 per cent of the total area of the peaks; disregard any peak with a retention time greater than that of the peak due to insulin glargine.

Related proteins. Determine by liquid chromatography (2.4.14) using the normalisation procedure. Maintain the solutions at 2° to 8°.

Test solution. Dissolve 15.0 mg of the substance under examination in 1.5 ml of *0.01M hydrochloric acid* and dilute to 10.0 ml with *water*.

Reference solution (a). Dissolve the contents of a vial of *insulin glargine IPRS* in 1.5 ml of a *0.01M hydrochloric acid* and dilute the solution to 10 ml with *water*.

Reference solution (b). Dissolve the contents of a vial of *insulin glargine IPRS* (containing 0^A-Arg-insulin glargine) in 0.3 ml of *0.01M hydrochloric acid* and add 1.7 ml of *water*.

Buffer solution. A 2.07 per cent w/v of *anhydrous sodium dihydrogen phosphate* in *water*, adjust pH to 2.5 with *orthophosphoric acid*.

Chromatographic system

- a stainless steel column 25 cm x 3.0 mm, packed with spherical end-capped octadecylsilane bonded to porous silica (4 µm),
- column temperature: 35°,
- mobile phase: A. a mixture of 18.4 g of *sodium chloride* in 250 ml of the buffer solution and 250 ml of *acetonitrile*; dilute to 1000 ml with *water*;
  B. a mixture of 3.2 g of *sodium chloride* in 250 ml of buffer solution and 650 ml of *acetonitrile*; dilute to 1000 ml with *water*;
- flow rate: 0.55 ml per minute,
- a gradient program using the conditions given below,
- spectrophotometer set at 214 nm,
- injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	96	4
20	83	17
30	63	37
33	96	4
40	96	4

Inject reference solution (b). The test is not valid unless peak-to-valley ratio calculated between height above the baseline of the peak due to 0^A-Arg-insulin glargine and height above the baseline of the lowest point of the curve separating this peak from the peak due to insulin glargine is not less than 2 per cent.

Inject reference solution (a) and test solution, in the chromatogram obtained with the test solution, the area of any peak other than the principal peak is not more than 0.4 per cent for each peak. The sum of the areas of all the peaks other than the principal peak is not more than 1.0 per cent of total areas of all the peaks.

Zinc. Not more than 1.0 per cent.

Determine by Atomic absorption spectrometry (2.4.2)

Test solution. Dissolve 45.0 mg of the substance under examination in 50.0 ml of *0.01M hydrochloric acid* and dilute 10.0 ml of this solution to 100 ml with *0.01M hydrochloric acid*.

Reference solution. Prepare reference solutions containing 0.2 µg, 0.4 µg and 0.6 µg zinc per ml by diluting *zinc standard solution* (10 ppm Zn) with *0.01M hydrochloric acid*.

Measure the absorbance at 213.9 nm using zinc hollow-cathode lamp or electrodeless-discharge-lamp as a source of radiation and air-acetylene flame of suitable composition (for example, 11 litre of air and 2 litre of acetylene per minute).

Water (2.3.43). Not more than 8.0 per cent, determined on 90.0 mg by means of coulometric titration.

Bacterial endotoxins (2.2.3). Not more than 10 IU per mg of protein.

Assay. Determine by liquid chromatography (2.4.14) as described under the test for Related proteins.

Inject the test solution and reference solution (a). Calculate the content of insulin glargine, $C_{267}H_{404}N_{72}O_{78}S_6$ taking into account the assigned content of *insulin glargine IPRS*.

Storage. Store in an airtight container, protected from light at a temperature of -20 ° ± 5°.

Insulin Glargine Injection

Insulin Glargine Injection is sterile, aqueous solution of insulin glargine.

Insulin Glargine Injection contains not less than 95 per cent and not more than 105 per cent of the stated amount of insulin glargine.

Insulin Glargine Injection complies with the following additional requirements.

Usual strength. 100 units per ml.

Description. A clear colourless solution, free from turbidity and foreign matter.

Identification

In the Assay, the principal peak in the chromatogram obtained with the test solution corresponds to the principal peak in the chromatogram obtained with the reference solution.

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Tests

pH (2.4.24). 3.5 to 4.5.

Dimers and related substances of higher molecular mass. Determine by size-exclusion chromatography (2.4.16) using normalisation procedure.

Test solution. Dilute the preparation under examination with *water* to obtain a concentration of about 40 units per ml of insulin glargine.

Reference solution (a). Dry about 200 mg of the substance under examination in an oven at 100° for 1.5 to 3 hours. Dissolve 15.0 mg of the dried substance in 1.5 ml of *0.01M hydrochloric acid* and dilute to 10.0 ml with *water.*

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *water.* Dilute 3.0 ml of this solution to 20.0 ml with *water.*

Chromatographic system
- two columns coupled in series, the coupling volume between the two columns being kept to a minimum,
- a stainless steel column 30 cm x 8 mm, packed with hydrophilic silica gel for chromatography (5 µm) with a pore size of about 15 nm, of a grade suitable for fractionation of global proteins in the relative molecular mass range of 2000 to 80000,
- mobile phase: a mixture of 200 volumes of *anhydrous acetic acid*, 300 volumes of *acetonitrile* and 500 volumes of *water*, adjust pH to 3.0 with *concentrated ammonia*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 276 nm,
- injection volume: 100 µl; if splitting of the principal peak is observed, the injection volume may be decreased.

Inject reference solution (a). The test is not valid unless symmetry factor is not more than 2.0 for the peak due to Insulin glargine; peak-to-valley ratio calculated between height above the baseline of the peak due to high molecular mass proteins and height above the baseline of the lowest point of the curve separating this peak from the peak due to insulin glargine is not less than 2 per cent. Inject reference solution (b). The test is not valid unless signal-to-noise ratio is not less than 10 for the principal peak.

Inject the test solution. The total of impurities with a retention time less than that of Insulin glargine is not more than 0.3 per cent of the total area of the peaks; disregard any peak with a retention time greater than that of the peak due to insulin glargine.

Related proteins. Determine by liquid chromatography (2.4.14) using the normalisation procedure. Maintain the solutions at 2° to 8°.

Test solution. Dilute the preparation under examination with *water* to obtain a concentration of about 40 units per ml of insulin glargine.

Reference solution (a). Dissolve the contents of a vial of *insulin glargine IPRS* in 1.5 ml of a *0.01M hydrochloric acid* and dilute the solution to 10 ml with *water.*

Reference Solution (b). Dissolve the contents of a vial of *insulin glargine IPRS* (containing 0^A-Arg-insulin Glargine) in 0.3 ml of *0.01M hydrochloric acid* and add 1.7 ml of *water.*

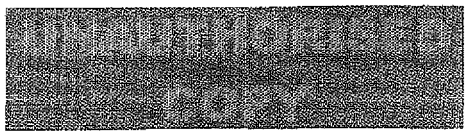
Buffer solution. Dissolve 20.7 g of anhydrous *sodium dihydrogen phosphate* in 900 ml of *water*, adjust to pH 2.5 with *orthophosphoric acid* and dilute to 1000 ml with *water.*

Chromatographic system
- a stainless steel column 25 cm x 3.0 mm, packed with spherical end-capped octadecylsilane bonded to porous silica (4 µm),
- column temperature: 35°,
- mobile phase: A. a mixture of 18.4 g of *sodium chloride* in 250 ml of the buffer solution and 250 ml of *acetonitrile*; dilute to 1000 ml with *water*,
  B. a mixture of 3.2 g of *sodium chloride* in 250 ml of buffer solution and 650 ml of *acetonitrile*; dilute to 1000 ml with *water*,
- flow rate: 0.55 ml per minute,
- a gradient program using the conditions given below,
- spectrophotometer set at 214 nm,
- injection volume: 5 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	96	4
20	83	17
30	63	37
33	96	4
40	96	4

Inject reference solution (b). The test is not valid unless peak-to-valley ratio calculated between height above the baseline of the peak due to 0^A-Arg-insulin glargine and height above the baseline of the lowest point of the curve separating this peak from the peak due to insulin glargine is not less than 2 per cent.

Inject reference solution (a) and test solution. In the chromatogram obtained with the test solution, the area of any peak other than the principal peak is not more than 0.5 per cent for each peak. The sum of the areas of all the peaks other than the principal peak is not more than 2.0 per cent of total areas of all the peaks.



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Total Zinc. Not less than 27 µg per 100 units and not more than 33 µg per 100 units of insulin glargine, determined by atomic absorption spectrometry (2.4.2) as stated under Insulin Preparations.

Test solution. Dilute the preparation under examination with *water* to obtain a concentration of about 1 units per ml of insulin glargine.

Reference solution. Prepare reference solutions containing 0.2 µg, 0.4 µg and 0.6 µg zinc per ml by diluting *zinc standard solution* (10 ppm Zn) with *0.01M hydrochloric acid.*

Measure the absorbance at 213.9 nm using zinc hollow-cathode lamp or electrodeless-discharge-lamp as a source of radiation and air-acetylene flame of suitable composition (for example, 11 litre of air and 2 litre of acetylene per minute).

Bacterial Endotoxins (2.2.3). Not more than 80 Endotoxin Units per 100 units of insulin glargine.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14) as described in the test for related proteins.

Inject the test solution and reference solution. Calculate the content of insulin glargine ($C_{267}H_{404}N_{72}O_{78}S_6$) taking into account the assigned content of *insulin glargine IPRS.*

Storage. Store protected from light at 2° to 8° and avoid freezing.

Labelling. The label states (1) the strength in terms of insulin glargine units per ml; (2) the material is produced by microbial synthesis via recombinant DNA technology; (3) the storage conditions.

Interferon Alfa-2 Concentrated Solution

CDLPQTHSLG SRRTLMLLAQ MRX$_1$ISLFSCL KDRHDFGFPQ
EEFGNQFQKA ETIPVLHEMI QQIFNLFSTK DSSAAWDETL
LDKFYTELYQ QLNDLEACVI QGVGVTETPL MKEDSILAVR
KYFQRITLYL KEKKYSPCAW EVVRAEIMRS FSLSTNLQES
LRSKE

alfa-2a: $C_{860}H_{1353}N_{227}O_{255}S_9$ Mol. Wt.19,241
alfa-2b: $C_{860}H_{1353}N_{229}O_{255}S_9$ Mol. Wt.19,269

Interferon alfa-2 concentrated solution is a solution of an rDNA derived therapeutic protein which exhibits non-specific antiviral activity, at least in homologous cells. Interferon alfa-2 concentrated solution also exerts antiproliferative and immunomodulator activity. Two different types of alfa-2 interferon, varying in the amino acid residue at position 23, are found and are named as alfa-2a and alfa-2b.

Designation	Residue at position 23 (X_1)
alfa-2a	Lys
alfa-2b	Arg

This monograph applies to interferon alfa-2a and 2b concentrated solutions.

Interferon alfa-2 concentrated solution contains not less than 1.4×10^8 IU per mg of protein and not less than 2×10^8 IU of Interferon alfa-2 per ml.

Production

Interferon alfa-2 concentrated solution is produced by a method based on recombinant DNA technology using bacteria as host cells. It is produced under controlled conditions designed to minimise microbial contamination of the product.

Interferon alfa-2 concentrated solution complies with the following additional requirements.

Host-cell-derived proteins. Not more than 100 ppm.

Host cell or vector derived DNA. Not more than 10 ng per dose.

Description. A clear, colourless or slightly yellowish liquid.

Identification

A. It shows the expected biological activity as described under Assay for potency.

B. Determine by isoelectric focusing (2.4.33).

Test solution. Dilute the preparation under examination with *water* to obtain a solution containing 1 mg protein per ml.

Reference solution. A 1 mg per ml solution of *interferon alfa-2 IPRS* in *water.*

Isoelectric point calibration solution pI range 3.0 to 10.0. Prepare and use according to the manufacturer's instructions.

Isoelectric focusing is carried out using either horizontal electrophoresis system or by vertical electrophoresis system as per the procedure described below or by any appropriate validated method.

Horizontal Electrophoresis

Select and use a suitable horizontal isoelectric focusing apparatus with facility for connecting a circulating bath chiller or any suitable equipment capable of maintaining 10°. Select gels for isoelectric focusing with a pH gradient from 3.5 to 9.5.

Use *phosphoric acid* as anode solution (98 g per litre *orthophosphoric acid*) and *1 M sodium hydroxide* as the

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cathode solution. Using filter paper apply 15 µl of the test solution and the reference solution to the gel close to the cathode.

Start the isoelectric focusing at 1500 V and 50 mA. Turn off the power after 30 minutes. Remove the application filters and reconnect the power supply for 1 hour. Keep the power constant during the focusing process.

Immerse the gel in a solution containing 115 g per litre of *trichloroacetic acid* and 34.5 g per litre of *sulphosalicylic acid* in *water* and agitate the container gently for 60 minutes.

Prepare a mixture of 32 volumes of *glacial acetic acid*, 100 volumes of *ethanol* and 268 volumes of *water*. Transfer the gel to the mixture and soak for 5 minutes.

Immerse the gel for 10 minutes in a staining solution prewarmed to 60°. The staining solution is prepared by adding 1.2 g per litre of *acid blue 83* to the mixture of *glacial acetic acid*, *ethanol* and *water*.

Wash the gel several times to destain with the mixture of *glacial acetic acid*, *ethanol* and *water* and keep the gel in this mixture for about 12-24 hours until the background is clear.

Add *glycerol*, 10 per cent v/v to the mixture of *glacial acetic acid*, *ethanol* and *water*. Soak the gel for 1 hour in the solution.

The principal bands of the electropherogram obtained with the test solution correspond in position to the principal bands of the electropherogram obtained with the reference solution. Plot the migration distances of the isoelectric point markers versus their isoelectric points and determine the isoelectric points of the principal components of the test solution and the reference solution. They do not differ by more than 0.2 pI unit. The test is not valid unless the isoelectric point markers are distributed along the entire length of the gel and the isoelectric points of the principal bands in the electropherogram obtained with the reference solution are between 5.8 and 6.3.

C. Examine the electropherograms obtained under reducing conditions in the test for impurities of molecular masses differing from that of interferon alfa-2. The principal band in the electropherogram obtained with test solution (a) corresponds in position to the principal band in the electropherogram obtained with reference solution (a).

D. Determine by peptide mapping (2.3.47).

Test solution. Dilute the preparation under examination with *water* to produce a solution containing 1.5 mg protein per ml. Transfer 25 µl to a microfuge tube. Add 1.6 µl of *1 M phosphate buffer solution pH 8.0*, 2.8 µl of a freshly prepared 1 mg per ml solution of *trypsin* in *water* (suitable for peptide mapping) and 3.6 µl of *water* and mix vigorously. Cap the tube and place it in a water-bath at 37° for 18 hours. Add 100 µl of a 573 g per litre solution of *guanidine hydrochloride* and mix well. Add 7 µl of 154.2 g per litre solution of *dithiothreitol* and mix well. Place the capped tube in boiling water for 1 minute and cool to room temperature.

Reference solution. A 1.5 mg per ml solution of the appropriate *interferon alfa-2 IPRS* prepared at the same time and in the same manner as the test solution.

Chromatographic system

- a stainless steel column 100 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) with a pore size of 30 nm,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 1000 ml with *water*,

  B. Add 1 ml of *trifluroacetic acid* to 100 ml of *water* and dilute to 1000 ml with *acetonitrile*,
- flow rate: 1 ml per minute,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 214 nm,
- injection volume: 100 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
8	100	0
68	40	60
72	40	60
75	100	0

Inject the test solution and the reference solution. The chromatogram obtained with each solution should be qualitatively similar to the chromatogram of *interferon alfa-2 IPRS*. The profile of the chromatogram obtained with the test solution should also correspond to that of the chromatogram obtained with the reference solution.

Tests

Impurities of molecular masses differing from that of interferon alfa-2. Determine by electrophoresis (sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12). The test is performed under both reducing and non-reducing conditions, using resolving gels of 14 per cent *acrylamide* and silver staining as the detection method.

Sample buffer (non-reducing conditions). Mix equal volumes of *water* and *concentrated SDS-PAGE sample buffer*.

Sample buffer (reducing conditions). Mix equal volumes of *water* and concentrated *SDS-PAGE sample buffer for reducing conditions* containing *2-mercaptoethanol* as the reducing agent.

Test solution (a). Dilute the preparation under examination in the sample buffer to obtain a solution containing a concentration of 0.5 mg protein per ml.

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Test solution (b). Dilute 0.2 ml of test solution (a) to 1 ml with the sample buffer.

Reference solution (a). Prepare a 0.625 mg per ml solution of the appropriate *interferon alfa-2 IPRS* in the sample buffer.

Reference solution (b). Dilute 0.2 ml of reference solution (a) to 1 ml with the sample buffer.

Reference solution (c). Dilute 0.2 ml of reference solution (b) to 1 ml with the sample buffer.

Reference solution (d). Dilute 0.2 ml of reference solution (c) to 1 ml with the sample buffer.

Reference solution (e). Dilute 0.2 ml of reference solution (d) to 1 ml with the sample buffer.

Reference solution (f). Use a solution of molecular mass standards suitable for calibrating SDS-PAGE gels in the range. 15 to 67 kDa.

Place the test and reference solutions, contained in covered test-tubes, on a water-bath for 2 minutes.

Apply 10 µl of reference solution (f) and 50 µl of each of the other solutions to the stacking gel wells. Perform the electrophoresis under the conditions recommended by the manufacturer of the equipment. Detect proteins in the gel by silver staining.

The test is not valid unless (1) the validation criteria are met; (2) a band is seen in the electropherogram obtained with reference solution (e); (3) a gradation of intensity of staining is seen in the electropherograms obtained, respectively, with test solution (a) and test solution (b) and with reference solutions (a) to (e).

The electropherogram obtained with test solution (a) under reducing conditions may show, additional bands but no such band should be more intense than the band obtained with reference solution (d). Further, not more than 3 such bands should be more intense than the principal band obtained with reference solution (e).

The electropherogram obtained with test solution (a) under non-reducing conditions may show, in addition to the principal band, less intense bands with molecular masses higher than the principal band. No such band is more intense than the principal band in the electropherogram obtained with reference solution (d) and not more than three such bands are more intense than the principal band in the electropherogram obtained with reference solution (e).

Related proteins. Determine by liquid chromatography (2.4.14).

0.25 per cent w/w hydrogen peroxide solution. Dilute *hydrogen peroxide solution* with *water* to obtain 0.25 per cent w/w solution.

Test solution. Dilute the preparation under examination with *water* to obtain a solution containing 0.5 mg protein per ml.

Reference solution. To a volume of the test solution, add a suitable volume of the 0.25 per cent *hydrogen peroxide solution* to give a final hydrogen peroxide concentration of 0.005 per cent, and allow to stand at room temperature for 1 hour, to generate about 5 per cent oxidised interferon. Add 12.5 mg of *L-methionine* per ml of the solution. Allow to stand at room temperature for 1 hour. Store the solutions for not longer than 24 hours at a temperature of 2° to 8°.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) with a pore size of 30 nm,
- mobile phase: A. to 700 ml of *water* add 2 ml of *trifluoroacetic acid* and 300 ml of *acetonitrile*,
  B. to 200 ml of *water* add 2 ml of *trifluoroacetic acid* and 800 ml of *acetonitrile*,
- flow rate: 1 ml per minute,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 210 nm,
- injection volume. 100 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	72	28
1	72	28
5	67	33
20	63	37
30	57	43
40	40	60
42	40	60
50	72	28
60	72	28

Inject 50 µl alternatively the test solution and the reference solution.

Interferon alfa-2 elutes at a retention time of about 20 minutes in the chromatogram. With the reference solution a peak related to oxidized interferon appears at a retention time of about 0.9 relative to the principal peak.

The test is not valid unless the resolution between the peaks corresponding to oxidised interferon and interferon is at least 1.0. Consider only the peaks whose retention time is 0.7 to 1.4 relative to that of the principal peak.

In the chromatogram obtained with the test solution, the area of any peak, apart from the principal peak, is not greater than 3.0 per cent of the total area of all of the peaks. The sum of the

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areas of any peaks other than the principal peak is not greater than 5.0 per cent of the total area of all of the peaks.

Bacterial endotoxins (2.2.3). Not more than 100 Endotoxin Units per mg of protein.

Assay

A. *Protein*

Test solution. Dilute the preparation under examination with *water* to obtain a concentration of about 0.5 mg of interferon alfa-2 per ml.

Reference solutions. Prepare a stock solution of 0.5 mg per ml of *bovine albumin.* Prepare eight dilutions of the stock solution containing between 3 µg per ml and 30 µg per ml of *bovine albumin.*

Prepare 30-fold and 50-fold dilutions of the test solution.

Prepare a mixture of 2.0 ml of a 2.0 per cent w/v solution of *copper sulphate* in *water*, 2.0 ml of a 4.0 per cent w/v solution of *sodium tartrate* in *water* and 96.0 ml of a 4.0 per cent w/v solution of *sodium carbonate* in *0.2 M sodium hydroxide.*

Add 1.25 ml of the above mixture to the test-tube containing 1.5 ml of *water* to prepare the blank, 1.25 ml to the test tube containing different dilutions of the sample and 1.25 ml to the test tube with the reference solution.

Mix after each addition and after approximately 10 minutes, add to each test-tube 0.25 ml of a mixture of equal volumes of *water* and *phosphomolybdotungstic reagent.* Mix after each addition. After 30 minutes, measure the absorbance of each solution at 750 nm (2.4.7) using the blank as the compensation liquid.

Draw a calibration curve from the absorbances of the eight reference solutions and the corresponding protein contents and read from the curve the content of protein in the test solution.

B. *Potency*

The potency of interferon alfa-2 is estimated based on its ability to protect cells against a viral cytopathic effect compared to the protection accorded by an appropriate International Standard of human recombinant interferon alfa-2 or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Carry out the assay by a suitable method, based on the following design.

Use an established cell line sensitive to the cytopathic effect of a suitable virus responsive to interferon. The following cell cultures and virus have shown to be suitable: MDBK cells (ATCC No. CCL22), or Mouse L cells (NCTC clone 929; ATCC No.CCL 1) as the cell culture and vesicular stomatitis virus VSV, Indiana strain (ATCC No. VR-158) as the infective agent; or human diploid fibroblast FS-71 cells responsive to interferon as the cell culture, encephalomyocarditis virus (ATCC No.VR-129B) as the infective agent and WISH (ATCC No. CCL-25) and Encephalomyocarditis Virus (EMC virus ATCC No. VR-129B).

Incubate in at least four groups, cells with three or more different concentrations of the preparation under examination and one with the reference preparation in a microtitre plate. Include appropriate controls of untreated cells in each group.

Choose the concentrations of the preparations such that the lowest concentration produces some protection and the largest concentration produces less than maximal protection against the viral cytopathic effect.

Add the cytopathic virus after the cells have established to all wells except the control wells.

Determine the cytopathic effect of virus quantitatively and calculate the potency of the preparation to be examined by the usual statistical methods for a parallel line assay.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits of the estimated potency (P= 0.95) are not less than 64 per cent and not more than 156 per cent of the stated potency.

Storage. Store protected from light, at or below -20°.

Labelling. The label states (1) The type of interferon (alfa-2a or alfa-2b); (2) the type of production.

Interferon Alfa-2a Injection

Interferon Alfa-2a Injection is a dilution of Interferon Alfa-2a Concentrated Solution in a suitable liquid. It is supplied as a ready-to-use solution.

Usual strengths. 3 MIU per vial, 4.5 MIU per vial, 5 MIU per vial, 6 MIU per vial, 44 µg per vial.

Description. A clear, colourless or slightly yellowish solution virtually free from particles.

Identification

A. It gives the appropriate response when examined using the conditions under the Assay.

B. In the test for Related proteins, the retention time of the principal peak in the chromatogram obtained with test solution (a) corresponds to that of the principal peak in the chromatogram obtained with test solution (b).

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C. In the test for Impurities of molecular weights differing from that of Interferon Alfa-2a, the principal band in the gel obtained with test solution (a) corresponds to that in the gel obtained with reference solution (a).

Tests

pH (2.4.24). Comply with the limits as approved by National Regulatory Authority.

Impurities of molecular weights differing from that of Interferon Alfa-2a. Determine by polyacrylamide gel electrophoresis (2.4.12) under reducing and non-reducing conditions, using slab gels 1.0 mm thick and 14 per cent acrylamide as the resolving gel.

Sample buffer (non-reducing conditions). Mix equal volumes of *water* and *concentrated SDS-PAGE sample buffer.*

Sample buffer (reducing conditions). Mix equal volumes of *water* and concentrated *SDS-PAGE sample buffer for reducing conditions* containing *2-mercaptoethanol* as the reducing agent.

Test solution (a). A 5.5 MIU per ml solution of interferon alfa-2a preparation under examination in sample buffer, if necessary.

Test solution (b). Dilute 0.2 ml of test solution (a) to 1 ml with the sample buffer.

Reference solution (a). Prepare a 25 µg per ml solution of the appropriate *interferon alfa-2 IPRS* in the sample buffer.

Reference solution (b). Dilute 0.2 ml of reference solution (a) to 1 ml with the sample buffer.

Reference solution (c). Dilute 0.2 ml of reference solution (b) to 1 ml with the sample buffer.

Reference solution (d). Dilute 0.2 ml of reference solution (c) to 1 ml with the sample buffer.

Reference solution (e). Dilute 0.2 ml of reference solution (d) to 1 ml with the sample buffer.

Reference solution (f). Use a solution of molecular mass standards suitable for calibrating SDS-PAGE gels in the range 15 to 67 kDa.

Place the test and reference solutions, contained in covered test-tubes on a water-bath for 2 minutes.

Apply 10 µl of reference solution (f) and 50 µl of each of the other solutions to the stacking gel wells. Perform the electrophoresis under the conditions recommended by the manufacturer of the equipment. Detect proteins in the gel by silver staining.

The test is not valid unless (1) the validation criteria are met for reference solution (f); (2) a band is seen in the electropherogram obtained with reference solution (e); (3) a gradation of intensity of staining is seen in the electropherograms obtained, respectively, with test solution (a) and test solution (b) and with reference solutions (a) to (e).

The electrophoretogram obtained with test solution (a) under reducing conditions may show, in addition to the principal band, less intense bands with molecular weights lower than the principal band. No such band is more intense than the principal band in the electrophoretogram obtained with reference solution (d) (1 per cent) and not more than three such bands are more intense than the principal band in the electrophoretogram obtained with reference solution (e) (0.4 per cent).

Related Proteins. Determine by liquid chromatography (2.4.14) using the normalisation procedure. Store the solutions for not longer than 24 hours at a temperature of 2° to 8°.

Test solution (a). A 5.5 MIU per ml solution of interferon alfa-2a preparation under examination in *water*, if necessary.

Test solution (b). Add a suitable volume of 0.25 per cent w/w *hydrogen peroxide solution* to a volume of the test solution (a) to give a final hydrogen peroxide concentration of 0.005 per cent w/w and allow to stand at room temperature for 1 hour or for the length of time that will generate about 5 per cent oxidised interferon. Add 12.5 mg of *L-methionine* per ml of solution. Allow to stand at room temperature for 1 hour.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilyl silica gel for chromatography (5 µm),
- mobile phase: A. dissolve 0.2 ml of *trifluoroacetic acid* and 30 ml of *acetonitrile* in 70 ml of *water*,
  B. dissolve 0.2 ml of *trifluoroacetic acid* and 20 ml of *water* in 80 ml of *acetonitrile*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 100 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	72	28
1	72	28
5	67	33
20	63	37
30	57	43
40	40	60
42	40	60
50	72	28
60	72	28

The retention time of the peak due to interferon alfa-2a is about 20 minutes. The relative retention times with reference

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to Interferon Alfa-2a are: impurity A (oxidised interferon alfa-2a) about 0.7; impurity B (*N*-acetyl interferon) about 1.1; impurity C (interferon with broken disulfide bridge) about 1.3.

The test is not valid unless, in the chromatogram obtained with reference solution (b), the resolution factor between the peaks corresponding to oxidised interferon (impurity A) and interferon is at least 1.0.

In the chromatogram obtained with test solution (a), the area of any peak corresponding to impurity A is not greater than 5.0 percent; the area of any peak corresponding to impurity B is not greater than 3.0 per cent; the sum of the areas of any other secondary peaks is not greater than 5.0 per cent; the sum of the areas of any secondary peaks is not greater than 12.0 per cent.

Disregard any peak with a retention time less than 0.7 times, or greater than 1.4 times, of that of the principal peak in the chromatogram obtained with test solution (b).

Bacterial endotoxins (2.2.3). Not more than 2 IU in a volume containing 1 MIU of interferon alfa-2a.

Other tests. Complies with the tests stated under Parenteral Preparations (Injections).

Assay. The potency of interferon alfa-2a is calculated by comparing its effect to protect cells against a viral cytopathic effect with the same effect of the appropriate International Standard of human recombinant interferon alfa-2a or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Carry out the assay by a suitable method, based on the following design.

Use in standard culture conditions, an established cell line sensitive to the cytopathic effect of a suitable virus (a human diploid fibroblast cell line, free of microbial contamination, responsive to interferon and sensitive to encephalomyocarditis virus, is suitable).

The following cell cultures and virus have shown to be suitable: MDBK cells (ATCC No. CCL22), or Mouse L cells (NCTC clone 929; ATCC No. CCL 1) as the cell culture and vesicular stomatitis virus VSV, Indiana strain (ATCC No. VR-158) as the infective agent; or human diploid fibroblast FS-71 cells responsive to interferon as the cell culture, and encephalomyocarditis virus (ATCC No. VR-129B) as the infective agent.

Incubate in at least four series, cells with three or more different concentrations of the preparation being examined and the reference preparation in a microtitre plate and include in each series appropriate controls of untreated cells. Choose the concentrations of the preparations such that the lowest concentration produces some protection and the largest concentration produces less than maximal protection against the viral cytopathic effect. Add at a suitable time the cytopathic virus to all wells with the exception of a sufficient number of wells in all series, which are left with uninfected control cells. Determine the cytopathic effect of virus quantitatively with a suitable method. Calculate the potency of the preparation being examined by the standard statistical methods for a parallel line assay.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The fiducial limits of error are not less than 64 per cent and not more than 156 per cent of the stated potency.

Storage. Interferon Alfa-2a Injection should be stored at a temperature of 2° to 8°. It should not be allowed to freeze.

Labelling. The label of the sealed container states the number of MIU (Million Units) contained in it.

Interferon Alpha 2b Injection

Interferon Alpha-2b is in ready to use presentation prepared from interferon alpha-2b bulk solution in a solution appropriate for its stability.

Usual strength. 3 MIU, 5 MIU

Description. A clear, essentially particulate free, colourless or slightly yellowish liquid filled in appropriate container.

Identification

A. It shows the expected biological activity using the condition under the assay.

B. In the test for Related proteins, the retention time of the principal peak in the chromatogram obtained with test solution corresponds to that of the principal peak in the chromatogram obtained with reference solution.

C. In the test for Impurities of molecular weights differing from that of Interferon Alfa-2b, the principal band in the gel obtained with test solution (a) corresponds to that of the principal band in the gel obtained with reference solution (a).

Tests

pH (2.2.24). 6.5 to 7.1

Impurities of molecular masses differing from that of Interferon Alfa-2b. Determine by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12) under reducing and non reducing conditions.

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Resolving gel. 14 per cent acrylamide

Detection method. Silver staining

Sample buffer (non-reducing conditions). Mix equal volumes of *water* and concentrated *SDS-PAGE sample buffer.*

Sample buffer (reducing conditions). Mix equal volumes of *water* and concentrated *SDS-PAGE sample buffer for reducing conditions* containing *2-mercaptoethanol* as the reducing agent.

Test solution (a). A 5.5 MIU per ml solution of interferon alfa-2b preparation under examination in sample buffer, if necessary.

Test solution (b). Dilute 0.2 ml of test solution (a) to 1 ml with the sample buffer.

Reference solution (a). Prepare a 0.625 mg per ml solution of the appropriate *interferon alfa-2b IPRS* in the sample buffer.

Reference solution (b). Dilute 0.2 ml of reference solution (a) to 1 ml with the sample buffer.

Reference solution (c). Dilute 0.2 ml of reference solution (b) to 1 ml with the sample buffer.

Reference solution (d). Dilute 0.2 ml of reference solution (c) to 1 ml with the sample buffer.

Reference solution (e). Dilute 0.2 ml of reference solution (d) to 1 ml with the sample buffer.

Reference solution (f). Use a solution of molecular mass standards suitable for calibrating SDS-PAGE gels in the range. 15 to 67 kDa.

Place the test and reference solutions, contained in covered test-tubes, on a water-bath for 2 minutes.

Apply 10 µl of reference solution (f) and 50 µl of each of the other solutions to the stacking gel wells. Perform the electrophoresis under the conditions recommended by the manufacturer of the equipment. Detect proteins in the gel by silver staining.

The test is not valid unless (1) the validation criteria are met for solution (f); (2) a band is seen in the electropherogram obtained with reference solution (e); (3) a gradation of intensity of staining is seen in the electropherograms obtained, respectively, with test solution (a) and test solution (b) and with reference solutions (a) to (e).

The electropherogram obtained with test solution (a) under reducing conditions may show, additional bands but no such band should be more intense than the band obtained with reference solution (d). Further, not more than 3 such bands should be more intense than the principal band obtained with reference solution (e).

The electropherogram obtained with test solution (a) under non-reducing conditions may show, in addition to the principal band, less intense bands with molecular masses higher than the principal band. No such band is more intense than the principal band in the electropherogram obtained with reference solution (d) and not more than three such bands are more intense than the principal band in the electropherogram obtained with reference solution (e).

Related proteins. Determine by liquid chromatography (2.4.14).

0.25 per cent w/w hydrogen peroxide solution. Dilute *hydrogen peroxide solution* with *water* to obtain 0.25 per cent w/w solution.

Test solution. A 5.5 MIU per ml solution of interferon alfa-2b preparation under examination in *water*, if necessary

Reference solution. To a volume of the test solution, add a suitable volume of the 0.25 per cent *hydrogen peroxide solution* to give a final hydrogen peroxide concentration of 0.005 per cent, and allow to stand at room temperature for 1 hour, to generate about 5 per cent oxidised interferon. Add 12.5 mg of *L-methionine* per ml of the solution. Allow to stand at room temperature for 1 hour. Store the solutions for not longer than 24 hours at a temperature of 2° to 8°.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm) with a pore size of 30 nm,
- mobile phase: A. To 700 ml of *water* add 2 ml of *trifluoroacetic acid* and 300 ml of *acetonitrile*,
  B. To 200 ml of *water* add 2 ml of *trifluoroacetic acid* and 800 ml of *acetonitrile*,
- flow rate: 1 ml per minute,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 210 nm,
- injection volume: 100 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	72	28
1	72	28
5	67	33
20	63	37
30	57	43
40	40	60
42	40	60
50	72	28
60	72	28

Inject 50 µl alternatively the test solution and the reference solution.

Interferon alfa-2b elutes at a retention time of about 20 minutes in the chromatogram. With the reference solution a peak related to oxidized interferon appears at a retention time of about 0.9 relative to the principal peak.

The test is not valid unless the resolution between the peaks corresponding to oxidized interferon and interferon is at least 1.0. Consider only the peaks whose retention time is 0.7 to 1.4 relative to that of the principal peak.

In the chromatogram obtained with the test solution, the area of any peak, apart from the principal peak, is not greater than 3.0 per cent of the total area of all of the peaks. The sum of the areas of any peaks other than the principal peak is not greater than 5.0 per cent of the total area of all of the peaks.

Bacterial endotoxins (2.2.3). Not more than 2 Endotoxin Units in a volume containing 1 MIU of interferon alpha-2b.

Other tests. Complies with the test stated under Parenteral Preparations (Injections).

Assay

Potency. The potency of interferon alfa-2b is estimated based on its ability to protect cells against a viral cytopathic effect compared to the protection accorded by an appropriate International Standard of human recombinant interferon alfa-2 or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Carry out the assay by a suitable method, based on the following design.

Use an established cell line sensitive to the cytopathic effect of a suitable virus responsive to interferon. The following cell cultures and virus have shown to be suitable: MDBK cells (ATCC No. CCL22), or Mouse L cells (NCTC clone 929; ATCC No. CCL 1) as the cell culture and vesicular stomatitis virus VSV, Indiana strain (ATCC No. VR-158) as the infective agent; or human diploid fibroblast FS-71 cells responsive to interferon as the cell culture, encephalomyocarditis virus (ATCC No. VR-129B) as the infective agent or WISH (ATCC No. CCL-25) and Encephalomyocarditis Virus (EMC virus ATCC No. VR-129B).

Incubate in at least three groups, cells with three or more different concentrations of the preparation under examination and one with the reference preparation in a microtitre plate. Include appropriate controls of untreated cells in each group.

Choose the concentrations of the preparations such that the lowest concentration produces some protection and the largest concentration produces less than maximal protection against the viral cytopathic effect.

Add the cytopathic virus after the cells have established to all wells except the control wells.

Determine the cytopathic effect of virus quantitatively by any suitable method and calculate the potency of the preparation to be examined by the usual statistical methods for a parallel line assay.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits of the estimated potency (P= 0.95) are not less than 64 per cent and not more than 156 per cent of the stated potency.

Storage. Store protected from light, at a temperature of 2-8°.

Labelling. The label of the sealed container states the number of MIU (million International Units) contained in it.

Interferon Beta 1a Concentrated Solution

MSYNLLGFLQ RSSNFQCQKL LWQLNGRLEY CLKDRMNFDI
PEEIKQLQQF QKEDAALTIY EMLQNIFAIF RQDSSSTGWN*
ETIVENLLAN VYHQINHLKT VLEEKLEKED FTRGKLMSSL
HLKRYYGRIL HYLKAKEYSH CAWTIVRVEI LRNFYFINRL
TGYLRN

* glycosylation site

$C_{908}H_{1406}N_{246}O_{252}S_7$ Mol. Wt. 22,500

Interferon Beta 1a Concentrated Solution is a solution of a glycosylated protein having the same amino acid sequence and disulphide bridge and a similar glycosylation pattern as interferon beta produced by human diploid fibroblasts in response to viral infections and various other inducers.

Interferon Beta-1a Concentrated Solution is produced by a method based on recombinant DNA (rDNA) technology, using mammalian cells in culture.

Interferon Beta-1a Concentrated Solution contains not less than 0.20 mg of protein per ml and potency not less than 1.5×10^8 IU per milligram of protein.

Category. Antiviral-cytokine; antiproliferative and immunomodulatory.

Host-cell-derived proteins. Not more than 100 ppm.

Host-cell or vector-derived DNA. The limit is 10 ng per therapeutic dose.

***N*-terminal truncated forms.** Examination for specific *N*-terminal truncated forms should be performed using a suitable technique such as *N*-terminal sequence determination.

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Total high molecular weight impurities, including dimer. Not more than 2.0 per cent, using an appropriate validated liquid chromatography method.

Description. A clear or slightly opalescent, colourless or slightly yellowish liquid.

Identification

1. It shows the biological activity as described under Assay.

2. Determine isoform distribution by using method A or B.

A. Determine by using mass spectrometry (2.4.39).

Introduction of the sample. Direct inflow of a desalted preparation to be examined or liquid chromatography-mass spectrometry combination.

Mode of ionization. Electrospray.

Signal acquisition. Complete spectrum mode from 1100 to 2400.

Calibration. Use myoglobin in the *m/z* range of 600-2400; set the instrument within validated instrumental settings and analyse the sample; the deviation of the measured mass does not exceed 0.02 per cent of the reported mass.

Interpretation of results. A typical spectrum consists of 6 major glycoforms (A to F), which differ in their degree of sialylation and/or antennarity type as shown in below.

MS peak	Glycoform*	Expected M	Sialylation Level
A	2A2S1F	22375	Disialylated
B	2A1S1F	22084	Monosialylated
C	3A2S1F and/or 2A2S1F+1 Hex NacHex Repeat	22739	Disialylated
D	3A3S1F	23031	Trisialylated
E	4A3S1F and/or 3A3S1F+1 Hex NacHex Repeat	23400	Trisialylated
F	2A0S1F	21793	Non-sialylated

*2A = biantennary complex type oligosaccharide; 3A = triantennary complex type oligosaccharide; 4A = tetraantennary complex type oligosaccharide; 0S = non-sialylated; 1S = monosialylated; 2S = disialylated; 3S = trisialylated; 1F = fucosylated.

The mass spectrum obtained with the preparation to be examined corresponds, with respect to the 6 major peaks, to the mass spectrum obtained with reference *interferon beta-1a IPRS*.

B. Determination of glycan distribution by using liquid chromatography (2.4.14).

Use N glycosidase F (PNGase F) to cleave the glycans from the protein and separate the cleaved glycans from the digest mixture by reversed phase solid phase extraction. The glycans are then labelled by reductive amination with a fluorescent dye (2 aminobenzoic acid, 2 AA). The resulting labelled glycans are separated by high performance ion exchange liquid chromatography and detected by fluorescence. The glycans are identified by retention time and quantified. Because 2-AA is bound to a single location (the reducing end) on each released glycan, the relative percent distribution of the fully sialylated glycans distinguished by antennary structure can be determined by peak area calculations for the purposes of identity testing.

The chromatogram obtained with the preparation under examination corresponds, with respect to the 6 major peaks (listed in 2A), to the chromatogram obtained with *interferon beta-1a IPRS*. Any new glycan peak above the quantitation limit of the method not present in the *interferon beta-1a IPRS* needs to identified and the relevance to the safety and efficacy of interferon beta-1a needs to be described.

3. Determine by peptide mapping (2.3.47).

Test solution. Add 5 µl of a 24.2 per cent w/v solution of *tris(hydroxymethyl)aminomethane* and a volume of the preparation under examination containing 20 µg of protein to a polypropylene tube of 0.5 ml capacity. Add 4 µl of a 0.1 per cent w/v solution of *endoprotease Lys C* in *0.05M tris-hydrochloride buffer solution pH 9.0*. Mix gently and incubate at 30° for 2 hours. Add 10 µl of a 1.54 per cent w/v solution of *dithiothreitol*. Dilute the solution with the same volume of a 57.3 per cent w/v solution of *guanidine hydrochloride*. Incubate at 4° for 3 to 4 hours.

Reference solution. Prepare at the same time and in the same manner as for the test solution but using *interferon beta-1a IPRS* instead of the preparation under examination.

Chromatographic system

- precolumn. a stainless steel column 2 cm x 2.1 mm, packed with octadecylsilyl silica gel (5 µm) with a pore size of 30 nm,
- column. a stainless steel column 25 cm x 2.1 mm, packed with octadecylsilyl silica gel (5 µm) with a pore size of 30 nm.
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 1000 ml with *water*,
  B. dilute 1 ml of *trifluoroacetic acid* in 700 ml of *acetonitrile* and dilute to 1000 ml with *water*,
- flow rate: 0.2 ml per minute,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 214 nm,
- injection volume. contains 20 µg of digested protein.

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
30	64	36
45	55	45
50	40	60
70	0	100
83	0	100
85	100	0

Inject the reference solution and the test solution. The test is not valid unless the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of interferon beta-1a digest supplied with *interferon beta-1a IPRS*.

The profile of the chromatogram obtained with the test solution corresponds to that obtained with the reference solution.

Tests

Impurities of molecular masses differing from that of interferon beta-1a. Determine by method A or B.

A. Determine by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (2.4.12) under reducing condition.

Resolving gel. 12 per cent acrylamide.

Concentrated SDS-PAGE sample buffer. Dissolve 125 mM *Tris-hydrochloride* (pH 6.8), 4 per cent SDS, 20 per cent glycerol, 0.004 per cent bromophenol blue, and 200 mM dithiothreitol as the reducing agent. 2-mercaptoethanol at 10 per cent may also be used to substitute for dithiothreitol.

Sample buffer. A mixture of equal volumes of *concentrated SDS-PAGE sample buffer* for reducing conditions and *water.*

Test solution (a). Concentrate the preparation under examination using a suitable method to obtain a protein concentration of 1.5 mg per ml.

Test solution (b). Mix equal volumes of test solution (a) and the concentrated sample buffer.

Test solution (c). Dilute test solution (a) to obtain a protein concentration of 0.6 mg per ml. Mix equal volumes of this solution and the concentrated sample buffer.

Test solution (d). Mix 8 µl of test solution (c) and 40 µl of the sample buffer.

Test solution (e). Mix 15 µl of test solution (d) and 35 µl of the sample buffer.

Test solution (f). Mix 18 µl of test solution (e) and 18 µl of the sample buffer.

Test solution (g). Mix 12 µl of test solution (f) and 12 µl of the sample buffer.

Reference solution (a). Dissolve the solution of relative molecular mass markers suitable for calibrating SDS-PAGE gels in the range of 15-67 kDa in the sample buffer.

Reference solution (b). A solution containing 0.075 per cent w/v of *interferon beta-1a IPRS* in sample buffer.

Boil the sample for 3 minutes. Apply 20 µl of test solutions (b) to (g) and reference solutions (a) and (b).

Detection. Coomassie staining (or equivalent), carried out as follows: immerse the gel in Coomassie staining solution (or equivalent) at 33-37° for 90 min with gentle shaking, then remove the staining solution; destain the gel with a large excess of a mixture of 1 volume of *glacial acetic acid*, 1 volume of *2-propanol* and 8 volumes of *water.*

Apparent molecular masses of interferon beta-1a, under-glycosylated interferon beta-1a, deglycosylated interferon beta-1a, interferon beta-1a dimer are about 23,000, 21,000, 20,000 and 46,000 respectively.

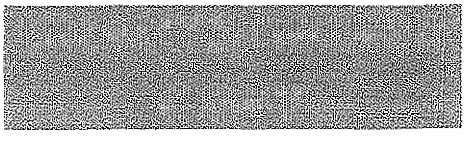
For identification of bands, use the electropherogram provided with *interferon beta-1a IPRS.*

The test is not valid unless the validation criteria are met; a band is seen in the electropherogram obtained with test solution (g); a gradation of intensity of staining is seen in the electropherograms obtained with test solutions (b) to (g).

In the electropherogram obtained with test solution (c), the band corresponding to underglycosylated interferon beta 1a is not more intense than the principal band in the electropherogram obtained with test solution (e) (5.0 per cent); in the electropherogram obtained with test solution (b), the band corresponding to deglycosylated interferon beta-1a is not more intense than the principal band in the electropherogram obtained with test solution (e) (2.0 per cent); any other band corresponding to an impurity of a molecular mass lower than that of interferon beta-1a, apart from the band corresponding to underglycosylated interferon beta-1a is not more intense than the principal band in the electropherogram obtained with test solution (f) (1.0 per cent), total impurities not more than 4.0 per cent.

B. Determine by gel chip capillary electrophoresis under reducing conditions using the Protein 80 kit and bioanlyzer or equivalent kit and system.

Samples are concentrated then diluted to ~2 mg per ml. Samples and reference are mixed with reducing sample buffer. Samples, reference standard and molecular weight marker are then heat denatured in a 100° boiling water-bath in the presence of a reducing agent and a denaturant in the sample buffer. Each of Gel-Dye Mix, destaining solution, the samples and the molecular weight ladder are applied onto the designated well in a gel chip.



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Electrophoresis is performed with the bioanalyzer for analysis. The bioanalyzer and the gel chip form a closed circuit that applies an electrokinetic force moving the sample proteins through the gel matrix inside the separation capillary based on size. The gel matrix inside the capillary contains a fluorescent dye that binds to the SDS coated proteins. As proteins travel further through the capillary to the wells, the background is destained. The proteins are then detected by laser induced fluorescence and the raw data are displayed in the form of electropherograms. An upper and lower marker present in the gel dye matrix act as internal standards and are used to align the molecular weight ladder data with the data from the sample wells, compensating for drift effects that may occur during the course of the run. The bioanalyzer software calculates the molecular weight of the protein by comparison to the protein ladder. The software then normalizes the peak areas against the area of the upper marker to generate a relative concentration of the analyte peaks with respect to the internal markers.

Results for underglycosylated (limit not more than 5.0 percent), nonglycosylated (limit not more than 2.0 per cent) and other impurities (limit not more than 1.0 per cent for individual impurity, total impurities not more than 4.0 per cent) are reported as a relative percent of the total relative concentration, which excludes the system peaks and the upper and lower markers.

Oxidised interferon beta-1a. Not more than 6.0 per cent. Determine by peptide mapping (2.3.47) as described in identification.

Use the chromatogram obtained with the test solution used in Identification test 3. Locate the peaks due to the peptide fragment comprising amino acids 34-45 and its oxidised form using the chromatogram of oxidised interferon beta-1a digest supplied with *interferon beta-1a IPRS*.

Calculate the percentage of oxidation of interferon beta-1a using the following expression:

$$\frac{A_{34-45ox}}{A_{34-45} + A_{34-45ox}} \times 100$$

$A_{34-45ox}$ = area of the peak corresponding to the oxidised peptide fragment 34-45;

A_{34-45} = area of the peak corresponding to the peptide fragment 34-45.

Deamidated Interferon beta-1a. Determine by peptide mapping (2.3.47).

Test solution. Buffer exchange minimum of 60 µg of sample into *100 mM sodium phosphate, 200 mM sodium chloride*, pH 7 with final volume approximately 250 µl. Deliver 100 µl of buffer exchanged sample into vial. Prepare a fresh mixture of DTT and EDTA with following proportions 8.8 µl 50 mM DTT with 4 µl of 20 mM EDTA. Add 12.8 µl of the DTT/EDTA mixture to sample. Allow to sit at ambient temperature for 15 - 30 minutes. Add 5.0 ml of 0.20 mg per ml Endo Lys C enzyme solution and mix by drawing up and down several times. Incubate the aliquots at 2°- 8° for 18 - 24 hours. Add 175 ml of fresh mixture of 155 µl *8 M guanidine hydrochloride* and 20 ml 50 mM DTT to each aliquot; mix by drawing up and down several times. Aliquots must be injected within 24 hours of the final addition of 50 mM DTT unless frozen at -70°.

Reference solution. Prepare at the same time and in the same manner as for the test solution but using *interferon beta-1a IPRS* instead of the preparation under examination.

Chromatographic system

- column. a stainless steel column 15 cm x 4.6 mm, packed with C18 hydrophobic bonded phase on silica support of 3 µm with a pore size of 120Å.
- mobile phase: A. dilute 1 gram of *trifluoroacetic acid* to 1000 ml with *water*,
  B. dilute 1 gram of *trifluoroacetic acid* in 700 ml of *acetonitrile* then dilute to 1000 ml with *water*,
- a linear gradient programme using the conditions given below,
- flow rate: 1.0 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: contains 20 µg of digested protein.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	65	35
2	60	40
15	50	50
17	10	90

Label the peaks AP7, Asp AP7 and Iso AspAP7 (Refer to representative chromatogram)

$$\text{per cent deamination} = \frac{\text{area iso AspAP7} + \text{area AspAP7}}{(\text{area iso AspAP7} + \text{area AspAP7} + \text{area AP7}} \times 100$$

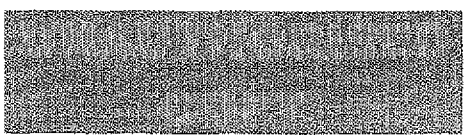
When determining per cent deamidated (Asn-25) interferon beta-1a, no peaks should be present in the regions of AP7, isoAsp AP7, or AspAP7 in the enzyme digest blank sample.

Asp: Hydrolysis product of the cyclic imide intermediate caused by deamidation of asparagine in which polypeptide backbone is attached by an alpha-carboxyl linkage.

Iso Asp: Hydrolysis product of the cyclic imide intermediate caused by deamidation of asparagine in which polypeptide backbone is attached by a beta-carboxyl linkage.

AP7: Peptide of amino acid sequence 20-33 of interferon beta-1a

(Name assigned as per the elution order of the major fragments of the interferon beta-1a peptide map)



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Per cent biantennary sialylation. Not less than 90 per cent.

Use the chromatogram obtained with the test solution used in identification test 2B (2-AA IEC) or mass spectra obtained using method 2A. The sialylation of biantennary glycans on the molecule is determined using the area data from the identified biantennary glycans to calculate the percentage of sialic acid occupancy (per cent biantennary sialylation).

Locate the peaks for BiNA0 (2A0S1F), BiNA1 (2A1S1F), BiNA2 (2A2S1F) and BiNA3 (2A3S1F, with calculated mass of 22670 Da).

Determine the percent biantennary sialylation using the following equation

$$\%\text{Biantennary Sialylation} = \frac{(\text{Area of BiNA1}) + \{(\text{Area of BiNA2} + \text{Area of BiNA3}) \times 2\}}{(\text{Area of BiNA0} + \text{Area of BiNA1} + \text{Area of BiNA2} + \text{Area of BiNA3}) \times 2} \times 100$$

Dimers and related substance of higher molecular mass. Determine by size exclusion chromatography (2.4.16).

Test solution. Dilute the injection, if necessary, with mobile phase to give a solution containing 25 µg of interferon beta-1a.

Reference solution. Prepare a solution of 0.05 per cent w/v of *bovine albumin* and 0.02 per cent w/v of *cytochrome c* in the mobile phase.

Chromatographic System

- a stainless steel column 30 cm x 7.8 mm packed with appropriate porous silica beads (5 µm) with a fractionation range for proteins of approximately 5,000 to 150,000 fitted with a guard column (4 cm x 6 mm) packed with the same material,
- mobile phase: to 800 ml of *water* add 2.77 g *sodium phosphate monobasic monohydrate*, 21.9 g *sodium phosphate dibasic heptahydrate*, and 12.0 g *sodium chloride*, then dilute with *water* to 1000 ml. Adjust to pH 7.1. If the pH is not within specification, adjust with *orthophosphoric acid* or 50 per cent *sodium hydroxide*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm.

Inject 100 µl of the blank solution (mobile phase). The test is not valid unless, in the chromatogram obtained with the blank solution no peaks are observed between the retention time of the peak due to bovine albumin and the retention time of peak due to cytochrome c. Initial blank injections may contain peaks as the solution is used to condition the column.

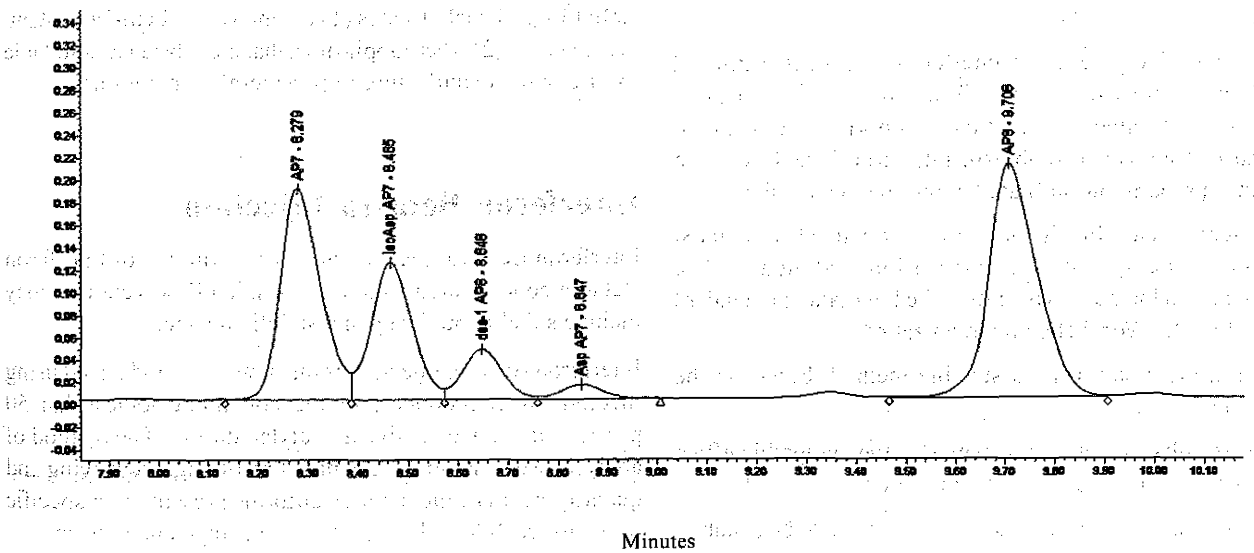
Inject 40 µl of reference solution and a quantity of test solution containing 25 µg of interferon beta-1a. The test is not valid unless, in the chromatogram obtained with reference solution, the resolution between peaks due to bovine albumin and cytochrome c is at least 4.0. In the chromatogram obtained with the test solution, the total area of any peaks eluting before the principal peak is not more than 2.0 per cent.

Bacterial endotoxins (2.2.3). Not more than 0.7 Endotoxin Unit in the volume that contains 1×10^6 IU of interferon beta-1a.

Assay

A. Determine by liquid chromatography (2.4.14). Prepare 3 independent dilutions for each solution.

Test solution. Dilute the preparation under examination to obtain a concentration of 100 µg per ml.



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Reference solution. Dissolve the contents of a vial of *interferon beta-1a IPRS* to obtain a concentration of 100 µg per ml.

Chromatographic system

- precolumn: a stainless steel column 2 cm x 2.1 mm, packed with butylsilyl silica gel (5 µm) with a pore size of 30 nm,
- column: a stainless steel column 25 cm x 2.1 mm, packed with butylsilyl silica gel (5 µm) with a pore size of 30 nm,
- mobile phase: A: a 0.1 per cent v/v solution of *trifluoroacetic acid* in *water*,
  B: Dilute 1 ml *trifluoroacetic acid* and 300 ml of *water* to 1000 ml with *acetonitrile*,
- flow rate: 0.2 ml per minute,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 214 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
20	0	100
25	0	100
26	100	0
40	100	0

Inject the reference solution. The test is not valid unless the symmetry factor for the peak due to interferon beta-1a is between 0.8 and 2.0; and the relative standard deviation between the peak areas obtained after injection of the 3 independent dilutions is not more than 3.0 per cent.

Inject the test solution. The retention time of interferon beta-1a is about 20 minutes.

Calculate the content of interferon beta-1a ($C_{908}H_{1406}N_{246}O_{252}S_7$), from the declared content of *interferon IPRS*.

B. *Potency*. The potency of interferon beta-1a is estimated based on its ability to protect cells against a viral cytopathic effect with the same ability of the appropriate International Standard of human recombinant interferon beta-1a or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Carry out the assay using a suitable method, based on the following design.

Use an established cell line sensitive to the cytopathic effect of a suitable virus responsive to interferon.

The following cell cultures and virus are shown to be suitable: WISH cells (ATCC No. CCL-25) and vesicular stomatitis virus VSV, Indiana strain (ATCC No. VR-158) as infective agent; A549 cells (ATCC No. CCL-185) and encephalomyocarditis virus EMC (ATCC No. VR-129B) as infective agent.

Incubate in at least 4 series, cells with 3 or more different concentrations of the preparation under examination and the reference preparation in a microtitre plate and include in each series appropriate controls of untreated cells. Choose the concentrations of the preparations such that the lowest concentration produces some protection and the largest concentration produces less than maximal protection against the viral cytopathic effect.

Add at a suitable time the cytopathic virus to all wells with the exception of a sufficient number of wells in all series, which are left with uninfected control cells.

Determine the cytopathic effect of the virus quantitatively with a suitable method. Calculate the potency of the preparation to be examined by the usual statistical methods.

The estimated potency is not less than 80 per cent and not more than 130 per cent of the stated potency. The confidence limits (P = 0.95) are not less than 64 per cent and not more than 156 per cent of the estimated potency.

Cytopathtic effect assay (CPE assay described above) has been used as the standard assay for potency determination of interferon beta-1a. If an assay other than CPE assay is used to determine potency of interferon beta-1a, crossover study is to be performed between CPE assay and the new assay to demonstrate equivalence. Limits equivalent to those for CPE assay are to be established for the new assay based on the outcome of the crossover study.

Storage. Store protected from light and moisture, at a temperature below -70°. If the substance is sterile, store in a sterile, tamper proof, air tight container.

Labelling. The label states (1) the interferon beta-1a content, in mg per ml; (2) where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

Interferon Beta-1a Injection

Interferon Beta-1a Injection is a sterile solution of Interferon Beta-1a concentrated solution in a suitable solvent that may include suitable buffering and stabilizing agents.

Interferon Beta-1a Injection is manufactured and tested using methods which produce a product containing not less than 50 per cent of the main active di-sialylated form. The method of analysis used should be capable of resolving, identifying and quantifying all of the main glycoforms present, with specific limits for each form being set by the competent authority.

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Interferon Beta-1a Injection contains not less than 80 per cent and not more than 125 per cent of the stated potency.

Usual strengths. 22 µg per 0.5 ml; 44 µg per 0.5 ml, 66 µg (18 MIU) per 1.5 ml; 132 µg (36 MIU) per 1.5 ml.

Description. A clear, colorless solution.

Identification

1. It complies with the biological activity as described under Assay.

2. Determine by using method A or B.

A. Determine isoform distribution by using mass spectrometry.

Introduction of the sample. Direct inflow of a desalted preparation under examination or liquid chromatography-mass spectrometry combination.

Mode of ionization. Electrospray.

Signal acquisition. Complete spectrum mode from 1100 to 2400.

Calibration. Use myoglobin in the *m/z* range of 600-2400; set the instrument within validated instrumental settings and analyse the sample; the deviation of the measured mass does not exceed 0.02 per cent of the reported mass.

Interpretation of results. A typical spectrum consists of 6 major glycoforms (A to F), which differ in their degree of sialylation and/or antennarity type as shown.

MS peak	Glycoform*	Expected M	Sialylation Level
A	2A2S1F	22375	Disialylated
B	2A1S1F	22084	Monosialylated
C	3A2S1F and/or 2A2S1F+1 Hex NacHex Repeat	22739	Disialylated
D	3A3S1F	23031	Trisialylated
E	4A3S1F and/or 3A3S1F+1 Hex NacHex Repeat	23400	Trisialylated
F	2A0S1F	21793	Non-sialylated

*2A = biantennary complex type oligosaccharide; 3A = triantennary complex type oligosaccharide; 4A = tetraantennary complex type oligosaccharide; 0S = non-sialylated; 1S = monosialylated; 2S = disialylated; 3S = trisialylated; 1F = fucosylated.

The mass spectrum obtained with the preparation under examination corresponds, with respect to the 6 major peaks, to the mass spectrum obtained reference *interferon beta-1a IPRS.*

B. Determine glycan distribution by using liquid chromatography (2.4.14).

Use N glycosidase F (PNGase F) to cleave the glycans from the protein and separate the cleaved glycans from the digest mixture by reversed phase solid phase extraction. The glycans are then labelled by reductive amination with a fluorescent dye (2 aminobenzoic acid, 2 AA). The resulting labelled glycans are separated by high performance ion exchange liquid chromatography and detected by fluorescence. The glycans are identified by retention time and quantified. Because 2-AA is bound to a single location (the reducing end) on each released glycan, the relative percent distribution of the fully sialylated glycans distinguished by antennary structure can be determined by peak area calculations for the purposes of identity testing.

The chromatogram obtained with the preparation under examination corresponds, with respect to the 6 major peaks (listed in 2A), to the chromatogram obtained with *interferon beta-1a IPRS.* Any new glycan peak above the quantitation limit of the method not present in the *interferon beta-1a IPRS* needs to be identified and the relevance to the safety and efficacy of interferon beta-1a needs to be described.

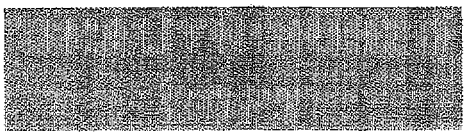
3. Determine by peptide mapping (2.3.47).

Test solution. Add 5 µl of a 24.2 per cent w/v solution of *tris(hydroxymethyl)aminomethane* and a volume of the preparation under examination containing 20 µg of protein to a polypropylene tube of 0.5 ml capacity. Add 4 µl of a 0.1 per cent w/v solution of *endoprotease LysC* in *0.05M tris-hydrochloride buffer solution pH 9.0.* Mix gently and incubate at 30° for 2 hours. Add 10 µl of a 1.54 per cent w/v solution of *dithiothreitol.* Dilute the solution with the same volume of a 57.3 per cent w/v solution of *guanidine hydrochloride.* Incubate at 4° for 3-4 hours.

Reference solution. Prepare at the same time and in the same manner as for the test solution but using *interferon beta-1a IPRS* instead of the preparation under examination.

Chromatographic system

- a stainless steel precolumn 2 cm x 2.1 mm, packed with octadecylsilyl silica gel (5 µm) with a pore size of 30 nm,
- a stainless steel column 25 cm x 2.1 mm, packed with octadecylsilyl silica gel (5 µm) with a pore size of 30 nm,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 1000 ml with *water,*
  B. dilute 1 ml of *trifluoroacetic acid* in 700 ml of *acetonitrile* and dilute to 1000 ml with *water,*
- flow rate: 0.2 ml per minute,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 214 nm,
- injection volume: contains 20 µg of digested protein.



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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
30	64	36
45	55	45
50	40	60
70	0	100
83	0	100
85	100	0

Inject the reference solution and the test solution. The test is not valid unless the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of Interferon Beta-1a digest supplied with *interferon beta-1a IPRS*.

The profile of the chromatogram obtained with the test solution corresponds to that obtained with the reference solution.

Tests

pH (2.4.24). 4.5 to 5.1

Osmolality (2.4.23). 261 to 349 mosmol per kg

Impurities of molecular masses differing from that of Interferon Beta-1 a. Determine by method A or B.

A. Determine by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (2.4.12) under reducing conditions.

Resolving gel. 12 per cent acrylamide.

Concentrated SDS-PAGE sample buffer. Dissolve *125 mM Tris-hydrochloride* (pH 6.8), 4 per cent *SDS*, 20 per cent *glycerol*, 0.004 per cent *bromophenol blue*, and 200 *mM dithiothreitol* as the reducing agent. *2-mercaptoethanol* at 10 per cent may also be used to substitute for *dithiothreitol*.

Sample buffer. A mixture of equal volumes of *concentrated SDS-PAGE sample buffer* for reducing conditions and *water*.

Test solution (a). Concentrate the preparation under examination using a suitable method to obtain a protein concentration of 1.5 mg per ml.

Test solution (b). Mix equal volumes of test solution (a) and the concentrated sample buffer.

Test solution (c). Dilute test solution (a) to obtain a protein concentration of 0.6 mg per ml. Mix equal volumes of this solution and the concentrated sample buffer.

Test solution (d). Mix 8 µl of test solution (c) and 40 µl of the sample buffer.

Test solution (e). Mix 15 µl of test solution (d) and 35 µl of the sample buffer.

Test solution (f). Mix 18 µl of test solution (e) and 18 µl of the sample buffer.

Test solution (g). Mix 12 µl of test solution (f) and 12 µl of the sample buffer.

Reference solution (a). Dissolve the solution of relative molecular mass markers suitable for calibrating SDS-PAGE gels in the range of 15-67 kDa in the sample buffer.

Reference solution (b). A solution containing 0.075 per cent w/v of *Interferon Beta-1a IPRS* in sample buffer.

Boil the sample for 3 minutes. Apply 20 µl of test solutions (b) to (g) and reference solutions (a) and (b).

Detection. Coomassie staining (or equivalent).

Apparent molecular masses of interferon beta-1a, underglycosylated interferon beta-1a, deglycosylated interferon beta-1a, interferon beta-1a dimer are about 23000, 21000, 20000 and 46000 respectively.

For identification of bands, use the electropherogram provided with *interferon beta-1a IPRS*.

The test is not valid unless the validation criteria are met; a band is seen in the electropherogram obtained with test solution (g); a gradation of intensity of staining is seen in the electropherograms obtained with test solutions (b) to (g).

The electropherogram obtained with test solution (c) may show, in addition to the principal band, less intense bands with molecular weights lower than the principal band. The band corresponding to underglycosylated interferon beta-1a is not more intense than the principal band in the electropherogram obtained with test solution (e) (5.0 per cent).

The electropherogram obtained with test solution (b) may show, in addition to the principal band, less intense bands with molecular weights lower than the principal band. The band corresponding to deglycosylated interferon beta-1a is not more intense than the principal band in the electropherogram obtained with test solution (e) (2.0 per cent); any other band corresponding to an impurity of a molecular mass lower than that of interferon beta-1a, apart from the band corresponding to underglycosylated interferon beta-1a is not more intense than the principal band in the electropherogram obtained with test solution (f) (1.0 per cent), total impurities not more than 4.0 per cent.

B. Determine by gel chip capillary electrophoresis under reducing conditions using suitable kit and system.

Preparation under exam is concentrated then diluted to approximately 2 mg per ml. Preparation under exam and *interferon beta-1a IPRS* are mixed with reducing sample buffer. Preparation under exam, *interferon beta-1a IPRS* and molecular weight marker are then heat denatured in a 100° boiling water-bath in the presence of a reducing agent and a denaturant in the sample buffer. Each of Gel-Dye Mix, destaining solution,

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the preparation under exam and the molecular weight ladder are applied onto the designated well in a gel chip.

Electrophoresis is performed with the bioanalyzer for analysis. The bioanalyzer and the gel chip form a closed circuit that applies an electrokinetic force moving the sample proteins through the gel matrix inside the separation capillary based on size. The gel matrix inside the capillary contains a fluorescent dye that binds to the SDS coated proteins. As proteins travel further through the capillary to the wells, the background is destained. The proteins are then detected by laser induced fluorescence and the raw data are displayed in the form of electropherograms. An upper and lower marker present in the gel dye matrix act as internal standards and are used to align the molecular weight ladder data with the data from the sample wells, compensating for drift effects that may occur during the course of the run. The bioanalyzer software calculates the molecular weight of the protein by comparison to the protein ladder. The software then normalizes the peak areas against the area of the upper marker to generate a relative concentration of the analyte peaks with respect to the internal markers.

Identity of interferon beta-1a is confirmed by comparing the molecular weight of the main interferon beta-1a peak in the preparation under exam to the molecular weight of the main interferon beta-1a peak in the *nterferon beta-1a IPRS*.

Results for underglycosylated (not more than 5.0 per cent), nonglycosylated (not more than 2.0 per cent) and other impurities (not more than 1.0 per cent for individual impurity, total impurities not more than 4.0 per cent) are reported as a relative per cent of the total relative concentration, which excludes the system peaks and the upper and lower markers.

Oxidized interferon beta-1a. Not more than 9.0 per cent.

Use the chromatogram obtained with the test solution used in identification test 3. Identify and integrate the peptide fragment peak comprising amino-acids 34-45 and its oxidized form by comparing to the reference chromatogram of oxidized interferon beta-1a digest supplied with *interferon beta-1a IPRS*.

Calculate the percentage oxidation of interferon beta-1a in the test solution using the following expression:

$$\frac{A_{34-45ox}}{A_{34-45} + A_{34-45ox}} \times 100$$

$A_{34-45ox}$ = area of the peak corresponding to the oxidised peptide fragment 34-45;

A_{34-45} = area of the peak corresponding to the peptide fragment 34-45.

Per cent biantennary sialylation. Not less than 90 per cent.

Use the chromatogram obtained with the test solution used in identification test 2. The sialylation of biantennary glycans on the molecule is determined using the area data from the identified biantennary glycans to calculate the percentage of sialic acid occupancy (per cent biantennary sialylation).

Identify and integrate the peaks for BiNA0 (2A0S1F), BiNA1 (2A1S1F), BiNA2 (2A2S1F) and BiNA3 (2A3S1F) by comparison to the reference chromatogram for glycan distribution analysis of *interferon beta-1a IPRS*. Determine the per cent biantennary sialylation using the following expression:

$$\%\text{Biantennary Sialylation} = \frac{(\text{Area of BiNA1}) + \{(\text{Area of BiNA2} + \text{Area of BiNA3}) \times 2\}}{(\text{Area of BiNA0} + \text{Area of BiNA1} + \text{Area of BiNA2} + \text{Area of BiNA3}) \times 2} \times 100$$

Dimers and related substance of higher molecular mass. Determine by size exclusion chromatography (2.4.16).

Test solution. Dilute the injection, if necessary, with mobile phase to give a solution containing 25 µg of interferon beta-1a.

Reference solution. Prepare a solution of 0.05 per cent w/v of *bovine albumin* and 0.02 per cent w/v of *cyctochrome c* in the mobile phase.

Chromatographic System

- a stainless steel column 30 cm x 7.8 mm packed with appropriate porous silica beads (5 µm) with a fractionation range for proteins of approximately 5,000 to 150,000 fitted with a guard column (4 cm x 6 mm) packed with the same material,
- mobile phase: to 800 ml of *water* add 2.77 g *sodium phosphate monobasic monohydrate*, 21.9 g *sodium phosphate dibasic heptahydrate*, and 12.0 g *sodium chloride*, then dilute with *water* to 1000 ml. Adjust to pH 7.1. If the pH is not within specification, adjust with *orthophosphoric acid* or 50 per cent *sodium hydroxide*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 280 nm.

Inject 100 µl of the blank solution (mobile phase). The test is not valid unless, in the chromatogram obtained with the blank solution no peaks are observed between the retention time of the peak due to bovine albumin and the retention time of peak due to cytochrome c. Initial blank injections may contain peaks as the solution is used to condition the column.

Inject 40 µl of reference solution and a quantity of test solution containing 25 µg of interferon beta-1a. The test is not valid unless, in the chromatogram obtained with reference solution, the resolution between peaks due to bovine albumin and cytochrome c is at least 4.0. In the chromatogram obtained with the test solution, the total area of any peaks eluting before the principal peak is not more than 2.0 per cent.

Bacterial endotoxins (2.2.3): Not more than 0.7 Endotoxin Unit in the volume that contains 1 x 10^6 IU of interferon beta-1a.

Other tests. Comply with the tests stated under Parenteral Preparations (Injections).

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Assay

A. Determine by liquid chromatography (2.4.14). Prepare 3 independent dilutions for each solution.

Test solution. Dilute the injection, if necessary, to obtain a concentration of 100 µg per ml of interferon beta-1a.

Reference solution. Dissolve the contents of a vial of *interferon beta-1a IPRS* to obtain a concentration of 100 µg per ml.

Chromatographic system

- precolumn. a stainless steel column 2 cm x 2.1 mm, packed with butylsilyl silica gel (5 µm) with a pore size of 30 nm,
- column. a stainless steel column 25 cm x 2.1 mm, packed with butylsilyl silica gel (5 µm) with a pore size of 30 nm,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* in 1000 ml of *water,*

  B. dilute 1 ml of *trifluoroacetic acid* and 300 ml of water to 1000 ml with *acetonitrile,*
- flow rate: 0.2 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 214 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
20	0	100
25	0	100
26	100	0
40	100	0

Inject the reference solution. The test is not valid unless the symmetry factor for the peak due to interferon beta-1a is between 0.8-2.0; and the relative standard deviation between the peak areas obtained after injection of the 3 independent dilutions is not more than 3.0 per cent.

Inject the test solution. The retention time of interferon beta-1a is about 20 minutes.

Calculate the content of interferon beta-1a ($C_{908}H_{1406}N_{246}O_{252}S_7$), from the declared content of *interferon beta-1a IPRS.*

B. *Potency.* The potency of interferon beta-1a is estimated based on its ability to protect cells against a viral cytopathic effect (CPE) with the same ability of the appropriate International of human recombinant interferon beta-1a or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Carry out the assay using a suitable method based on following design.

WISH cells (ATCC No. CCL-25) and vesicular stomatitis virus, VSV, Indiana strain (ATCC No. VR-158) as infective agent; A549 cells (ATCC No. CCL-185) and encephalomyocarditis virus EMC (ATCC No. VR-129B) as infective agent.

Incubate in at least 4 series, cells with 3 or more different concentrations of the preparation under examination and the reference preparation in a microtitre plate and include in each series appropriate controls of untreated cells. Choose the concentrations of the preparations such that the lowest concentration produces some protection and the largest concentration produces less than maximal protection against the viral cytopathic effect.

Add at a suitable time the cytopathic virus to all wells with the exception of a sufficient number of wells in all series, which are left with uninfected control cells.

Determine the cytopathic effect of the virus quantitatively with a suitable method. Calculate the potency of the preparation under examination by the usual statistical methods.

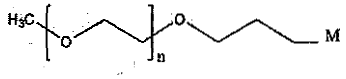
The estimated potency is not less than 80 per cent and not more than 130 per cent of the stated potency. The confidence limits (P = 0.95) are not less than 64 per cent and not more than 156 per cent of the estimated potency.

Cytopathtic effect assay (CPE assay described above) has been used as the standard assay for potency determination of interferon beta-1a. If an assay other than CPE assay is used to determine potency of interferon beta-1a, crossover study is to be performed between CPE assay and the new assay to demonstrate equivalence. Limits equivalent to those for CPE assay are to be established for the new assay based on the outcome of the crossover study.

Storage. Interferon Beta-1a Injection should be protected from light and stored at a temperature of 2° to 8°. It should not to be allowed to freeze.

Labelling. The label of the sealed container states the µg per ml, and the number of MIU (Million International Units).

Pegfilgrastim



TPLGPASSLP QSFLLKCLEQ VRKIQGDGAA LQEKLCATYK

LCHPEELVLL GHSLGIPWAP LSSCPSQALQ LAGCLSQLHS

GLFLYQGLLQ ALEGISPELG PTLDTLQLDV ADFATTIWQQ

MEELGMAPAL QPTQGAMPAF ASAFQRRAGG VLVASHLQSF

LEVSYRVLRH LAQP

$(C_2H_4O)_nC_{845}H_{1339}N_{223}O_{253}S_9$ Mol. Wt. 39,000 Da

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Pegfilgrastim is granulocyte colony-stimulating factor (human), 3-hydroxypropyl-*N*-methionyl-1-ether with α-methyl-Ω-hydroxypoly (oxy-1,2-ethanediyl).

Pegfilgrastim is a monopegylated form of filgrastim-recombinant human granulocyte colony-stimulating factor. It is a single chain, 175 amino acid, non-glycosylated polypeptide. It is prepared by coupling a linear polyethylene glycol (PEG) molecule, of an average molecular weight of 20 kDa, to the *N*-terminus of the filgrastim protein.

Pegfilgrastim contains *N*-terminally pegylated recombinant human methionyl granulocyte colony-stimulating factor. Pegfilgrastim contains not less than 80 per cent and not more than 125 per cent of the stated potency.

Host cell derived proteins (HCP). Not more than 100 ppm.

Host cell or vector derived DNA. Not more than 10 ng per dose.

Category. Haemopoietic growth factor.

Description. A clear, colourless liquid.

Identification

A. It shows the biological activity as described under Assay.

B. Determine by isoelectric focusing (2.4.33).

In the test for Impurities with charges different from that of pegfilgrastim, the principal band in the electropherogram obtained with the test solution is similar in position to the principal band in the electropherogram obtained with the reference solution (a).

C. Determine by size-exclusion chromatography (2.4.16).

In the test for Impurities with molecular mass higher than that of pegfilgrastim, the retention time of the principal peak obtained with the test solution is similar to that of the principal peak obtained with the reference solution.

D. Determine by polyacrylamide gel electrophoresis under non-reducing conditions (2.4.12).

In the test for Impurities with molecular masses differing from that of pegfilgrastim under non-reducing conditions, the principal band in the electropherogram obtained with the test solution is similar in position to the principal band in the electropherogram obtained with reference solution (d).

E. Determine by peptide mapping (2.3.47).

Solution (a). Dissolve 6.7 g of *guanidine hydrochloride* and 0.36 g of *tris hydrochloride* in 4 ml of *water*, adjust pH to 8.4 with *hydrochloric acid* and dilute to 10 ml with *water*.

Solution (b). Dissolve 10 µg of *endoprotease Lys C* or *glutamyl endopeptidase (V8 protease)* in 40 µl of *20 mM tris hydrochloride pH 8.0*.

Test solution (a). Dilute 100 µl of 10 mg per ml of preparation under examination in 900 µl of *solution (a)* in a polypropylene tube, add 10 µl of *0.5 M β-mercaptoethanol* and mix. Incubate at 37° for 1 hour. Cool to ambient temperature, add 10 µl of *1M iodoacetamide* and mix. Incubate the tube at 37° for 1 hour in dark.

Test solution (b). Equilibrate any desalting column with *20 mM tris hydrochloride pH 8.0*. Add about 5 ml of *20 mM tris hydrochloride pH 8.0* to the column and centrifuge at 1000 *g* for 1 minute. Repeat 4-5 times. For elution, place the column outlet in a fresh tube. Load test solution (a) on the column, and spin at 1000 g for 1 minute. Measure the absorbance of eluted sample at 280 nm. Dilute the eluate using *20 mM tris hydrochloride pH 8.0* to get an absorbance of 0.4-0.5.

Test solution (c). To 250 µl of *test solution (b)* add 20 µl of *solution (b)* and mix. Incubate for 24 hour at 37° and stop the reaction by adding 10 µl of 10 per cent v/v of *formic acid*.

Reference solution. Prepare at the same time and in the same manner as for the test solution but use *pegfilgrastim IPRS* instead of the preparation under examination.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with butylsilyl silica gel (5 µm),
- column temperature. 45°,
- mobile phase: A. a 0.1 per cent v/v solution of *trifluroacetic acid* in *water*,
  B. a 0.1 per cent v/v solution of *trifluroacetic acid* in 1000 ml of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 0.2 ml per minute,
- spectrophotometer set at 215 nm,
- injection volume: 100 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	98.2	1.8
2	98.2	1.8
30	73.0	27.0
75	46.0	54.0
90	11.8	88.2
100	11.8	88.2
101	98.2	1.8
120	98.2	1.8

Inject the reference solution. Test is not valid unless the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram supplied with *pegfilgrastim IPRS*. Inject the test solution (c). The profile of chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

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Tests

Impurities with molecular mass higher than that of pegfilgrastim. Determine by size-exclusion chromatography (2.4.16).

Formulation buffer pH 4.0. Dissolve 5 g *D-sorbitol* in 40 ml of *water* and add 4.2 ml of *0.2 M acetic acid.* Adjust the pH 4.0 with *0.2 M sodium acetate trihydrate.* Add 330 µl of 10 mg per ml *polysorbate 20* and sufficent *water* to produce 100 ml.

Test solution. Dilute the preparation under examination (if necessary) with formulation buffer to obtain a concentration of 1 mg per ml.

Reference solution (a). Dilute *pegfilgrastim IPRS* with formulation buffer to obtain a concentration of 1 mg per ml.

Reference solution (b). Incubate an appropriate amount of the reference solution (a) at 55° for 15 minutes in a polypropylene tube. Cool it to room temperature after incubation.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm, packed with hydrophilic silica gel, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10000 to 50000,
- mobile phase: a mixture of 6.8 ml of 85 per cent v/v solution of *orthophosphoric acid* in 800 volumes of *water*, adjust pH to 2.5 with *10 M sodium hydroxide*, 50 volumes of *ethanol* and 150 volumes of *water*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 30 µl.

Inject reference solution (b). The test is not valid unless the per cent aggregate in reference solution (b) is not more than 5 per cent and the relative standard deviation for the per cent area of aggregate is not more than 10 per cent. Relative retention times with reference to pegfilgrastim monomer for aggregates is about 0.8 and for multi-pegylated is about 0.9.

Inject reference solution (a) and test solution. Relative per cent of all peaks eluting with retention times less than that of the principal peak is not more than 3 per cent.

Impurities with molecular masses differing from that of pegfilgrastim. Determine by electrophoresis (sodium dodecyl sulphate polyacrylamide gel electrophoresis) (SDS-PAGE) (2.4.12) under non-reducing conditions.

Resolving gel. 4-12 per cent *polyacrylamide* gradient *bis-tris* gel of 1 mm thickness.

Sample buffer (non-reducing conditions). Mix *60 mM Tris* pH 6.8, 25 per cent *glycerol*, 2 per cent *SDS* and 0.2 per cent *bromophenol blue* (or equivalent).

Test solution. Dilute the preparation under examination with *water* to obtain a protein concentration of 1 mg per ml.

Reference solution (a). Dilute *pegfilgrastim IPRS* with *water* to obtain a concentration of 1 mg per ml.

Reference solution (b). Dilute *pegfilgrastim IPRS* with *water* to obtain a concentration of 0.1 mg per ml.

Reference solution (c). A solution of molecular markers suitable for calibrating SDS-polyacrylamide gels in the range 15 - 100 kDa.

Reference solution (d). Add 10 µl of Reference solution (a).

Reference solution (e). Add 2 µl of Reference solution (b).

Reference solution (f). Add 1 µl of Reference solution (b).

Place the test solution, in boiling water bath for 5 minutes.

Apply reference solution (d), reference solution (e), reference solution (f), 5 µl of reference solution (c) and 10 µl of test solution to the stacking gel wells.

Detection: Silver staining.

The test is not valid unless (1) the proteins of the molecular weight marker are distributed along 80 per cent of the gel and over the required separation range and are clearly visible; (2) the principal band of test solution and reference solution appears between 45 to 66 kDa marker bands; (3) the principal band reference solution (f) is clearly visible.

In the electropherogram obtained with the test solution, no band other than the principal band is more intense than the principal band in the electropherogram obtained with reference solution (e).

Impurities with charges differing from that of pegfilgrastim. Determine by isoelectric focusing (2.4.33).

Test solution. Dilute the preparation under examination to produce a solution containing 0.3 mg per ml.

Reference solution (a). A solution of *pegfilgrastim IPRS* containing 0.3 mg per ml.

Reference solution (b). A solution of *pegfilgrastim IPRS* containing 0.03 mg per ml.

Reference solution (c). Use an isoelectric point (pI) calibration solution, in the pI range of 3.5 - 9.5, prepared according to manufacturer's instructions.

Focusing:

- pH gradient: 4.5 - 8.0,
- catholyte: *1 M sodium hydroxide*,
- anolyte: *0.04 M glutamic acid* in a 0.0025 per cent v/v solution of *orthophosphoric acid*,
- application: 20 µl.

Detection. Proceed as described in Isoelectric Focusing (2.4.33).

Detect the product and its related impurities.

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In the electropherogram obtained with reference solution (c), relevant isoelectric point markers are distributed along the entire length of the gel. In the electropherogram obtained with reference solution (a), the pI of the principal band is 5.7- 6.3.

In the electropherogram obtained with test solution no band other than the principal band is more intense than the principal band in the electropherogram obtained with reference solution (b) (10 per cent).

Related proteins. Determine by liquid chromatography (2.4.14)

Sample buffer. A mixture of *10 mM sodium acetate buffer solution* pH 4.0, 5 per cent *D-sorbitol* and 0.004 per cent *polysorbate 20*.

Test solution. Dilute preparation under examination in sample buffer to the concentration of 1.0 mg per ml.

Reference solution (a). Dilute *pegfilgrastim IPRS* in sample buffer to the concentration of 1 mg per ml.

Reference solution (b). Dilute 100 µl of 10 mg per ml *pegfilgrastim IPRS* in 800 µl of diluent. Add 100 µl of *0.1M hydrogen peroxide* and incubate at 37° for 5 hours. Dissolve 30 mg of *L-methionine* to quench the reaction.

Reference solution (c). Mix equal volumes of reference solution (a) and reference solution (b).

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with polystyrene and divinylbenzene (5 µm),
- column temperature: 45°,
- mobile phase: A. a 0.1 per cent v/v solution of *trifluroacetic acid* in *water*,
  B. a 0.1 per cent v/v solution of *trifluroacetic acid* in 1000 ml of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 0.7 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	53	47
7	53	47
43	44	56
43.1	0	100
46	0	100
47	53	47
55	53	47

Inject reference solution (c), the profile of the chromatogram obtained shows two oxidized peaks corresponding to oxidized peak 1 with relative retention time of about 0.9 and oxidized peak 2 with relative retention time of about 0.95 with respect to the pegfilgrastim main peak (retention time of about 27 minutes). The test is not valid unless resolution between oxidized peak 1 and 2 is not less than 1.5, and resolution between oxidized peak 2 and main peak is not less than 0.9.

Inject reference solution (a) and test solution. In the chromatogram obtained with the test solution, the sum of the areas of all the peaks other than the principal peak is not more than 5.0 per cent of the total area of all the peaks.

Test for free mPEG. Determine by electrophoresis (sodium dodecyl sulphate polyacrylamide gel electrophoresis) (SDS-PAGE) (2.4.12) under non-reducing condition.

Resolving gel. 4 to 12 per cent polyacrylamide gradient bis-tris gel of 1 mm thickness.

Sample buffer (5X). A mixture of *60 mM tris hydrochloride* pH 6.8, 25 per cent *glycerol*, 2 per cent *sodium dodecyl sulphate*, 0.2 per cent *bromophenol blue* (or equivalent).

Test solution. Dilute the preparation under examination with *water* to obtain a protein concentration of 1 mg per ml.

Reference solution (a). Dilute *pegfilgrastim IPRS* with *water* to obtain a concentration of 1 mg per ml.

Reference solution (b). Dilute 20 kDa PEG in *water* to obtain a concentration of 0.1 mg per ml.

Reference solution (c). Solution of molecular markers suitable for calibrating SDS-polyacrylamide gels in the range 14 to 100 kDa.

Reference solution (d). Mix 10 µl of reference solution (a) with 4 µl of reference solution (b).

Reference solution (e). Add 4 µl of reference solution (b).

Reference solution (f). Add 2 µl of reference solution (b).

Sample treatment. Heat the test solution and reference solutions at 70° for not more than 2 minutes after adding 1X sample buffer separately.

Load reference solution (d), reference solution (e), reference solution (f), 5 µl of reference solution (c) and 10 µl of test solution to the stacking gel wells.

Detection: Iodine staining.

After electrophoresis immerse the gel in 20 ml of *0.1 M perchloric acid* for 15 minutes. Remove the solution and add 5 ml of 5 per cent *barium chloride* solution followed by 2 ml of 0.1 g of *resublimed iodine crystal* dissolved in 2 ml of *isopropanol*. Incubate the gel for about 5 minutes at room temperature. After the bands have developed, rinse the gel with water to remove colloidal *iodine* particles and immediately scan the gel.

The test is not valid unless (1) the bands of all proteins with molecular weight markers between 14 and 100 kDa are visible

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and well resolved in the gel; (2) the principal band and the PEG band in the reference solution (d) do not show an overlap and (3) the principal PEG band in reference solution (f) is clearly visible.

In the electropherogram obtained with the test solution, no band other than the principal band is more intense than the principal band in the electropherogram obtained with reference solution (e).

Residual Filgrastim. Determine by size exclusion chromatography (2.4.16) as described under test for Impurities with molecular mass higher than that of pegfilgrastim with following modification.

Reference solution (c). Dilute *filgrastim IPRS* in suitable diluent to a concentration of 0.3 mg per ml.

Inject reference solutions (a), (c) and test solution. Determine per cent area of filgrastim to estimate the per cent purity of the test solution. In the chromatogram obtained with the test solution, the per cent free filgrastim is not more than 0.5 per cent.

Bacterial endotoxin (2.2.3). Not more than 350 EU per dose of pegfilgrastim.

Assay

A. *Protein.* Determine by liquid chromatography (2.4.14) as described under the test for related proteins.

Calculate the content of pegfilgrastim from the declared content of *pegfilgrastim IPRS.*

B. *Potency.* The potency of the preparation is determined by comparison of the dilutions of preparation under examination with the dilutions of *pegfilgrastim IPRS.*

Determine the potency using a filgrastim responsive cell line (e.g., NFS-60 or its variant, M-NFS-60) in a cell-based proliferation assay with a suitable read out. Perform a comparison of a dilution series of preparation under examination with a dilution series of *pegfilgrastim IPRS.* The reference and test concentrations should be adjusted so that the fluorescence values are normalized. Use a validated protein estimation procedure.

Determination of the biological activity of pegfilgrastim solution is based on its property of stimulation of proliferation of M-NFS-60 cells (ATCC No.CRL 1838). The following method uses the *resazurin (sodium)* or any other suitable staining method for assessing the proliferation of cells.

The following method uses the conversion of tetrazolium bromide (MTT) or any other suitable dye as a staining method. Alternative methods based on luminescence or fluroscence detection may also be used as the assay readout, subject to appropriate validation.

M-NFS-60 (ATCC No. CRL-1838) or any other suitable Myeloblastic cell line are incubated with varying dilution of test and reference preparations of pegfilgrastim. They are then incubated with a solution of MTS, MTT or any other suitable dye. The potency of test preparation is determined by comparision of the dilutions of the test preparation with the dilutions of appropriate International Standard of pegfilgrastim or with a reference preparation calibrated in International Units, which yield the same response.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Add 100 µl of the dilution medium to all wells of a 96-well microtitre plate. Add an additional 150 µl of this solution to the blank wells. Add 100 µl of each solution under examination in triplicate (test preparation and reference preparation at a concentration of about 10 ng per ml, plus a series of two fold dilutions to obtain a standard curve). Prepare a suspension of M-NFS-60 or any other suitable Myeloblastic cell line containing 5 x 10^5 cells per ml immediately before use, add 50 ul of prepared cell suspension to each well, maintaining the cells in a uniform suspension during addition.

Incubate the plate at 37° ± 1° for a minimum of 46 ± 2 hours in a humidified incubator using 6 ± 1 per cent carbon dioxide. Add 20 ul of suitable dye to each well and re-incubate for 4 hours. Estimate the quantity of formazan produced using a microtitre well plate reader at 490 nm.

Assess the slope, and parallelism for each sample compared to the standard using a validated parallel line assay data analysis software or alternate equivalent software. If system suitability criteria are met and sample passes parallelism criteria to reference standard, calculate the relative potency of the sample.

Alternative methods of quantifying cell proliferation, such as measurement of intracellular ATP by luciferase bioluminescence may be used as the assay readout, subjected to appropriate development and validation. The assay conditions such as cell concentration, incubation time and dilution are then adapted accordingly.

The ratio between the relative fluorescence units of highest and lowest concentrations should be ≥ 2.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency.

Storage. Store at 2° to 8° in an airtight container.

Labelling .The label of the sealed container states (1) the name: (2) content in mg per ml, and (3) potency of the drug substance.

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Recombinant Streptokinase Bulk Solution

Streptokinase Concentrated Solution

IAGPEWLLQR PSVNNSQLVV SVAGTVEGTN QDISLKFFEI DLTSRPAHGG KTEQGLSPKS KLFATDSGAM PHKLEKADLL KAIQEQLIAN VHSNDDYFEV IDFASDATIT DRNGKVYFAD KDGSVTLPIQ PVQEFLLKGH VRVRPYKEKP VQNQAKSVDV EYTVQFTPLN PDDDFRPALK DTKLLKTLAI GDTITSQELL AQAQSILNKN HPGYTIYERD SSIVTHDNDI FRTILPMDQE FTYHVKNREQ AYRINKKSGL NEEINNTDLI SEKYYVLKKG EKPYDPFDRS HLKLFTIKYV DVNTNELLKS EQLLTASERN LDFRDLYDPR DKAKLLYNNL DAFGIMDYTL TGKVEDNIDD TNRILTVYMG KRPEGENASY HLAYDKDRYT EEEREVYSYL RYTGTPIPDN PNDK

Recombinant Streptokinase Bulk Solution is a fibrinolytic enzyme produced by a method based on recombinant DNA technology using bacteria or suitable genetically engineered host cells.

Recombinant Streptokinase Bulk Solution has a potency of not less than 600 IU per microgram of nitrogen.

Category. Thrombolytic agent.

Production

If intended for use in the manufacture of parenteral preparations, the method of manufacture is validated to demonstrate that the product, if tested, would comply with the following test.

Host cell-derived proteins (HCP). Not more than 100 ppm.

Host cell and vector-derived DNA. Not more than 10 ng per dose.

Description. A clear, colourless to slightly yellowish liquid.

Identification

A. Clot Lysis method: Place 0.5 ml of citrated human plasma in a haemolysis tube maintained in a water-bath at 37°. Add 0.1 ml of a solution of the contents of the container containing 10000 IU of streptokinase activity per ml in phosphate buffer pH 7.2 and 0.1 ml of a solution of human thrombin containing 20 IU per ml in phosphate buffer pH 7.2. Mix immediately. A clot forms and lyses within 30 minutes. Repeat the procedure using citrated bovine plasma. The clot does not lyse within 1 hour.

B. Determine by western blotting method, Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12) under reducing condition.

Run a 12 per cent SDS-PAGE gel after loading the lanes with preparation under examination equivalent to 10-20 µg Recombinant Streptokinase Bulk Solution and bovine serum albumin as negative control. Set up the transfer blotting apparatus. Develop the blot with suitable developer. The end result is the development of colour only in the sample protein lane (Recombinant Streptokinase main band) which is identified and confirmed by the corresponding antibody.

C. Determine purity by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12) under reducing condition.

In SDS-PAGE the mobility depends on the protein's molecular size. Run a 12 per cent resolving gel mix and 5 per cent stacking gel after loading the lanes with 10-20 µg equivalents Recombinant Streptokinase Bulk Solution. Stain the gel with coomassie blue and destain.

No artefacts are visualized in the sample protein lane. Protein band intensity is appropriate to the protein load. Main band in same position as standard and in line with the molecular weight markers. Main band ≥ 97 per cent of total integrated bands.

D. Determine purity by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)(2.4.12) under non-reducing condition.

In native polyacrylamide gel electrophoresis the mobility depends on both the protein's charge and its hydrodynamic size. The electric charge driving the electrophoresis is governed by the intrinsic charge on the protein at the pH of the running buffer.

Run 8 per cent native resolving gel after loading the lanes with 10-20 µg equivalents Recombinant Streptokinase Bulk Solution. Stain the gel with coomassie blue and destain.

No artefacts are visualized in the sample protein lane. Protein band intensity is appropriate to the protein load. Main band same position as standard and main band ≥ 98 per cent of total integrated bands.

E. Determine by isoelectric focussing polyacrylamide gel electrophoresis (PAGE) (2.4.33) under non-reducing condition.

Isoelectric focusing is a method of electrophoresis that separates proteins according to their net charge (pI). Separation is carried out in poly acrylamide gel which contains the mixture of amphoteric electrolytes called poly ampholytes (small multicharged polymers that have many pI values).

Run 6 per cent resolving gel mix with ampholytes (3-10, Broad range pI) after loading the lanes with 10-20 µg equivalents Recombinant Streptokinase Bulk Solution. Stain the gel with coomassie blue and destain.

No artefacts are visualized in the sample protein lane. Protein band intensity is appropriate to the protein load. The sample should have single main band at R_f position pI 5.0 - 5.3 is observed.

Tests

pH (2.4.24). 6.8 to 7.5, determined in a solution prepared by diluting the preparation under examination in *carbon dioxide-*

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free water to produce a solution containing at least 10^5 IU of streptokinase activity per ml.

Protein. Determine by nitrogen content (2.3.30). 1 mg of N is equivalent to 6.25 mg of protein.

Assay

Potency. The potency of streptokinase is determined by comparing its capacity to activate plasminogen to form plasmin with the same capacity of a reference preparation of streptokinase calibrated in International Units; the formation of plasmin is determined using a suitable chromogenic substrate.

The International Unit is the activity of a stated amount of the International Standard for streptokinase. The equivalence in International Units of the International Standard is as stated by the World Health Organization.

Reference and test solutions: Prepare 2 independent series of 4 dilutions of each of the substance under examination and of the reference preparation of streptokinase in *tris (hydroxymethyl) aminomethane sodium chloride buffer pH 7.4*, in the range of 0.5-4.0 IU per ml. Prepare and maintain all solutions at 37°.

Substrate solution: Mix 1.0 ml of *tris (hydroxymethyl) aminomethane buffer pH 7.4* with 1.0 ml of chromophore substrate. Add 5 µl of a 10 per cent w/v solution of *polysorbate 20*. Keep at 37° in a water-bath. Immediately before commencing the activation assay, add 45 µl of a 1 mg per ml solution of human plasminogen.

Analyse each streptokinase dilution, maintained at 37°, in duplicate. Initiate the activation reaction by adding 60 µl of each dilution to 40 µl of substrate solution. For blank wells, use 60 µl of *tris (hydroxymethyl) aminomethane sodium chloride buffer solution pH 7.4* instead of the reference and test solutions. Allow the reaction to proceed at 37° for 20 minutes and read the absorbance at 405 nm. If a suitable thermostated plate reader is available, this may be used to monitor the reaction. Alternatively, it may be necessary to stop the reaction after 20 minutes using 50 µl of a 50 per cent v/v solution of *glacial acetic acid*. Best results are obtained when the absorbance for the highest streptokinase concentration is between 0.1 and 0.2 (after blank subtraction). If necessary, adjust the time of incubation in order to reach this range of absorbances.

Calculate the regression of the absorbance on log concentrations of the solutions of the substance under examination and of the reference preparation of streptokinase and calculate the potency of the substance under examination using the usual statistical methods for parallel-line assays.

The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits (P = 0.95) of the estimated potency are not less than 80 per cent and not more than 125 per cent of the stated potency.

Recombinant Streptokinase Bulk Solution intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins complies with the following additional requirement.

Bacterial endotoxins (2.2.3). Not more than 0.02 Endotoxin Unit per 100 IU of streptokinase activity.

Storage. Store protected from light and at a temperature of about -20°. If it is intended for the manufacture of parenteral preparations, the container should be sterile and sealed so as to exclude micro-organisms.

Labeling. The label states (1) the number of International Units of streptokinase activity per mg, calculated on the dried basis; (2) the name and quantity of any added substance; (3) where applicable, that the substance is free from bacterial endotoxins; (4) where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

Recombinant Streptokinase for Injection

Recombinant Streptokinase for Injection is a sterile preparation in a sealed container consisting of Streptokinase with or without auxiliary agents.

The injection is constituted by dissolving the contents of the sealed container in the requisite amount of sterile water for injection, immediately before use.

Usual strengths. 750,000 IU per vial and 1,500,000 IU per vial.

Description. A white powder or a white friable solid, hygroscopic.

Identification

Determine by clot lysis method. Place 0.5 ml of citrated human plasma in a haemolysis tube maintained in a water-bath at 37°. Add 0.1 ml of a solution of the contents of the container containing 10 000 IU of streptokinase activity per millilitre in phosphate buffer pH 7.2 and 0.1 ml of a solution of human thrombin containing 20 IU per ml in phosphate buffer pH 7.2. Mix immediately. A clot forms and lyses within 30 min. Repeat the procedure using citrated bovine plasma. The clot does not lyse within 1 hour.

Tests

Physical Form. Lyophilized powder.

Particulate contamination (2.5.9). The reconstituted solution complies with the limit test for particulate contamination.

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Other tests. The content of the preparation complies with the tests stated under Parenteral preparations (Injections).

Residual moisture content (2.3.43). Determine on the individual contents of three containers by titrimetric (Karl Fischer) method.

pH (2.4.24). 6.8 to 7.5. Determined in a solution prepared by diluting the preparation under examination in *carbon dioxide-free water* to produce a solution containing at least 10^5 IU of streptokinase activity per ml.

Potency. Determine on the individual and mixed contents of three containers.

The potency of streptokinase is determined by comparing its capacity to activate plasminogen to form plasmin with the same capacity of a reference preparation of streptokinase calibrated in International Units; the formation of plasmin is determined using a suitable chromogenic substrate.

The International Unit is the activity of a stated amount of the International Standard for streptokinase. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Reference and test solutions. Prepare 2 independent series of 4 dilutions of each of the substance under examination and of the reference preparation of streptokinase in *tris (hydroxymethyl) aminomethane sodium chloride buffer pH 7.4*, in the range of 0.5-4.0 IU per ml. Prepare and maintain all solutions at 37°.

Substrate solution. Mix 1.0 ml of *tris (hydroxymethyl) aminomethane buffer pH 7.4* with 1.0 ml of chromophore substrate. Add 5 µl of a 10 per cent w/v solution of *polysorbate 20*. Keep at 37° in a water-bath. Immediately before commencing the activation assay, add 45 µl of a 1 mg per ml solution of human plasminogen.

Analyse each streptokinase dilution, maintained at 37°, in duplicate. Initiate the activation reaction by adding 60 µl of each dilution to 40 µl of substrate solution. For blank wells, use 60 µl of *tris (hydroxymethyl) aminomethane sodium chloride buffer solution pH 7.4* instead of the reference and test solutions. Allow the reaction to proceed at 37° for 20 minutes and read the absorbance at 405 nm. If a suitable thermostated plate reader is available, this may be used to monitor the reaction. Alternatively, it may be necessary to stop the reaction after 20 minutes using 50 µl of a 50 per cent v/v solution of *glacial acetic acid*. Best results are obtained when the absorbance for the highest streptokinase concentration is between 0.1 and 0.2 (after blank subtraction). If necessary, adjust the time of incubation in order to reach this range of absorbances.

Calculate the regression of the absorbance on log concentrations of the solutions of the substance under examination and of the reference preparation of streptokinase and calculate the potency of the substance under examination using the usual statistical methods for parallel-line assays.

The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits (P = 0.95) of the estimated potency are not less than 80 per cent and not more than 125 per cent of the stated potency.

Bacterial endotoxins (2.2.3). Not more than 23.3 Endotoxin Unit per 10000 IU per ml of streptokinase activity.

Sterility (2.2.11). Complies with the test for sterility.

Abnormal toxicity (2.2.1). Determine by using a solution containing 50,000 Units in 0.5 ml of water for injection administered in 15 to 20 minutes.

Storage. Store in sealed containers, protected from light and at a temperature of about 2°-8°. The container should be sterile and sealed so as to exclude micro-organisms.

Labelling. The label states (1) the number of International Units of streptokinase activity per vial; (2) the name of any added substance; (3) where applicable, that the substance is free from bacterial endotoxins.

Rituximab

Rituximab Heavy Chain Chimeric

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNGDTSY
NQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNVWGAGTTVTVS
AASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKAEPKSCDKTHTCPPCPAPELLG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Rituximab Light Chain Chimeric

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPVR
FSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRTVAAPSVFIFPPS
DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Structure of Rituximab Mol. Wt. 143859.7

Rituximab Drug Substance is a monoclonal antibody consisting of 1328 amino acid residues composed of two heavy chains of 451 amino acids and two light chains of 213 amino acidsthat targets the CD20 antigen, a specific protein found on the surface of normal and malignant B lymphocytes.

Rituximab (anti-CD20) is a genetically engineered chimeric mouse-human monoclonal antibody representing a

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glycosylated immunoglobulin with human IgG Kappa constant region (Fc domain) and mouse light and heavy chain variable regions (Fab domain). Rituximab contains not less than 80 per cent and not more than 125 per cent of the stated potency

Host cell proteins. Not more than 100 ppm.

Host cell DNA. Not more than 10 ng per dose.

Category. Anticancer

Description. A clear colorless to pale yellow liquid free from particles that can be observed by visual observation.

Identification

Determine by method B and any two methods from method A, C, D, E.

A. Bioassay

B. Determine by peptide mapping (2.3.47).

Test solution. Dilute the preparation under examination in *0.025 M Tris buffer solution pH 8.0* to obtained a solution having concentration of 10 mg per ml. Add 900 mg of *guanidine hydrochloride*, to 900 µl of test solution, and mix. Add 20 µl of 2 M of Tris base and 27 µl of 1 M DTT and incubate at 50° for 1 hour. Add 54 µl of *1M Iodoaceticacid* to the above solution and incubate at room temperature for 45 minutes. Desalt the above solution using K9 column filled with sephadex G25 resin or 3kD a cut off membrane filters using the desalting buffer (0.025 M Tris base, *3 M Urea*, adjusted to pH 7.5 with *hydrochloric acid* using Hydrochloride). Collect the peaks respectively and check for protein concentration using Bradford assay. Adjust the concentration of sample to 1.2 mg per ml. Add Trypsin enzyme in the ratio of 1:20 and incubate at 37° for 16 hours and analyze on HPLC.

Reference solution. Prepare in the same manner as the test solution using *rituximab IPRS*.

Blank control solution. Prepare in the same manner as the test solution using suitable diluent.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm) such as Supelco, Biowide (300 Å),
- column temperature: 60°,
- mobile phase: A. a 0.01 per cent v/v of *trifluoroacetic acid* in *water*;
  B. a 0.01 per cent v/v of *trifluoroacetic acid* in *acetonitrile*;
- a linear gradient programme using conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 215 nm,

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
3	100	0
118	59	41
130	0	100
133	0	100
135	100	0
145	100	0

Inject the test solution and reference solution and reagent blank. Integrate all peaks that elute between approximately 10 and 120 minutes. A suitable resolution criterion should be established for a critical peak pair for the reference solution.

Visually compare the elution profiles between the test solution and the adjacent reference solution in terms of peak retention times, number of peaks, and relative peak heights. The peaks in chromatogram obtained with the test solution correspond to that of the chromatogram obtained with the reference solution.

The retention time of established marker peaks should be within ± 0.7 minutes of the reference solution marker peaks.

NOTE— there may be some regions with variable peak height due to carbohydrate differences or oxidation products.

C. Determine by electrophoresis (sodium dodecyl sulphate – polyacrylamide gel electrophoresis) (SDS-PAGE) (2.4.12).

In the tests for Impurities with molecular masses differing from that of rituximab. The principal band in the electropherogram obtained with test solution is similar in position and intensity to the principal band in the electropherogram obtained with reference solution.

D. Determine by capillary electrophoresis (capillary zone electrophoresis) (2.4.32).

Test solution. Dilute the preparation under examination with water to obtain a concentration of 0.5 mg per ml.

Reference solution. Prepare in the same manner as the test solution using *rituximab IPRS*.

Blank control solution. Prepare in the same manner as the test solution using appropriate product diluent.

Capillary system

- neutral capillary (50 µm Ø x 560 mm) or N-CHO coated capillary (50 µm Ø x 650 mm) or other suitable capillary,
- temperature: 20° ± 2°,
- sample temperature 10° ± 2°,
- spectrophotometer set at 214 nm,
- injection volume: 100 µl.

CZE buffer. (0.04 Mε-amino-n-caproic acid containing 0.2 per cent w/v of hydroxypropylmethylcellulose, pH adjusted to 4.5 with *glacial acetic acid.*

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Preconditioning of the capillary

Rinse the capillary using *CZE buffer* until visual evaluations of the electropherograms demonstrate consistent profiles for pre-conditioning of capillary.

Inject suitable volume of the reference solution followed by the test solution. Complete the analysis with an injection of the reference solution and the reagent blank.

*Migration.*Apply field strength of 30 kV for 15 min using the *CZE buffer* as the electrolyte in both buffer reservoirs.The main peak elutes between 15 to 17 minutes.

The difference in the migration time for the main peak of the first and last reference solution injections bracketing each test solution must be less than or equal to 0.1 minute.

Positive identity is confirmed if the difference in migration time between the main peak of the reference solution and test solution is less than or equal to 0.1 minute.

E. Determine by isoelectric focusing (2.4.33) Capillary Electrophoresis (CE).

Solution (a). Catholyte: *0.2 M phosphoric acid.*

Solution(b). Anolyte: *0.3 M sodium hydroxide.*

Solution (c). Chemical: *0.35 M acetic acid.*

Solution (d). 0.5 M arginine.

Solution (e). 0.2 M iminodiacetic acid.

Solution (f). 4.3 M urea.

Solution (g) (3 M Urea- cIEF Gel). Weigh 1.80 g of urea and add cIEF Gel to the volumetric flask so that the final volume is 10 ml.

Test solution: Add 200 µl of *solution (f),* 12.0 µl of *Pharmalyte* 3-10 carrier ampholytes, 20.0 µl of *solution (d),* 2.0 µl of solution (e), *2.0* µl of pI marker 10.0, pI marker 7.0, pI marker 5.5 and pI marker 4.1 and add 10 µl of protein concentration within 5 mg per ml to 10 mg per ml.

Reference solution. Prepare in the same manner as the sample using *rituximab IPRS.*

Capillary System

- neutral capillary. 50 µm internal diameter and length about 40 cm,
- sample temperature. 10°,
- spectrophotometer set at 280 nm.

Turn on the UV lamp and allow the system to warm up for at least 30 minutes prior experimentation. The protein concentration within 5 mg per ml to 10 mg per ml. The protein sample must have a salt concentration below 0.05 M since high salt content is detrimental to cIEF separation. The protein sample can be buffer exchanged before use. The identity is established comparing the product with reference preparation. It complies with the limits approved by National Regulatory Authority.

In the electropherogram obtained with reference solution, the pI of principal band is 9.3 ± 0.2. In the electropherogram obtained with *test solution* no band other than the principal band is more intense than the principal band in the electropherogram obtained with reference solution.

It complies with the biological activity as described under Assay.

Tests

pH (2.4.24). 6.3 to 6.7.

Impurities with molecular masses differing from that of Rituximab. Determine by method A or B

A. Determine by using Capillary Electrophoresis by SDS (CE-SDS) (2.4.32) under both reducing and non-reducing conditions using IgG heterogeneity kit or equivalent.

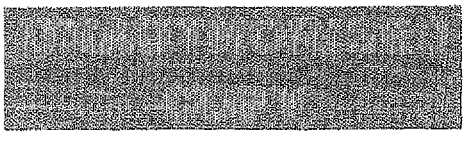
Procedure. Analyse preparation under examination and *rituximab IPRS* against the molecular weight marker using an electrophoretic method capable of giving separation in the range 10- 225/250 kDa followed by UV detection. Dilute the preparation under examination and *rituximab IPRS* in an appropriate diluent to obtain a minimum concentration to be detected by the capillary electrophoresis for example the total protein concentration should be within the range of 0.5 mg per ml to 4 mg per ml and use a validated method for reducing and non-reducing condition.

Test solution for CE-SDS under reducing condition. Add 68.0 µl *water,* 100 µl SDS sample buffer, 2 µl of 10 kDa internal standard and 10 µl mercaptoethanol to 20 µl of preparation under examination in a 1.5 ml eppendorf tubes. Vortex it and heat the mixture at 70° for 3 minutes. Cool it down at room temperature. Transfer it to vials for capillary electrophoresis analysis.Test solution may also be prepared as per application note.

Reference solution for CE-SDS under reducing condition. Prepare in the same manner as the *test solution for CE-SDS under reducing condition* using *rituximab IPRS.*

Tests solution for CE-SDS under non- reducing condition: Mix 68.0 µl of water, 100 µl of SDS sample buffer, 2 µl of 10 kDa internal standard, 10 µl *0.25 M iodoacetamide*, 20 µl of preparation under examination. Vortex it and heat the mixture at 70° for 5 minutes. Cool it down at room temperature. Transfer it to PCR vials for capillary electrophoresis analysis. Test solution may also be prepared as per application note.

Reference solutionfor CE-SDS under non-reducing condition. Prepare in the same manner as the *test solution for CE-SDS under non-reducing condition* using *rituximab IPRS.*



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Capillary system
- material. Bare fused silica coated,
- size:effective length = about20 cm, internal diameter =50 µm,
- capillary: 30.2 cm × 50 µm,
- effective length: 20 cm,
- capillary Temperature: 25,
- spectrophotometer set at 220 nm,
- detector: PDA 220 nm,
- capillary temperature: 25°,
- sample temperature: 10°,
- polarity: Anode at the inlet and Cathode at the outlet,
- Injection time: 5.0 kV for 20.0 seconds at the electrode polarity reverse,
- separation voltage: 15.0 kV for 30.0 minutes at polarity reverse.

Followed by pre-conditioning of capillary with suitable buffer, inject *test solution* and *reference solution* by keeping between-run rinsing using suitable buffer.

Migration. Apply a field strength 5.0 kV for 20.0 seconds at the electrode polarity reverse at the time of injection and: 15.0 kV for 30.0 minutes at polarity reverse as separation voltage. Maintain polarity anode at the inlet and cathode at the outlet.

Under reducing condition, in electropherogram obtained with reference solution and test solution identify the peaks corresponding to light chain, non-glycosylated heavy chain and heavy chain. Calculate the percentage of non-glycosylated heavy chain in the solution. Calculate the corrected percentage area of light chain, non-glycosylated heavy chain and heavy chain. The corrected percentage area of non-glycosylated heavy chainis not more than 2.0 per cent.

Under non-reducing condition, in electropherogram obtained with reference solution and test solution identify the peaks corresponding tolow molecular weight impurities and IgG. Calculate the percentage of impurities in the sample solution. Calculate the corrected percentage area of low molecular weight impurities and IgG. The corrected percentage are a low molecular weight impurities is not more than 10.0 per cent.

B. Determine by electrophoresis (sodium dodecyl-sulfate polyacrylamide gel electrophoresis) (SDS-PAGE) (2.4.12).

2x Non-reducing sample buffer. Mix 2.5 ml 0.5 M TRIS-HCl, pH 6.8, 2.0 ml glycerol, 4.0 ml of 10 per cent w/v of SDS, 0.5 mlof 0.1 per cent bromphenol blue and dilute to 10.0 ml with *water* or equivalent commercially available validated buffers may be used .

2x Reducing sample buffer. Mix 100 µl of 0.5 M dithiothreitol and 400µl of 2x non-reducing sample buffer or or equivalent commercially available validated buffers may be used.

10x SDS-PAGE mini-gel running buffer. 29 g *TRIS base*, 144 g *glycine*, 10 g SDS in 1000 ml of *water*.

Test solution. Dilute suitable quantity of the substance under examination with formulation buffer to obtained solution having concentration 1 mg per ml. Mix equal volume of test solution and 2x non-reducing or 2x reducing sample buffer, as required. Heat the samples in a closed microcentrifuge tube at 90° for 2 min. Load 10 µl per gel lane for non-reducing gel and 20 µl per gel lane for reducing gel.

*Reference solution.*Prepare in the same manner as the test solution using *rituximab IPRS*. Load 10 µl per gel lane.

Blank control solution. Prepare in the same manner as the test solution using formulation buffer.Load 10 µl per gel lane.

Sample treatment. Heat test, blank and reference solutions at 90° for 2 minutes

Application. 10 µl, in the following order: reference solution, test solution and blank control solution

Molecular weight marker. Mix equal volumes of molecular weight marker solution (e.g., 6000 to 200,000) and 1x non-reducing sample buffer or any other commercially available equivalent molecular weight marker may be used. Load 5 µl per lane.

Load test solutions, reference solution, control samples, and molecular weight markers on a 4-20 per cent *TRIS-glycine*gel or equivalent and analyze using a suitable SDS-PAGE apparatus. Once the separation is complete, stain the gel using a suitable staining procedure. Dry the gel using a suitable technique.

System suitability. All of the bands of the molecular weight standard must be detected and clearly separated. 2.0 per cent w/v *Rituximab IPRS* may be used as sensitivity control.The band(s) observed in the test solution and reference solution match in position and intensity on visual observation.

Related substances. Determine by size-exclusion chromatography (2.4.16).

Test solution. Dissolve the preparation under examination with *water* to obtain a concentration of 10 mg per ml.

*Reference solution.*Prepare in the same manner as the test solution using *rituximab IPRS*.

*Blank control solution.*Prepare in the same manner as the test solution using formulation buffer.

Chromatographic system
- a stainless steel column 7.8 mm x 300 mm, packed with silica gel (5µm) 250Å pores,
- mobile phase: *0.2 M dipotassium hydrogen phosphate solution* containing *0.25 M potassium chloride*, pH adjusted to 7.0 with *hydrochloric acid*.

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0.2 M dibasic sodium phosphate, pH 7.0, *0.25 M sodium chloride*, pH adjusted to 7.0 with *monobasic sodium phosphate*,
- flow rate. 0.5 ml per minute,
- spectrophotometer set at 280 nm or 215 nm,
- injection volume.:20 µl,
- run time: 30 minutes.

The test is not valid unless the resolution (R, using width at half height) between the monomer peak and nearest aggregate peak must be equal to or more than 1.5.

Integrate all peaks not present in the formulation buffer chromatogram. Calculate the content of dimer, oligomers and aggregates. The sum of the peaks with retention times less than that of the principal peak is not more than 2.0 per cent,the sum of the peaks with retention times higher than that of the principal peak is not more than 7.0 per cent and the sum of the peaks with retention times lesser and higher than that of the principal peak is not more than 9.0 per cent.

Calculate the percent of monomer. It complies with the limits approved for the particular product.

Charged variants. Determine by ion-exchange liquid chromatography (2.4.14) using method A or method B

Method A

Test solution. Mix 900 µl of *water*, 150 µl of *1.0 M TRIS*, 0.04 *M ethylenediaminetetraacetic acid* (pH 7.4) and 150 µl of *0.01 M cysteine* and 150 µl of preparation under examination. Digest the mixture by adding 150 µl of 0.1 mg per ml *papain* (activity 39-46 units per mg, adjusted accordingly). Incubate at 37° for 2 to 2.5 hours, followed by storage of the digest on ice prior to buffer exchange.

Reference solution. Prepare in the same manner as the test solution using *rituximab IPRS*.

Buffer exchange. Equilibrate Sephadex G-25 PD-10 columns with more than or equal to 25 ml of solvent composed of *0.01M 2-(N-morpholino)ethane sulfonic acid*, pH 6.0. Layer 1.0 ml of each digest onto separate PD-10 columns. Allow the solution to completely enter the bed. Discard the eluent. Add 1.5 ml of the same solvent onto each column. Allow the solution to fully enter the bed. Discard the eluent. Layer 2.0 ml of the same solvent onto each column and collect the eluent.

Blank control solution. Prepare in the same manner as the test solution using 150 µl of *formulation buffer*.

Chromatographic system
- a stainless steel column 4.6mm x 150 mm length, packed with strong cation exchange (8µm), 1000 Å pore size
- mobile phase A. 0.01*M 2-(N-morpholino)ethane sulphonic acid*, pH 6.0,
  B.*0.01M 2-(N-morpholino)ethane sulphonic acid*, 0.2 M *sodium chloride*, pH 6.0,
- flow rate.1 ml per minute,
- a gradient program using the conditions given below,
- spectrophotometer set at 280 nm, column equilibration 95 per cent mobile Phase A,
- injection volume. 200 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
5	95	5
45	15	85
45.1	0	100
47	0	100
48	95	5
60	95	5

Inject the mobile phase, reagent blank, reference solution, test solution. Test is not valid unless the chromatogram obtained with the blank solution must not contain peaks > 1 per cent as compared to the reference solution that elute between 12-50 minutes. A suitable resolution (R) criterion should be established for critical fragment peaks..

Integrate all *Rituximab*-related peaks that elute between approximately 12 and 50 minutes. Calculate and report the percent area for each *rituximab*-related peak. Calculate and report the percent peak area for the summed total Fab peaks and for the summed total Fc peaks.

Acidic variants ≤ 30 per cent.

Main peak ≥ 40 per cent.

Method B

Enzyme. Carboxypeptidase-B enzyme 2.1 mg *per ml*

Test solution.

Intact samples. Dilute the preparation under examination with *water* to obtain the concentration of 1.0 mg per ml. Transfer 200 µl of the test solution in sterile HPLC vials and analyse it using HPLC.

Carboxypeptidase-B(CP-B) Treated Samples. Dissolve the preparation under examination with *water* to obtain the concentration to 1.0 mg per ml. Take 350 µl of the test samples in 1.5 ml microfuge tube and add 5 µl of 2.1 mg per ml CP-B to the tube incubate the CP-B treated samples at 37° for two hours. After incubation place the samples 2-8° to stop the reaction immediately. Transfer 200 µl of CP-B treated-test samples in sterile HPLC vials and analyse it.

Reference Solution. Prepare as described for the test solution using of *Rituximab IPRS*.

Blank solution. Prepare in the same manner as the test solution using *formulation buffer*.

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Chromatographic System:
- a column 25 cm x 4.6 mm (10 µm) Weak Cation exchange,
- column temperature: 30 °,
- sample temperature: 2 to 8°,
- mobile phase: A. a mixture of *0.01M monobasic sodium phosphate* and *0.06 M sodium chloride*, adjusted to pH 4.5,
- a mixture of *0.01M disodium hydrogen phosphate dehydrate* and *0.06 M solium chloride*, adjusted to pH 9.0,
- flow rate: 1.0 ml per minute,
- a gradient programme using the conditions given below,
- spectrophotometer set at 214 nm,
- column Equilibration: 93 per cent of mobile phase A and 7 per cent mobile phase B,
- injection volume: 50 µl,
- run time: 45 minutes.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	93	7
2	93	7
32	0	100
38	0	100
39	93	7
45	93	7

Inject blank, reference solution, and the test solution, and end the sequence with an injection of the reference solution.

Integrate all rituximab related peaks that elute between approximately 10-40 minutes. Calculate and report the percent area for each rituximab related peaks. The resolution between the main peak and basic peak 1 should not less than 2.0 and the relative retention time between main peak and basic peak 1 should not less than 1.0.

Acidic variants. Acidic variants ≤ 30 per cent

Main peak. Main peak ≥ 40 per cent,

Glycan distribution. Determine by capillary electrophoresis with fluorescence detection (2.4.32). Determine by method A or method B.

Method A

PNGase F incubation buffer. 0.02 M sodium phosphate containing *0.05 M* EDTA and 0.02 per cent sodium azide, pH 7.5.

Internal standard. Dissolve 50 mg of maltoheptaose in *water* and dilute to 1000.0 ml with *water*.

8-aminopyrene-1,3,6-trisulfonic acid (APTS) solution. Add 0.5 ml of 15 per cent acetic acid to the original dye container per 5 mg of *APTS* listed as content in the label claim and mix well.

Test solution. Dilute the preparation under examination with formulation buffer to obtain a concentration 10 mg per ml using formulation buffer. Add 120 µl of *water* to a Microcon 30 concentrator. Place 30 µl of test solution into the Microcon 30 concentrator and spin in a microcentrifuge at 6000 rpm for approximately 15 minutes. Add 40 µl of PNGase F incubation buffer to the Microcon concentrator. Vortex briefly, then place concentrator upside down into a new 1.5 ml centrifuge tube, centrifuge at 2000 rpm for 2 minutes to recover the sample. Transfer all of the sample to a new 1.5 ml screwcap polypropylene tube. Prepare a dilute solution of PNGase F enzyme using incubation buffer. Exact dilution will depend on the activity and concentration of the enzyme used, but will be 10-40 fold dilution. Add 10 µl of the diluted PNGase F to the sample tube and vortex the mixtureand incubate at 37° for 13-17 hours. Add 50 µl of internal standard and mix well by vortexing. Precipitate protein by heating at 95° for 5 minutes. Centrifuge at 10,000 rpm for approximately 10 minutes. Transfer 80 µl to a new 1.5-ml screwcap polypropylene tube. Dry supernatant at low heat until a translucent oligosaccharide pellet is obtained. Add 15 µl of APTS fluorophore labeling reagent solution and mix well until pellet is dissolved. Add 5 µl of *1 M sodium cyanoborohydride* in THF and mix well. Incubate at 55° for approximately 2 hours. Add 500 µl HPLC grade *water* and mix well.

Reference solution. Prepare in the same manner as the test solution using *Rituximab IPRS*.

Blank control solution. Prepare in the same manner as the test solution using formulation buffer, except do not add PNGase F.

Capillary system
- coated capillary with reduced electroendosmotic flow, 50 µm ID, or any other suitable column
- capillary length. 21.0 cm to detector, 31.2 cm total length,
- separation voltage. 20.0 kV,
- polarity. Negative to positive,
- temperature. 20°,
- detection. Arg-Ion laser, 488 nm excitation, 520 nm emission.

Separation buffer. Carbohydrate Separation Gel Buffer or equivalent.

Perform three injections of reference solution or until a visual evaluation of the electropherogram demonstrate consistent profiles for at least two consecutive injections. Inject reference solution, reagent blank, test solution(s), ending with an injection of reference solution.

Calculate the corrected peak area percent of each named peak and identify the peak corresponding to galactosylatedglycan. The corrected area percentage of each glycan should comply with the limits approved by National Regulatory Authority.

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The percent area of the peaks corresponding to galactosylatedglycan is not more than 35.0 per cent

Method B

Test solution: Aliquot the sample volume in order to have between 25-300 µg of protein. Dry the glycoprotein solution completely in centrifugal vacuum evaporator. Add 45 µl of 1XPBS buffer and add 1.5 µl of 1:10 diluted 2-mercaptoethanol. Heat the sample for 5 minutes at 100°. Cool the sample to room temperature and add 5 µl of NP40 detergent and 1.5 µl of PNGaseF enzyme (750 units for 300 µg of protein). Incubate the reaction at 37° for 15 hours. Add 150 µl of cold ethanol, vortex and place it in ice for complete protein precipitation. Centrifuge the sample at 15000 rpm for 5 minutes and withdraw the supernatant. Dry the supernatant solution completely in centrifugal vacuum evaporator. Add 2 µl of *1M cyanoborohydride* and 2 µl of APTS labeling reagent to the dried supernatant. Incubate the reaction mixture at 37° for 4 hours.

Reference Solution. Prepare in the same manner as the test solution using *Rituximab IPRS.*

Blank control solution. Prepare in the same manner as the test solution using formulation buffer, except do not add PNGase F.

Capillary System

- capillary: coated capillary with reduced endoosmotic flow (50 µm I.D. and 50.2 cm total length),
- voltage: 30 kV,
- current: 40 µA,
- capillary temperature: 20°,
- sample temperature: 10°,
- detector condition: Arg-Ion laser, Excitation-488nm, Emission-520 nm,
- separation: 30kV with reverse polarity for 20 minutes.

Calculate the corrected peak area percent of each named peak and identify the peak corresponding to galactosylatedglycan. The corrected area percentage of each glycan should comply with the limits approved by National Regulatory Authority. The percent area of the peaks corresponding to galactosylatedglycan is not more than 35.0 per cent.

Bacterial endotoxins (2.2.3). Not more than 1.0 EU per mg or equivalent to EU per ml.

Other tests.

Protein A leachetes. Comply with the limits as approved by National Regulatory Authority

Protein. Determine by ultraviolet/visible spectrophotometry (2.4.7) using Method A or B.

Method A

Test solution. Dilute the preparation under examination with *water* to obtained a solution having concentration of 0.5 mg per ml.

Reference solution. Dilute *Rituximab IPRS* with water to 0.5 mg per ml.

Blank control solution. The appropriate product formulation buffer is diluted in a comparable manner as the test solution.

Spectral range. 240 - 500 nm.

Absorption maximum. 278 nm.

Pipet the test and reference solutions into a quartz cuvette and scan the absorbance in the spectral range against the blank control solution. Record the absorbance A_{max} at (278 ± 2 nm) and A_{320} at 320 nm. Calculate $|A_{320}|/A_{max} \times 100$. Calculate the corrected A_{max} by subtracting the A_{320} value from A_{max}.

$|A320|/Amax \times 100$ must be less than or equal to 3.0 per cent for each sample measurement.

Calculate the protein concentration as follows.

$$\text{Protein concentration} = \frac{\text{corrected } A_{max} \text{ of sample} \times \text{concentration of reference}}{\text{corrected } A_{max} \text{ of reference}} \times \text{total dilution factor}$$

Alternately, an established extinction coefficient may be used to calculate protein concentration.

Acceptance criteria. Not less than 90 per cent and not more than 110 per cent of the stated amount of protein.

Method B

Determine the protein content as per appendix (2.7.1) or take reading at 280 nm and calculate the concentration of the sample(s) using following formula

$$\text{Concentration (mg/ml)} = \frac{\text{Average absorbance at 280 nm} \times \text{dilution factor}}{\text{Extinction coefficient}}$$

NOTE — Value of Extinction coefficient to be provided by the manufacturer.

Acceptance criteria: Not less than 90 per cent and Not more than 110 per cent of the stated amount of protein.

Assay

Potency. Determine by complement dependent cytotoxicity assay by using WIL2-S cells.

Medium. Dissolve 485 ml of *RPMI 1640*, 10 ml of *1M HEPES*, 6.8 ml of 6.5 g of *bovine serum albumin* and 5 ml of Penicillin and Streptomycin solution (Final concentration to be 100-120 units per ml of Penicillin and 0.10-0.12 mg per ml of streptomycin) or any suitable antibiotic in *water* or any other

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solvent, if indicated and filter aseptically through 0.22 µm filter using sterile filtration assembly.

Test solution. Dilute the preparation under examination with medium to obtained a solution of concentration of 0.0098 to 2.5 µg per ml.

Reference solutions (standard curve). Prepare several concentrations of *Rituximab IPRS* bracketing the test solution concentration to determine an assay calibration curve.

Assay control solution. Use a reference solution and/or qualified lot of drug product as an assay control. Dilute in the same manner as the test solution.

Human complement. Human complement is diluted appropriately based on titration assays.

Cell culture solution. Prepare cultures of WIL2-S cells, a human B-lymphoblastoid cell line (ATCC CRL-8885) or equivalent, in the medium.

Cell suspension. Adjust the cell concentration of 0.8 to 1.2 x 10^6 cells per ml 1 x 10^6 cells per ml in the medium.

To individual wells of a 96-well tissue culture plate, add 50 µl of each standard curve solution, test solution or assay control solution to be tested, followed by 50 µl of the cell suspension and then 50 µl diluted human complement. Incubate in a humidified CO_2 incubator for 1 - 2 hours at 37°. Add 50 µl resazurin reagent per well. Incubate plate for an additional 15-26 hours. Agitate plate for 5 to 10 minutes at room temperature on a mini orbital shaker. Evaluate using a fluorescence plate reader with excitation wavelength at 530 nm and emission wavelength at 590 nm.

Relative fluorescence units of the standards are plotted against standard concentrations to generate a standard curve using a 4-parameter curve fitting program. Sample and control potency values are calculated using the standard curve.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency obtained as a mean of a minimum of three independent determinations. The Confidence limits (P=0.95) of the estimated potency are Not less than 69.2 per cent and not more than 132.6 per cent of the stated potency.

Storage. Store at temperature as approved by NRA.

Rituximab Injection

Rituximab is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes. This IgG1 kappa antibody contains murine light and heavy chain variable regions, and human gamma 1 heavy chain and kappa light chain constant regions. Rituximab is composed of two heavy chains each having 451 amino acids and two light chains each having 213 amino acids.

Rituximab Injection is a sterile mixture of rituximab with suitable buffering and stabilizing agents.

Rituximab contains not less than 80 per cent and not more than 125 per cent of the stated potency

The injection complies with the requirements stated under Parenteral Preparations and the following requirements.

Usual strengths. 100 mg per vial, 500 mg per vial, 600 mg per vial.

Description. A clear to opalescent, colourless to pale yellow liquid.

Identification

By method A, B or D and C

A. It complies with the biological activity as described under assay.

B. Determine by capillary zone electrophoresis (2.4.32).

Test solution. Dilute a suitable volume of the injection with *water* to obtain a solution having concentration 0.05 per cent w/v of rituximab.

Reference solution. A 0.05 per cent w/v solution of *rituximab IPRS* in *water*.

Blank control solution. Dilute suitable volume of product diluent with *water* to obtained similar concentration to that of test solution.

Capillary system

- neutral capillary (50 µm ∅ x 560 mm) or N-CHO coated capillary (50 µm ∅ x 650 mm) or other suitable capillary,
- temperature: 20°,
- sample temperature. 10°,
- spectrophotometer set at 214 nm,
- injection volume: 100µl

CZE buffer. 0.04 M ε-amino-n-caproic acid containing 0.2 per cent w/v of *hydroxypropylmethylcellulose*, adjusted to pH 4.5 with *glacial acetic acid.*

Preconditioning of the capillary. Rinse the capillary using *CZE buffer* until visual evaluations of the electropherograms demonstrate consistent profiles for pre-conditioning of capillary. Inject blank, the reference solution and the test solution. Complete the analysis with an injection of the reference solution and the reagent blank.

Migration. Apply field strength of 30 kV for 15 minutes using the *CZE buffer* as the electrolyte in both buffer reservoirs. The main peak elutes in between 15 to 17 minutes

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The difference in the migration time for the main peak of the first and last reference solution injections must be equal or less than 0.1 minute

Positive identity is confirmed if the difference in migration time between the main peak of the reference solution and test solution is equal or less than 0.1 minute.

C. Determine by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (2.4.12).

In the tests for Impurities with molecular masses differing from that of *rituximab*. The principal band in the electropherogram obtained with test solution is similar in position and intensity to the principal band in the electropherogram obtained with reference solution.

D. Iso Electric Focusing (IEF) using capillary electrophoresis (CE) (2.4.33). Determine by Capillary Electrophoresis (2.4.32).

Solution (a). Catholyte (*0.2 M orthophosphoric acid*).

Solution (b). Anolyte (0.3 M sodium hydroxide).

Solution (c). Chemical mobiliser (0.35 M acetic scid).

Solution (d). *0.5 M arginine.*

Solution (e). 0.2 M iminodiacetic acid.

Solution (f). *4.3 M urea.*

Solution (g). 3 M Urea- cIEF Gel). A solution prepared by dissloving 1.80 g *urea* in cIEF Gel and dilute to 10.0 ml with cIEF gel.

Test solution. Prepared by mixing 200 µl of *solution(f)* 12.0 µl of pharmalyte 3-10 carrier ampholytes, 20.0 µl of *solution (d),* 2.0 µl of *solution(e), 2.0* µl of pI marker 10.0, pI marker 7.0, pI marker 5.5 and pI marker 4.1 and add 10 µl of protein concentration within 5 mg per ml to 10 mg per ml.

Reference solution. Prepared in the same manner as the test solution using *rituximab IPRS.*

Capillary System
- neutral Capillary. 50 µm,
- size: effective length is at least 40 cm,
- temperature: 10°,
- spectrophotometer set at 280 nm.

Turn on the UV lamp and allow the system to warm up for at least 30 minutes prior experimentation. The protein concentration within 5 mg per ml to 10 mg per ml. The protein sample must have a salt concentration below 0.05 M since high salt content is detrimental to cIEF separation. The protein sample can be buffer exchanged before use. The identity is established comparing the product with reference preparation. It complies with the limits approved by National Regulatory Authority.

In the electropherogram obtained with *reference solution*, the pI of principal band is 9.3 ± 0.2. In the electropherogram obtained with the *test solution,* no band other than the principal band is more intense than the principal band in the electropherogram obtained with *reference solution.*

Tests

pH (2.4.24). 6.3 to 6.7.

Osmolality (2.4.23). Not less than 250mosmol per kg

Impurities with molecular masses differing from that of rituximab

Determine by using Method A or B.

A. Determine by using Capillary Electrophoresis by SDS (CE-SDS) (2.4.32) under reducing and non-reducing conditions using IgG heterogeneity kit or equivalent

Procedure. Analyze the preparation under examination and *rituximab IPRS* against the molecular weight marker using an electrophoretic method capable of giving separation in the range 10- 225/250 kDa followed by UV detection.

Dilute the preparation under examination and *rituximab IPRS* in an appropriate diluent to obtain a minimum concentration to be detected by the capillary electrophoresis for example the total protein concentration should be within the range of 0.5mg per ml to 4mg per ml and use a validated method for reducing and non-reducing condition.

Test solution for CE-SDS under reducing condition. Mix 68.0 µl of *water*, 100 µl of SDS sample buffer, 2 µl of 10 kDa internal standard, 10 µl of mercaptoethanol and 20 µl of preparation under examination. Vortex it and heat the mixture at 70° for 3 minutes. Cool it down to room temperature. Transfer it to vials for capillary electrophoresis analysis. Test solution may also be prepared as per application note.

Reference solution for CE-SDS under reducing condition. Prepare in the same manner as the *test solution for CE-SDS under reducing condition* using *rituximab IPRS.*

Tests solution for CE-SDS under non-reducing condition. Mix 68.0 µl of *water*, 100 µl of SDS sample buffer, 2 µl of 10 kDa internal standard, 10 µl of *0.25M iodoacetamide*, 20 µl of preparation under examination. Vortex it and heat the mixture at 70° for 5 minutes. Cool it down at room temperature. Transfer it to PCR vials for analysis. Test solution may also be prepared as per application note.

Reference solutionfor CE-SDS under non-reducing condition. Prepare in the same manner as the *test solution for CE-SDS under non-reducing condition* using *rituximab IPRS.*

Capillary system
- material. Bare fused silica coated,
- capillary: 30.2 cm × 50 µm,
- effective length: 20 cm,
- detector: PDA 220 nm,

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- capillary temperature: 25°,
- sample temperature: 10°,
- polarity. Anode at the inlet and Cathode at the outlet,
- injection time: 5.0 kV for 20.0 seconds at the electrode polarity reverse,
- separation voltage: 15.0 kV for 30.0 minutes at polarity reverse.

Followed by pre-conditioning of capillary with suitable buffer, inject *test solution* and *reference solution* by keeping between-run rinsing using suitable buffer.

Migration. Apply a field strength 5.0 kV for 20.0 seconds at the electrode polarity reverse at the time of injection and 15.0 kV for 30.0 minutes at polarity reverse as separation voltage. Maintain polarity anode at the inlet and cathode at the outlet.

Under reducing condition, in electropherogram obtained with reference solution and test solution identify the peaks corresponding to light chain, non-glycosylated heavy chain and heavy chain.

Calculate the percentage of non-glycosylated heavy chain in the solution. Calculate the corrected percentage area of light chain, non-glycosylated heavy chain and heavy chain. The corrected percentage area of non-glycosylated heavy chainis. Not more than 2.0 per cent.

Under non-reducing condition, in electropherogram obtained with reference solution and test solution identify the peaks corresponding tolow molecular weight impurities and IgG. Calculate the percentage of impurities in the sample solution. Calculate the corrected percentage area of low molecular weight impurities and IgG.. The corrected percentage of low molecular weight impurities is not more than 10.0 per cent.

B. Determine by sodium dodecylsulfate – polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12).

2x Non-reducing sample buffer. Mix 2.5 ml of *0.5 M TRIS-HCl*, pH 6.8, 2.0 ml of glycerol, 4.0 ml of 10 per cent w/v of SDS, 0.5 ml of 0.1per cent w/v of bromphenol blue; and dilute to 10.0 ml with *water* or equivalent commercially available validated buffers may be used .

2x Reducing sample buffer. Mix100 µl of *0.5 M dithiothreitol* and 400 µl of 2x non-reducing sample buffer or equivalent commercially available validated buffers may be used..

10x SDS-PAGE mini-gel running buffer. 29 g TRIS base, 144 g glycine, 10 g SDS in 1000 ml of *water*.

Test solution. Dilute a suitable volume of preparation under examination with formulation buffer to obtain a solution of 1 mg per ml. Mix equal volume of test solution and 2x non-reducing or 2x reducing sample buffer, as required. Heat the samples in a closed microcentrifuge tube at 90° for 2 minutes. Load 10 µl per gel lane for non-reducing gel and 20 µl per gel lane for reducing gel.

Reference solution. Prepare in the same manner as the test solution using *rituximab IPRS*. Load 10 µl per gel lane.

Blank control solution. Prepare in the same manner as the test solution using formulation buffer. Load 10 µl per gel lane.

Sample treatment. Heat test, blank and reference solutions at 90° for 2 minutes.

Application. 10 µl, in the following order:, reference solution,test solution and blank control solution

Molecular weight marker. Mix equal volumes of molecular weight marker solution (e.g., 6000 to 200,000) and 1x non-reducing sample buffer or any other commercially available equivalent molecular weight marker may be used. Load 5 µl per lane.

Load test solutions, reference solution, control samples, and molecular weight markers on a 4-20per cent *TRIS-glycine* gel or equivalent and analyze using a suitable apparatus. Once the separation is complete, stain the gel using a suitable staining procedure. Dry the gel using a suitable technique.

System suitability. All of the bands of the molecular weight standard must be detected and clearly separated. 2.0 percent w/v *rituximab IPRS* may also be used as sensitivity control. The band(s) observed in the test solution and reference solution match in position and intensity on visual observation.

Related substances. Determine by size-exclusion chromatography (2.4.16).

Test solution. Dissolve the preparation under examination in *water* to obtain a concentration of 10 mg per ml.

Reference solution. Prepare in the same manner as the test solution using *rituximab IPRS*.

Blank control solution. Prepare in the same manner as the test solution using formulation buffer

Chromatographic system

- a stainless steel column 7.8 mm x 300 mm, packed with silica gel (5µm) 250Å pores,
- mobile phase. *0.2 M dipotassium hydrogen phosphate solution* containing *0.25M potassium chloride*, adjusted to pH 7.0 using *hydrochloric acid*,
  Or
  0.2 M dibasic sodium phosphate, pH7.0, 0.25 M *sodium chloride*, adjusted to pH 7.0 with *monobasic sodium phosphate*,
- flow rate. 0.5 ml per minute,
- spectrophotometer set at 280 nm or 215 nm,
- injection volume: 20 µl,
- run time: 30 minutes.

The test is not valid unless the resolution (R, using width at half height) between the monomer peak and nearest aggregate peak must be equal or greater than 1.5.

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Integrate all peaks not present in the formulation buffer chromatogram. Calculate the content of dimer, oligomers and aggregates. The sum of the peaks with retention times less than that of the principal peak is not more than 2.0 per cent,the sum of the peaks with retention times higher than that of the principal peak is not more than 7.0 per cent and the sum of the peaks with retention times lesser and higher than that of the principal peak is not more than 9.0 per cent.

Charged variants. Determine by ion-exchange liquid chromatography (2.4.14) using method A or method B

Method A

Test solution. Mix 900 µl of *water*, 150 µl of *1.0 M TRIS, 0.04 M ethylenediaminetetraacetic acid* (pH 7.4), 150 µl of *0.01M cysteine* and 150 µl of preparation under examination. Digest the mixture by adding 150 µl of 0.1 mg per ml *papain* (activity 39-46 units per mg, adjusted accordingly). Incubate at 37° for 2 to 2.5 hours, followed by storage of the digest on ice prior to buffer exchange.

Reference solution. Prepare in the same manner as the test solution using *rituximab IPRS*. *Buffer exchange*: Equilibrate Sephadex G-25 PD-10 columns with not less than 25 ml of solvent composed of *0.01 M 2-(N-morpholino)ethane sulphonic acid*, pH 6.0. Layer 1.0 ml of each digest onto separate PD-10 columns. Allow the solution to completely enter the bed. Discard the eluent. Add 1.5 ml of the same solvent onto each column. Allow the solution to fully enter the bed. Discard the eluent. Layer 2.0 ml of the same solvent onto each column and collect the eluent.

Blank control solution. Prepare in the same manner as the test solution using *formulation buffer*.

Chromatographic system

- a stainless steel column 4.6 mm x 150 mm, packed with strong cation exchange (8µm) 1000 Å pore size,
- mobile phase A.*0.01M 2-(N-morpholino)ethane sulphonic acid*, pH 6.0,
  B. *0.01M 2-(N-morpholino)ethane sulphonic acid*, 0.2 M *sodium chloride*, pH 6.0,
- spectrophotometer set at 280 nm,
- flow rate: 1 ml per minute,
- a gradient programme using the conditions given below,
- column equilibration. 95 per cent mobile Phase A,
- injection volume: 200 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
5	95	5
45	15	85
45.1	0	100
47	0	100
48	95	5
60	95	5

Inject each of mobile phase A, reagent blank, reference solution, test solution, and end the sequence with an injection of the reference solution. Test in not valid unless the blank control solution must not contain peaks more than 1 per cent as compared to the reference solution that elute between 12-50 min. A suitable resolution (R) criterion should be established for critical fragment peaks.

Integrate all *Rituximab*-related peaks that elute between approximately 12 and 50 minutes. Calculate and report the percent area for each *Rituximab*-related peak. Calculate and report the percent peak area for the summed total Fab peaks and for the summed total Fc peaks.

Acidic variants ≤ 45 per cent

Main peak ≥ 35 per cent

Method B

Enzyme. Carboxypeptidase-B enzyme 2.1 mg per ml.

Test solution.

Intact samples. Dilute the preparation under examination with *water* to obtain a concentration of 1.0 mg per ml. Transfer 200 µl of test samples in sterile HPLC vials and analyse it using HPLC.

Carboxypeptidase-B (CP-B) treatedsamples. Dissolve the preparation under examination with water to obtain concentration of 1.0 mg per ml. Take 350 µl of test samples in 1.5 ml microfuge tube and add 5 µl of 2.1 mg per ml CP-B to the tube. Incubate the CP-B treated samples at 37° for two hours. After incubation place the samples 2-8° to stop the reaction immediately. Transfer 200 µl of CP-B treated test samples in sterile HPLC vials and analyse it.

Reference Solution. Prepare in the same manner as the test solution using *rituximab IPRS*.

Blank solution. Prepare in the same manner as the test solution using *formulation buffer*.

Chromatographic System

- column 25 cm x 4.6mm, Weak Cation exchange (WCX-10), (10 µm) particle size,
- mobile phase A: a mixture of *0.01M monobasic sodium phosphate* and *0.06 M soldium chloride*, pH adjusted to pH 4.5,
  B *0.01M a mixture of disodium hydrogen phosphate dehydrate* and *0.06 M sodium chloride*, adjusted to pH 9.0 ,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- column equilibration: 93 per cent of mobile Phase A and 7 per cent of Mobile phase B,
- injection volume: 50 µl,
- run time:45 minutes,

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– column temperature: 30°,
– sample temperature: 2 to 8°.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	93	7
2	93	7
32	0	100
38	0	100
39	93	7
45	93	7

Inject blank, reference solution, test solution, and end the sequence with an injection of the reference solution.

Integrate all *rituximab* related peaks that elute between approximately 10-40 minutes. Calculate and report the percent area for each *rituximab* related peaks. The resolution between the main peak and basic peak 1 should Not less than 2.0 and the relative retention time between main peak and basic peak 1 should Not less than 1.0.

Acidic variants ≤ 45 per cent.

Main peak ≥ 35 per cent.

Glycan distribution. Determine by capillary electrophoresis with fluorescence detection (2.4.32).

Determine by Method A or Method B.

Method A

PNGase F incubation buffer.0.02M sodium phosphate containing 0.05M *ethylenediaminetetraacetic acid* and 0.02 per cent sodium azide, pH 7.5.

Internal standard. Dissolve 50 mg of maltoheptaose w/v using *water*.

8-aminopyrene-1,3,6-trisulfonic acid (APTS) solution. Add 0.5 ml of 15 per cent acetic acid to the original dye container per 5 mg of *APTS* listed as content in the label claim and mix well.

Test solution. Dilute the preparation under examination to obtain a concentration 10 mg per ml using formulation buffer. Add 120 µl of *water* to a Microcon 30 concentrator. Place 30 µl of sample into the Microcon 30 concentrator and spin in a microcentrifuge at 6000 rpm for approximately 15 minutes. Add 40 µl of PNGase F incubation buffer to the Microcon concentrator. Vortex, then place concentrator upside down into a new 1.5 ml centrifuge tube. Spin at 2000 rpm for approximately 2 minutes to recover the sample. Transfer the entire sample to a new 1.5-ml screwcap polypropylene tube. Prepare a dilute solution of PNGase F enzyme using incubation buffer. Exact dilution will depend on the activity and concentration of the enzyme used, but will be approximately 10-40 fold dilution. Add 10 µl of the diluted PNGase F to the sample tube and vortex. Incubate at 37° for 13-17 hours. Add 50 µl of internal standard and mix well by vortexing. Precipitate protein by heating at 95° for 5 minutes. Centrifuge at 10,000 rpm for 10 minutes. Transfer 80 µl to a new 1.5-ml screwcap polypropylene tube. Dry supernatant at low heat until a translucent oligosaccharide pellet is obtained. Add 15 µl of APTS fluorophore labeling reagent solution and mix well until pellet is dissolved. Add 5 µl of 1 M sodium cyanoborohydride in THF and mix well. Incubate at 55° for approximately 2 hours. Add 500 µl HPLC grade water and mix well.

Reference solution. Prepare in the same manner as the test solution using *Rituximab IPRS.*

Blank control solution. Prepare in the same manner as the test solution using formulation buffer, except do not add PNGase F.

Capillary system
– coated capillary with reduced electroendosmotic flow, 50 µm ID, or any other suitable column,
– capillary length. 21.0 cm to detector, 31.2 cm total length,
– separation voltage. 20.0 kV,
– polarity. Negative to positive,
– temperature. 20°,
– detection. Arg-Ion laser, 488 nm excitation, 520 nm emission.

Separation buffer. Carbohydrate Separation Gel Buffer or equivalent.

Perform three injections of reference solution or until a visual evaluation of the electropherograms demonstrate consistent profiles for at least two consecutive injections. Inject reference solution, reagent blank, test solution(s), ending with an injection of reference solution.

Calculate the corrected peak area percent of each named peak and identify the peak corresponding to galactosylatedglycan. The corrected area percentage of each glycan should comply with the limits approved by National Regulatory Authority. The percent area of the peaks corresponding to galactosylatedglycan is not more than 35.0 per cent.

Method B

Test solution. Aliquot the sample volume in order to have between 25-300 µg of protein. Dry the glycoprotein solution completely in centrifugal vacuum evaporator. Add 45 µl of 1X PBS buffer and add 1.5 µl of 1:10 diluted 2-mercaptoethanol. Heat the sample for 5 minutes at 100°. Cool the sample to room temperature and add 5µl of NP40 detergent and 1.5 µl of PNGase F enzyme (750 units for 300 µg of protein). Incubate the reaction at 37° for 15 hours. Add 150 µl of cold ethanol, vortex and place it in ice for complete protein precipitation. Centrifuge the sample at 15000 rpm for 5 minutes and withdraw

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the supernatant. Dry the supernatant solution completely in centrifugal vacuum evaporator. Add 2µl of *1M cyanoborohydride* and 2µl of APTS labeling reagent to the dried supernatant. Incubate the reaction mixture at 37° for 4 hours.

Reference Solution. Prepare in the same manner as the test solution using *rituximab IPRS*.

Blank control solution. Prepare in the same manner as the test solution using formulation buffer, except do not add PNGase F.

Capillary System

- coated capillary with reduced endoosmoticflow (50 µm I.D. and 50.2 cm total length),
- voltage: 30kV,
- current: 40µA,
- capillary temperature: 20°,
- sample temperature: 10°,
- detector condition: Arg-Ion laser, Excitation-488nm, Emission-520 nm,
- separation: 30kV with reverse polarity for 20 minutes.

Calculate the corrected peak area percent of each named peak and identify the peak corresponding to galactosylatedglycan. The corrected area percentage of each glycan should comply with the limits approved by National Regulatory Authority. The percent area of the peaks corresponding to galactosylatedglycan is not more than 35.0 per cent.

Bacterial endotoxins (2.2.3). Not more than 1 EU per mg.

Other tests. Complies with the tests stated under Parenteral Preparations.

Protein. Determine by ultraviolet/visible spectrophotometry (2.4.7) using Method A or B.

Method A

Test solution. Dilute the preparation under examination with water to obtain a solution having concentration, 0.5 mg per ml.

Reference solution. Dissolve suitable quantity of *Rituximab IPRS* in *water* to obtained a solution having concentration, 0.5 mg per ml.

Blank control solution. The appropriate product formulation buffer is diluted in a comparable manner as the test solution.

Spectral range. 240 - 500 nm.

Absorption maximum. 278 nm.

Pipette the test and reference solutions into a quartz cuvette and scan the absorbance in the spectral range against the blank control solution. Record the absorbance A_{max} at (278 ± 2 nm) and A_{320} at 320 nm. Calculate $|A_{320}|/A_{max} \times 100$. Calculate the corrected A_{max} by subtracting the A_{320} value from A_{max}. $|A320|/Amax \times 100$ must be equal or less than 3.0 per cent for each sample measurement.

Calculate the protein concentration as follows.

$$\text{Protein concentration} = \frac{\text{corrected } A_{max} \text{ of sample} \times \text{concentration of reference}}{\text{corrected } A_{max} \text{ of reference}} \times \text{total dilution factor}$$

Alternately, an established extinction coefficient may be used to calculate protein concentration.

Acceptance criteria. Not less than 90 per cent and not more than 110 per cent of the stated amount of protein.

Method B

Determine the protein content as per appendix (2.7.1) or take reading at 280 nm and calculate the concentration of the sample(s) using following formula

$$\text{Concentration (mg/ml)} = \frac{\text{Average absorbance at 280 nm} \times \text{dilution factor}}{\text{Extinction coefficient}}$$

NOTE — Value of Extinction coefficient to be provided by the manufacturer.

Acceptance criteria. Not less than 90 per cent and not more than 110 per cent of the stated amount of protein.

Assay

Potency. Determine by complement dependent cytotoxicity assay by using WIL2-S cells.

Medium. Dissolve 485 ml of *RPMI 1640, 10 ml of 1M HEPES*, 6.8 ml of 6.5 g of *bovine serum albumin* and *5 ml of Penicillin and Streptomycin solution* (Final concentration to be 100-120 units per ml of Penicillin and 0.10-0.12 mg per ml of streptomycin) or any suitable antibiotic in water or any other solvent, if indicated and filter aseptically through 0.22 µm filter using sterile filtration assembly.

Mix 500 ml of *RPMI 1640*, 10 ml of *1M HEPES*, 6.8 ml of 7.5 per cent *bovine serum albumin* and 1 ml of *gentamycin sulphate* (50 mg per ml) or any suitable antibiotic in *water* or any other solvent, if indicated and filter aseptically through 0.22µm filter using sterile filtration assembly.

Test solution. Dilute the preparation under examination with medium to obtain a concentration of 0.0098 to 2.5 µg per ml.

Reference solutions (standard curve). Prepare several concentrations of *rituximab IPRS* bracketing the test solution concentration to determine an assay calibration curve.

Assay control solution. Use a reference solution and/or qualified lot of drug product as an assay control. Dilute in the same manner as the test solution.

Human complement. Human complement is diluted appropriately based on titration assays.

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Cell culture solution. Prepare cultures of WIL2-S cells, a human B-lymphoblastoid cell line (ATCC CRL-8885) or equivalent, in the medium.

Cell suspension. Adjust the cell concentration to 0.8 to 1.2 x 10^6 cells per ml in the medium.

To individual wells of a 96-well tissue culture plate, add 50 µl of each standard curve solution, test solution or assay control solution to be tested, followed by 50 µl of the cell suspension and then 50 µl diluted human complement. Incubate in a humidified CO_2 incubator for 1 - 2 hours at 37°. Add 50 µl resazurin reagent per well. Incubate plate for an additional 15-26 hours. Agitate plate for 5 to 10 minutes at room temperature on a mini orbital shaker. Evaluate using a fluorescence plate reader with excitation wavelength at 530 nm and emission wavelength at 590 nm.

Relative fluorescence units of the standards are plotted against standard concentrations to generate a standard curve using a 4-parameter curve fitting program. Sample and control potency values are calculated using the standard curve.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The Confidence limits (P=0.95) of the estimated potency are not less than 69.2 per cent and not more than 132.6 per cent of the stated potency

Storage. Store at 2 to 8° in an airtight container.

Labelling. The label states (1) Content of rituximab in mg per ml (2) name of product with generic name (3) drug product (injection) in mg per ml (4) Potency; (4) storage temperature.

Somatropin

Somatropinum

FPTIPLSRLF	DNAMLRAHRL	HQLAFDTYQE	FEEAYIPKEQ
KYSFLQNPQT	SLCFSESIPT	PSNREETQQK	SNLELLRISL
LLIQSWLEPV	QFLRSVFANS	LVYGASDSNV	YDLLKDLEEG
IQTLMGRLED	GSPRTGQIFK	QTYSKFDTNS	HNDDALLKNY
GLLYCFRKDM	DKVETFLRIV	QCRSVEGSCG	F

$C_{990}H_{1528}N_{262}O_{300}S_7$ Mol. Wt. 22,125

Somatropin is a protein having the structure (191 amino-acid residues) of the major component of growth hormone produced by the human pituitary. Somatropin contains not less than 91 per cent and not more than 105 per cent (anhydrous substance) of the stated potency. By convention, for the purpose of labelling somatropin preparations, 1 mg of anhydrous somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) is equivalent to 3.0 IU of biological activity.

Somatropin is produced by a method based on recombinant DNA (rDNA) technology. During the course of product development, it must be demonstrated that the manufacturing process produces a product having a biological activity of not less than 2.5 IU per mg, using a validated bioassay based on growth promotion and approved by the competent authority.

Host cell derived proteins (HCP). Not more than 100 ppm.

Host cell or vector derived DNA. Not more than 10 ng per dose.

Category. Growth Hormone

Description. A white to almost white powder.

Identification

A. Determine by capillary electrophoresis (capillary zone electrophoresis) (2.4.32) as described in the test for charged variants with the following modifications.

Inject test solution (b) under pressure or vacuum, in the following sequence: sample injection for at least 3 seconds then *CZE buffer* injection for 1 second.

In the electropherogram obtained with the test solution (b) only one principal peak corresponding to somatropin is detected and no doubling of this peak is observed.

B. Determine by Liquid chromatography (2.4.14) as described in the test for related proteins

In the chromatogram obtained with the test solution the retention time of principal peak is similar to that of the principal peak in the chromatogram obtained with reference solution.

C. Determine by peptide mapping (2.3.47).

Test solution. Prepare a solution of the substance under examination in *0.05M tris-hydrochloride buffer* solution pH 7.5 to obtain a solution containing 2.0 mg per ml of *somatropin* and transfer about 1.0 ml to a tube made from a suitable material such as polypropylene. Prepare a 1 mg per ml solution of *trypsin* for peptide mapping in *0.05M tris-hydrochloride buffer* solution pH 7.5 and add 30 µl to the solution of the preparation under examination. Cap the tube and place in a water-bath at 37° for at least 4 hours. Remove sample from the water-bath and stop the reaction immediately, for example by freezing. If analyzed immediately using an automatic injector, maintain at 2° to 8°.

Reference solution. Prepare at the same time and in the same manner as for the test solution, but using *somatropin IPRS* instead of preparation under examination.

Chromatographic system.

– a stainless steel column 25 cm x 4.6 mm, packed with octylsilyl silica gel (5-10 µm) with a pore size of 30 nm,

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- column temperature: 30°,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 1000 ml with *water*,
  B. to 100 ml of *water*, add 1 ml of *trifluoroacetic acid* and dilute to 1000 ml with *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 100 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
20	80	20
40	75	25
65	50	50
70	20	80

Inject the test solution and the reference solution. The chromatogram obtained with each solution is qualitatively similar to the reference chromatogram of *somatropin IPRS* digest.

The profile of chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with reference solution.

D. Determine by Size-exclusion chromatography (2.4.16) as described in assay.

In the chromatogram obtained with the test solution the principal peak is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

Tests

Related proteins. Determine by liquid chromatography (2.4.14) using the normalisation procedure.

Maintain the solutions at 2° to 8°and use within 24 hours. If an automatic injector is used, maintain it at 2° to 8°.

Test solution. Dilute preparation under examination with *0.05M tris-hydrochloride buffer solution* pH 7.5, to obtain a concentration of 2 mg per ml of somatropin.

Reference solution (a). Dilute *somatropin IPRS* in *0.05 M tris-hydrochloride buffer* solution pH 7.5, to obtain a concentration of 2 mg per ml of somatropin.

Reference solution (b). Dissolve the contents of a vial of *somatropin/desamidosomatropin IPRS* in *0.05M tris-hydrochloride buffer* solution pH 7.5 to obtain a concentration of 2 mg per ml of somatropin.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, singly end-capped butylsilylsilica gel (5 µm) and with pore size of 30 nm; a silica saturation column is placed between the pump and the injector valve,
- column temperature: 45°,
- mobile phase: a mixture of 29 volumes of *propanol* and 71 volumes of *0.05 M tris-hydrochloride buffer* solution pH 7.5
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Precondition the column with 200 ml to 500 ml of a 0.1 per cent v/v solution *of trifluoroacetic acid* in a 50 per cent v/v solution of *acetonitrile*; repeat as necessary, to improve column performance.

Inject reference solution (b). The test is not valid unless the resolution between the peaks corresponding to desamidosomatropin and somatropin is at least 1.0.

Inject reference solution (a) and test solution, the sum of the areas of all the peaks other than the principal peak is not more than 6.0 per cent of the total area of all the peaks. Relative retention with reference to somatropin: for desamidosomatropin is about 0.85.

Dimer and related substances of higher molecular mass. Determine by size-exclusion chromatography (2.4.16), using the normalization procedure.

Test solution. Dilute the substance under examination in *0.025M phosphate buffer solution pH 7.0*, to obtain a concentration of 1.0 mg per ml of somatropin.

Reference solution (a). Dissolve the contents of a vial of *somatropin IPRS* in *0.025 M phosphate buffer* solution pH 7.0 and dilute with the same solution to obtain a concentration of 1.0 mg per ml.

Reference solution (b). Place 1 vial of *somatropin IPRS* in an oven at 50° for a period sufficient to generate 1 to 2 per cent of dimer (typically 12-24 hours). Dissolve its contents in *0.025 M phosphate buffer solution pH 7.0* and dilute with the same solution to obtain a concentration of 1 mg per ml.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm, packed with hydrophilic silica gel, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 5000 to 150 000,
- mobile phase: a mixture of 3 volumes of 2-*propanol* and 97 volumes of *0.063M tris-hydrochloride buffer solution pH 7.0*,
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Inject reference solution (a) and test solution. Relative retention time with reference to somatropin monomer: related

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substances of higher molecular mass is about 0.65; somatropin dimer is about 0.9.

Inject reference solution (b), the test is not valid unless the ratio of the peak height of the dimer to the height above the baseline of the lowest point of the curve separating the dimer from the monomer peak is not less than 2.5.

Calculate the content of dimer, oligomers and aggregates. The sum of the peaks areas with retention times less than that of the principal peak is maximum 4.0 per cent of total area of all the peaks.

Charged variants. Determine by Capillary electrophoresis (2.4.32).

CZE buffer. Dissolve 13.2 gram of *ammonium phosphate* in 1000 ml of water and adjust the pH to 6.0 using *orthophosphoric acid.*

Test solution (a). Dilute the preparation under examination to obtain a concentration of 1 mg per ml of somatropin.

Test solution (b). Mix equal volumes of *test solution (a)* and the *reference solution.*

Reference solution. Dissolve the contents of a vial of *somatropin IPRS* in *water* and dilute with the same solvent to obtain a concentration of 1 mg per ml.

Capillary system
- material. uncoated fused silica,
- size: effective length is at least 70 cm, internal diameter is 50 µm,
- temperature: 30°,
- spectrophotometer set at 200 nm,

Set the autosampler to store the samples at 4° during analysis.

Preconditioning of the capillary. Rinse with *1M sodium hydroxide* for 20 minutes, with *water* for 20 minutes and with *CZE buffer* for 20 minutes at 20 psi.

Between-run rinsing. Rinse using *0.1M sodium hydroxide* for 2 minutes and with the *CZE buffer* for 6 minutes.

Rinsing times may be adapted according to the length of the capillary and the equipment used.

Inject test solution (a) and the reference solution under pressure or vacuum, using the following sequence. Inject with pressure the reference standard solution and each test sample for at least 3 seconds, then *CZE buffer* injection for 1 second. The injection time and pressure may be adapted in order to meet the system suitability criteria.

Migration. Apply a field strength of 217 V/cm (20 kV for capillaries of 92 cm total length) for 80 minutes using *CZE buffer* as the electrolyte in both buffer reservoirs. It is recommended to use fresh buffer for each separation to decrease the risk of ion depletion.

The electropherogram obtained with test solution (a) and reference solution are similar to the electropherogram of somatropin supplied with *somatropin IPRS.* Two peaks (I_1, I_2) eluting prior to the principal peak and at least 2 peaks (I_3,I_4) eluting after the principal peak are clearly visible. Peak I_2 corresponds to the cleaved form and peak I_4 corresponds to the deaminated forms, eluting as a doublet. Relative migration with reference to somatropin: deamidated forms is 1.02 to 1.11. In the electropherogram obtained with the test solution the area of the peak due to deamidated forms is not more than 5.0 per cent; the area of any other impurity peak is not be more than 2.0 per cent and sum of areas of any peaks other than the principal peak is not more than 10.0 per cent of the total area of all the peaks

$$\text{Relative Migration peak i} = \frac{\text{Migration time of peak i}}{\text{Migration time of Somatropin peak}}$$

where, i represents a peak number.

Water (2.3.43). Not more than 10.0 per cent w/w determined by the semi-micro determination of water

Bacterial endotoxins (2.2.3). Not more than 5 IU per mg of protein.

Assay

Determine by Size-exclusion chromatography (2.4.16) as described in the test for dimer and related substances of higher molecular mass.

Calculate the content of somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) from the declared content of $C_{990}H_{1528}N_{262}O_{300}S_7$ in *somatropin IPRS.*

Storage. Store in an airtight container, at a temperature of 2° to 8°. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

Labelling. The label states (1) the somatropin content in mg; (2) the activity in IU per mg; (3) the name and the concentration of any other excipients.

Somatropin Concentrated Solution

FPTIPLSRLF DNAMLRAHRL HQLAFDTYQE FEEAYIPKEQ
KYSFLQNPQT SLCFSESIPT PSNREETQQK SNLELLRISL
LLIQSWLEPV QFLRSVFANS LVYGASDSNV YDLLKDLEEG
IQTLMGRLED GSPRTGQIFK QTYSKFDTNS HNDDALLKNY
GLLYCFRKDM DKVETFLRIV QCRSVEGSCG F

$C_{990}H_{1528}N_{262}O_{300}S_7$ Mol. Wt. 22,125

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Somatropin Concentrated Solution is a solution containing a protein having the structure (191 amino-acid residues) of the major component of growth hormone produced by the human pituitary. It may contain buffer salts and other auxillary substances. Somatropin Concentrated Solution contains not less than 91 per cent and not more than 105 per cent of stated potency. By convention, for the purpose of labelling somatropin preparations 1 mg of anhydrous somatropin is equivalent to 3.0 IU of biological activity.

Somatropin is produced by a method based on recombinant DNA (rDNA) technology. During the course of product development, it must be demonstrated that the manufacturing process produces a product having a biological activity of not less than 2.5 IU per mg, using a validated bioassay based on growth promotion and approved by the competent authority.

Host cell derived proteins (HCP). Not more than 100 ppm.

Host cell or vector derived DNA. Not more than 10 ng per dose.

Category. Growth Hormone

Description. Clear or slightly turbid, colourless solution.

Identification

A. Determine by capillary electrophoresis (capillary zone electrophoresis) (2.4.32) as described in the test for charged variants with the following modifications.

Inject test solution (b) under pressure or vacuum, in the following sequence: sample injection for at least 3 seconds then *CZE buffer* injection for 1 second.

In the electropherogram obtained with the *test solution (b)* only 1 principal peak corresponding to somatropin, is detected and no doubling of this peak is observed.

B. Determine by Liquid chromatography (2.4.14) as described in the test for related proteins

In the chromatogram obtained with the test solution the retention time of principal peak is similar to that of the principal peak in the chromatogram obtained with reference solution.

C. Determine by Peptide mapping (2.3.47).

Test solution. Dilute the preparation under examination in *0.05M tris-hydrochloride buffer* solution pH 7.5 to a concentration of 2 mg per ml of somatropin in a tube made from a suitable material such as polypropylene. Prepare a 1 mg per ml solution of *trypsin* for peptide mapping in *0.05M tris-hydrochloride buffer* solution pH 7.5 and add 30 µl to the solution of the preparation under examination. Cap the tube and place in a water-bath at 37° for 4 hours. Remove the sample from the water-bath and stop the reaction immediately by freezing. At the end of the digestion period place digests in the refrigerator for later analysis (within 36 hours) or analyze immediately. Digests are stable at room temperature for 16 hours and at 5° for 48 hours.

Reference solution. Prepare reference solution as per the procedure given for the test solution, ensuring that all procedures are carried out simultaneously, and under identical conditions but using *somatropin IPRS* instead of the preparation under examination.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octylsilyl silica gel (5-10 µm) with a pore size of 30 nm,
- column temperature: 30°
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 1000 ml with *water*,
  B. to 100 ml of *water*, add 1 ml of *trifluoroacetic acid* and dilute to 1000 ml with *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 100 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
20	80	20
40	75	25
65	50	50
70	20	80

Inject the test solution and the reference solution. The chromatogram obtained with each solution is qualitatively similar to the reference chromatogram of *somatropin IPRS* digest.

The profile of chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with reference solution.

D. Determine by Size-exclusion chromatography (2.4.16) as described in assay.

In the chromatogram obtained with the test solution the principal peak is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

Tests

Related proteins. Determine by liquid chromatography (2.4.14) use the normalization procedure.

Maintain the solutions at 2° to 8°and use within 24 hours. If an automatic injector is used, maintain it at 2° to 8°.

Test solution. Dilute preparation under examination with *0.05M tris-hydrochloride buffer solution* pH 7.5, to obtain a concentration of 2 mg per ml of somatropin.

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Reference solution (a). Dilute *somatropin IPRS* in *0.05M tris-hydrochloride buffer* solution pH 7.5 to obtain a concentration of 2 mg per ml of somatropin.

Reference solution (b). Dissolve the contents of a vial of *somatropin/desamidosomatropin IPRS* in *0.05M tris-hydrochloride buffer* solution pH 7.5 to obtain a concentration of 2 mg per ml of somatropin.

Chromatographic system
- a stainless steel column 25 cm x 4.6 mm, packed with butylsilyl silica gel (5 µm),
- column temperature: 45°,
- mobile phase: a mixture of 29 volumes of *propanol* and 71 volumes of *0.05 M tris-hydrochloride buffer solution pH 7.5*,
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Precondition the column with 200-500 ml of a 0.1 per cent v/v solution of *trifluoroacetic acid* in a 50 per cent v/v solution of *acetonitrile*; repeat as necessary, to improve column performance.

Inject reference solution (b). The test is not valid unless the resolution between the peaks corresponding to desamidosomatropin and somatropin is at least 1.0.

Inject reference solution (a) and test solution, the sum of the areas of all the peaks other than the principal peak is not more than 6.0 per cent of the total area of all the peaks. Relative retention with reference to somatropin for desamidosomatropin is about 0.85.

Dimer and related substances of higher molecular mass. Determine by size-exclusion chromatography (2.4.16), using the normalization procedure.

Test solution. Dilute the preparation under examination with *0.025M phosphate buffer solution pH 7.0* to obtain a concentration of 1 mg per ml of somatropin.

Reference solution (a). Dissolve the contents of a vial of *somatropin IPRS* in *0.025M phosphate buffer solution* pH 7.0 and dilute with the same solution to obtain a concentration of 1 mg per ml.

Reference solution (b). Place 1 vial of *somatropin IPRS* in an oven at 50° for a period sufficient to generate 1 to 2 per cent of dimer (typically 12-24 hours). Dissolve its contents in *0.025M phosphate buffer solution pH 7.0* and dilute with the same solution to obtain a concentration of 1 mg per ml.

Chromatographic system
- a stainless steel column 30 cm x 7.8 mm, packed with hydrophilic silica gel, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 5000 to 150000,
- mobile phase: a mixture of 3 volumes of *2-propanol* and 97 volumes of *0.063M tris-hydrochloride buffer solution pH 7.0*,
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Inject reference solution (a) and test solution. Relative retention with reference to somatropin monomer: a related substance of higher molecular mass is about 0.65 and somatropin dimer is about 0.9.

Inject reference solution (b), the test is not valid unless the ratio of the peak height of the dimer to the height above the baseline of the lowest point of the curve separating the dimer from the monomer peak is not less than 2.5.

Calculate the content of dimers, oligomers and aggregates. The sum of the peaks area with retention times less than that of the principal peak is not more than 4.0 per cent of total area of al the peaks.

Charged variants. Capillary electrophoresis (2.4.32).

CZE buffer. Dissolve 13.2 gram of *ammonium phosphate* in 1000 ml of water and adjust the pH to 6.0 using *orthophosphoric acid.*

Test solution (a). Dilute the preparation under examination to obtain a concentration of 1 mg per ml of somatropin.

Test solution (b). Mix equal volumes of *test solution (a)* and the *reference solution.*

Reference solution. Dissolve the contents of a vial of *somatropin IPRS* in *water* and dilute with the same solvent to obtain a concentration of 1 mg per ml.

Capillary System
- material: Uncoated fused silica,
- size: effective length is at least 70 cm, internal diameter is 50 µm,
- temperature: 30°,
- spectrophotometer set at 200 nm,

Set the autosampler to store the samples at 4° during analysis.

Deactivation prior to capillary's first-time use. Rinse the capillary for 10 minutes with *acetonitrile* followed by 15 minutes of water at 50 psi. Flush the capillary at 50° using 50 psi with *0.1M nitric acid* for 30 minutes, *0.1M sodium hydroxide* for 30 minutes, *0.1M nitric acid* for 30 minutes, water for 15 minutes, *phosphate buffer* for 1 hour. Incubate the capillary for 2 hours in the *phosphate buffer.*

Preconditioning of the capillary. Rinse with *1M sodium hydroxide* for 20 minutes, with *water* for 20 minutes and with the *CZE buffer* for 20 min at 20 psi.

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Between-run rinsing. Rinse using *0.1M sodium hydroxide* for 2 minutes and with the *CZE buffer* for 6 minutes.

Rinsing times may be adapted according to the length of the capillary and the equipment used.

Inject test solution (a) and the reference solution under pressure or vacuum, using the following sequence: sample injection for atleast 3 seconds, then CZE *buffer* injection for 1 second. The injection time and pressure may be adapted in order to meet the system suitability criteria.

Migration. Apply a field strength of 217 V/cm (20 kV for capillaries of 92 cm total length) for 80 minutes using the *CZE buffer* as the electrolyte in both buffer reservoirs. It is recommended to use fresh buffer for each separation to decrease the risk of ion depletion.

The electropherogram obtained with test solution (a) and reference solution are similar to the electropherogram of somatropin supplied with *somatropin IPRS*. Two peaks (I_1,I_2) eluting prior to the principal peak and at least 2 peaks (I_3,I_4) eluting after the principal peak are clearly visible. Peak I_2 corresponds to the cleaved form and peak I_4 corresponds to the deamidated forms, eluting as a doublet. Relative migration with reference to somatropin: deamidated forms is 1.02 to 1.11. In the electropherogram obtained with the test solution the area of the peak due to deaminated forms is not more than 5.0 per cent; the area of the peak other than principal peak is not be more than 2.0 per cent and sum of areas of any peaks other than the principal peak is not more than 10.0 per cent of the total area of all the peaks.

$$\text{Relative Migration peak i} = \frac{\text{Migration time of peak i}}{\text{Migration time of Somatropin peak}}$$

where, i represents a peak number.

Bacterial endotoxins (2.2.3). Not more than 5 IU per mg of protein.

Assay

Determine by Size-exclusion chromatography (2.4.16) as described in the test for dimer and related substances of higher molecular mass.

Calculate the content of somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) from the declared content of $C_{990}H_{1528}N_{262}O_{300}S_7$ in *somatropin IPRS*.

Storage. In an airtight container, at a temperature below -20°. Avoid repeated freezing and thawing. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

Labelling. The label states (1) the somatropin content in mg per ml; (2) the activity in IU per mg; (3) the name and the concentration of any other excipients.

Somatropin for Injection

Somatropin for Injection is a freeze-dried, sterile preparation of a protein having the structure (191 amino-acid residues) of the major component of growth hormone produced by the human pituitary.

Somatropin for Injection contains not less than 89 per cent and not more than 105 per cent of the stated potency. By convention, for the purpose of labelling of somatropin preparations 1 mg of anhydrous somatropin is equivalent to 3.0 IU of biological activity.

Somatropin for Injection is prepared either from somatropin or from somatropin concentrated solution, or by a method based on recombinant DNA (rDNA) technology in which the injectable preparation is produced without the isolation of an intermediate solid or liquid bulk. In the later case, during the course of product development, it must be demonstrated that the manufacturing process produces a product having a biological activity of not less than 2.5 IU per mg, using a validated bioassay based on growth promotion and approved by the competent authority. The purified preparation, to which buffers and stabilizers may be added, is filtered through a bacteria-retentive filter, aseptically distributed in sterile containers of glass type I (6.2.1) and freeze-dried. The containers are immediately sealed so as to exclude microbial contamination and moisture.

Usual strength. 5 mg (15 IU), 6 mg (18 IU), 10 mg (30 IU) and 12 mg (36 IU).

Host cell derived proteins (HCP). Not more than 100 ppm.

Host cell or vector derived DNA. Not more than 10 ng per dose.

Description. White or almost white powder

Identification

Where Somatropin for injection is prepared from Somatropin or from Somatropin concentrated solution compliance with the requirements for host cell derived proteins, host cell and vector-derived DNA, identification test A, identification test C, and charged variants need not be reconfirmed by the manufacturer during subsequent production of Somatropin for injection.

A. Determine by capillary electrophoresis (capillary zone electrophoresis) (2.4.32) as described in the test for charged variants with the following modifications.

Inject test solution (b) under pressure or vacuum, in the following sequence: sample injection for at least 3 seconds then *CZE buffer* injection for 1 second.

In the electropherogram obtained with the test solution (b) only one principal peak corresponding to somatropin, is detected and no doubling of this peak is observed.

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B. Determine by Liquid chromatography (2.4.14) as described in the test for related proteins.

In the chromatogram obtained with the test solution the retention time of principal peak is similar to that of the principal peak in the chromatogram obtained with reference solution.

C. Determine by Peptide mapping (2.3.47).

Test solution. Prepare a solution of the substance under examination in *0.05M tris-hydrochloride buffer solution pH 7.5* to obtain a solution containing 2 mg per ml of somatropin and transfer about 1.0 ml to a tube made from a suitable material such as polypropylene. Prepare a 1 mg per ml solution of *trypsin* in *0.05M tris-hydrochloride buffer solution pH 7.5* and add 30 µl to the solution of the preparation under examination. Cap the tube and place in a water-bath at 37° for 4 hours. Remove sample from the water-bath and stop the reaction immediately, for example by freezing. If analyzed immediately using an automatic injector, maintain at 2° to 8°.

Reference solution. Prepare reference solution as per the procedure given for the test solution, ensuring that all procedures are carried out simultaneously, and under identical conditions but using *somatropin IPRS* instead of the substance under examination.

Chromatographic system

- stainless steel column 25 cm x 4.6 mm, packed with octylsilyl silica gel (5-10 µm) with a pore size of 30 nm,
- column temperature: 30°,
- mobile phase: A. dilute 1 ml of *trifluoroacetic acid* to 1000 ml with *water*,
  B. to 100 ml of water, add 1 ml of *trifluoroacetic acid* and dilute to 1000 ml with *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 100 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
20	80	20
40	75	25
65	50	50
70	20	80

Inject the reference solution and the test solution. The chromatogram obtained with each solution is qualitatively similar to the reference chromatogram of *somatropin IPRS* digest.

The profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

D. Determine by Size-exclusion chromatography (2.4.16) as described in assay.

The principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

Tests

Related Proteins. Determine by liquid chromatography (2.4.14), using the normalization procedure.

Maintain the solutions at 2° to 8° and use within 24 hours. If an automatic injector is used, maintain it at 2° to 8°.

Test solution. Prepare the solution of the preparation under examination in *0.05M tris-hydrochloride buffer solution pH 7.5* to obtain a concentration of 2 mg per ml of somatropin.

Reference solution (a). Prepare a solution of *somatropin IPRS* in *0.05M tris-hydrochloride buffer solution pH 7.5* to obtain a concentration of 2 mg per ml of somatropin.

Reference solution (b). Dissolve the contents of a vial of *somatropin/desamidosomatropin IPRS* in *0.05M tris-hydrochloride buffer solution pH 7.5* to obtain a concentration of 2 mg per ml of somatropin.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, singly end-capped butylsilylsilica gel (5 µm) and with pore size of 30 nm; a silica saturation column is placed between the pump and the injector valve,
- column temperature: 45°,
- mobile phase: a mixture of 29 volumes of *propanol* and 71 volumes of *0.05M tris-hydrochloride buffer* solution pH 7.5
- flow rate: 0.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 µl.

Precondition the column with 200 ml to 500 ml of a 0.1 per cent v/v solution *of trifluoroacetic acid* in a 50 per cent v/v solution of *acetonitrile*; repeat as necessary, to improve column performance.

Inject reference solution (b). The test is not valid unless the resolution between the peaks corresponding to desamidosomatropin and somatropin is at least 1.0.

Inject reference solution (a) and test solution, the sum of the areas of all the peaks other than the principal peak is not more than 13.0 per cent of the total area of all the peaks. Relative retention with reference to somatropin: for desamidosomatropin is about 0.85.

Dimer and related substances of higher molecular mass. Determine by size-exclusion chromatography (2.4.16). use the normalization procedure.

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Test solution. Dilute the preparation under examination in *0.025M phosphate buffer solution pH 7.0* to obtain a concentration of 1.0 mg per ml of somatropin.

Reference solution. Dissolve the contents of a vial of *somatropin IPRS* in *0.025M phosphate buffer solution pH 7.0* and dilute with the same solution to obtain a concentration of 1 mg per ml.

Reference solution (b). Place 1 vial of *somatropin IPRS* in an oven at 50° for a period sufficient to generate 1 to 2 per cent of dimer (typically 12 to 24 hours). Dissolve its contents in *0.025M phosphate buffer solution pH 7.0* and dilute with the same solution to obtain a concentration of 1 mg per ml.

Chromatographic system
- a stainless steel column 30 cm x 7.8 mm, packed with hydrophilic silica gel, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 5000 to 150000,
- mobile phase: a mixture of 3 volumes of *2-propanol* and 97 volumes of *0.063M tris-hydrochloride buffer solution pH 7.0*;
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Inject reference solution (a) and test solution. Relative retention time with reference to somatropin monomer: related substances of higher molecular mass is about 0.65; somatropin dimer is about 0.9.

Inject reference solution (b), the test is not valid unless the ratio of the peak height of the dimer to the height above the baseline of the lowest point of the curve separating the dimer from the monomer peak is not less than 2.5.

Calculate the content of dimers, oligomers and aggregates. The sum of the peaks with retention times less than that of the principal peak is not more than 6.0 per cent.

Charged variants. Determine by Capillary electrophoresis (2.4.32).

All the solutions should be filtered through 0.45 µm membrane filter before use.

CZE *buffer.* Dissolve 13.2 gram of *ammonium phosphate* in 1000 ml of water and adjust the pH to 6.0 using *orthophosphoric acid.*

Test solution (a). Dilute the preparation under examination to obtain a concentration of 1 mg per ml of somatropin.

Test solution (b). Mix equal volumes of *test solution (a)* and the *reference solution.*

Reference solution. Dissolve the contents of a vial of *somatropin IPRS* in *water* and dilute with the same solvent to obtain a concentration of 1 mg per ml

Capillary System
- material: Uncoated fused silica,
- size: effective length is at least 70 cm, internal diameter is 50 µm,
- temperature: 30°,
- spectrophotometer set at 200 nm,

Set the autosampler to store the samples at 4° during analysis.

Preconditioning of the capillary. Rinse with *1M sodium hydroxide* for 20 minutes, with *water* for 20 minutes and with the CZE *buffer* for 20 minutes at 20 psi.

Between-run rinsing. Rinse using *0.1M sodium hydroxide* for 2 minutes and with the *CZE buffer* for 6 minutes.

Rinsing times may be adapted according to the length of the capillary and the equipment used.

Inject test solution (a) and the reference solution under pressure or vacuum, using the following sequence: sample injection for at least 3 seconds then *CZE buffer* injection for 1 second. The injection time and pressure may be adapted in order to meet the system suitability criteria.

Migration. Apply a field strength of 217 V/cm (20 kV for capillaries of 92 cm total length) for 80 minutes using the *CZE buffer* as the electrolyte in both buffer reservoirs. It is recommended to use fresh buffer for each separation to decrease the risk of ion depletion.

The electropherogram obtained with test solution (a) and reference solution are similar to the electropherogram of somatropin supplied with *somatropin IPRS.* Two peaks (I_1,I_2) eluting prior to the principal peak and at least 2 peaks (I_3,I_4) eluting after the principal peak are clearly visible. Peak I_2 corresponds to the cleaved form and peak I_4 corresponds to the deamidated forms, eluting as a doublet. Relative migration with reference to somatropin: deamidated forms is 1.02 to 1.11. In the electropherogram obtained with the test solution the area of the peak due to deamidated forms is not more than 6.5 per cent; the area of any other impurity peak is not more than 2.0 per cent and sum of areas of any peaks other than the principal peak is not more than 11.5 per cent of the total area of all the peaks.

$$\text{Relative Migration peak i} = \frac{\text{Migration time of peak i}}{\text{Migration time of Somatropin peak}}$$

where, i represents a peak number.

Water (2.3.43). Not more than 3.0 per cent, determined by the semi-micro determination of water.

Bacterial endotoxins (2.2.3). Not more than 5 IU per mg of protein.

Other tests. Complies with the tests stated under Parenteral Preparations (Injections).

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Assay

Determine by Size-exclusion chromatography (2.4.16) as described in the test for dimer and related substances of higher molecular mass.

Calculate the content of somatropin from the declared content of *somatropin IPRS*.

Storage. Store in a sterile, airtight, tamper-proof container, at a temperature of 2° to 8°.

Labelling. The label states (1) the content of Somatropin in the container, in milligrams; (2) the composition and volume of the liquid to be added for reconstitution; (3) the time within which the reconstituted solution shall be used and the storage conditions during this period; (4) the name and quantity of any excipient; (5) the storage temperature; (6) that the preparation shall not be shaken during reconstitution.

Teriparatide

Recombinant Human Parathyroid Hormone ($rhPTH^{1-34}$)

SVSEIQLMHN LGKHLNSMER VEWLRKKLQD VHNF

$C_{181}H_{291}N_{55}O_{51}S_2$ Mol. Wt. approx. 4117.8 Da

Teriparatide is a biologically active N-terminal fragment of endogenous (native) human parathyroid hormone produced by recombinant DNA technology in bacteria as host cells. It is produced under conditions designated to minimize microbial contamination of the product.

Teriparatide contains not less than 90.0 per cent and not more than 110.0 per cent of teriparatide ($C_{181}H_{291}N_{55}O_{51}S_2$), calculated on anhydrous, acetic acid-free, and chloride-free basis.

Category. Bone forming agent.

Description. White to practically white solid (Lyophilized powder/cake).

Host cell-derived proteins. Not more than 100 ppm.

Host cell-derived DNA. Not more than 10 ng per dose.

Each batch of Teriparatide complies with the following requirements.

Identification

Test D and E may be omitted if tests A, B and C are carried out. Tests C and E may be omitted if tests A, B and D are carried out. Tests C and D may be omitted if tests A, B and E are carried out.

A. The biological activity of teriparatide is estimated to be equipotent to that *teriparatide IPRS* based on its comparative ability to stimulate the accumulation of cAMP in UMR-106 cells with respect to the appropriate reference standard of teriparatide.

Determination of the biological activity of teriparatide is based on the stimulation of adenylate cyclase activity in the rat osteosarcoma cell line UMR 106. Activation of the PTH receptor initiates a cascade event which culminates in an intracellular rise in cAMP concentration.

The method uses cAMP end-point measurement by ELISA. Incubate UMR-106 cells in tissue culture plate until an even monolayer is observed. Maintain the cells in serum free media for 18 to 26 hours in order to decrease the levels of endogenous cAMP. Suitably wash the cells and incubate with varying dilutions of test and reference preparations for up to 8 dilutions for the suitable time. Collect the cell lysate containing the cAMP and determine the cAMP accumulation by using a suitable ELISA or suitable detection assay.

Analyze the data by fitting a sigmoidal dose-response curve to the data obtained and by using a suitable statistical method, for example the 4-parameter or parallel line model.

The relative potency is not less than 60 per cent and not more than 120 per cent of the stated potency.

B. Determine by Peptide mapping (2.3.47). Use either method I or method II.

Method I

Test solution. Dilute the preparation under examination in *0.05 M tris-hydrochloride buffer solution pH 7.0* to obtain a concentration of 1 mg per ml and transfer 500 µl of it to a clean sample container. Add 10 µl of 1 mg per ml *trypsin solution* (in *water*) and incubate for 24 hours at 37°. Stop the reaction by freezing at or below -20°.

Reference solution. Dissolve the content of *teriparatide IPRS* in *0.05 M tris-hydrochloride buffer solution pH 7.0* to obtain a concentration of 1 mg per ml and transfer 500 µl of it to a clean sample container. Add 10 µl of 1 mg per ml *trypsin solution* (in *water*) and incubate for 24 hours at 37°. Stop the reaction by freezing at or below -20°.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilyl silica gel (3 µm) with a pore size of 20 nm,
- mobile phase: A. 0.05 per cent v/v solution of *trifluoroacetic acid*,
  B. to 800 ml of *acetonitrile*, add 0.5 ml of *trifluoroacetic acid* and 200 ml of *acetonitrile*,
- a linear gradient programme using the conditions given below,
- flow rate: 1 ml per minute,

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– spectrophotometer set at 214 nm,
– injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
30	45	55
30	0	100
35	0	100
35	95	5
45	95	5

Inject the reference solution. The test is not valid unless the chromatogram obtained with the reference solution shows eight prominent peaks as given in Fig.-1.

Inject the test solution. The profile of the chromatogram obtained with test solution corresponds to that of the chromatogram obtained with the reference solution.

Method II

Sample dilution buffer. 20 mM sodium phosphate, pH adjusted to 7.8 with *sodium hydroxide* or *orthophosphoric acid* .

Test solution. Dissolve the preparation under examination in dilution buffer to obtain a concentration of 1.5 mg per ml. To 0.15 ml of this solution, add 0.09 ml of 0.25 mg per ml enzyme solution. Mix and incubate at 37° for 18 to 24 hours. Quench the digest by adding 0.66 ml of mobile phase A to obtain a final digested protein concentration of 0.25 mg per ml.

Reference solution. Dissolve the contents of *teriparatide IPRS* in dilution buffer to obtain a concentration of 1.5 mg per ml. To 0.15 ml of this solution, add 0.09 ml of 0.25 mg per ml enzyme solution. Mix and incubate at 37° for 18 to 24 hours. Quench the digest by adding 0.66 ml of mobile phase A to obtain a final digested protein concentration of 0.25 mg per ml.

Blank solution: Combine 0.15 ml of *sample dilution buffer* to 0.09 ml of 0.25 mg per ml enzyme solution. Mix and incubate at 37° for 18 to 24 hours. Quench the digest by adding 0.66 ml of mobile phase A.

Chromatographic system
– a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilyl silica gel (3.5 µm),
– column temperature: 40°,
– mobile phase: A. 0.1 per cent v/v solution of *trifluoroacetic acid* in *water*,
B. 0.1 per cent v/v *trifluoroacetic acid in* a mixture of 40 volumes of *water* and 60 volumes of *acetonitrile*,
– a gradient programme using the conditions given below,
– flow rate: 1 ml per minute,
– spectrophotometer set at 214 nm,
– injection volume: 20 µl.

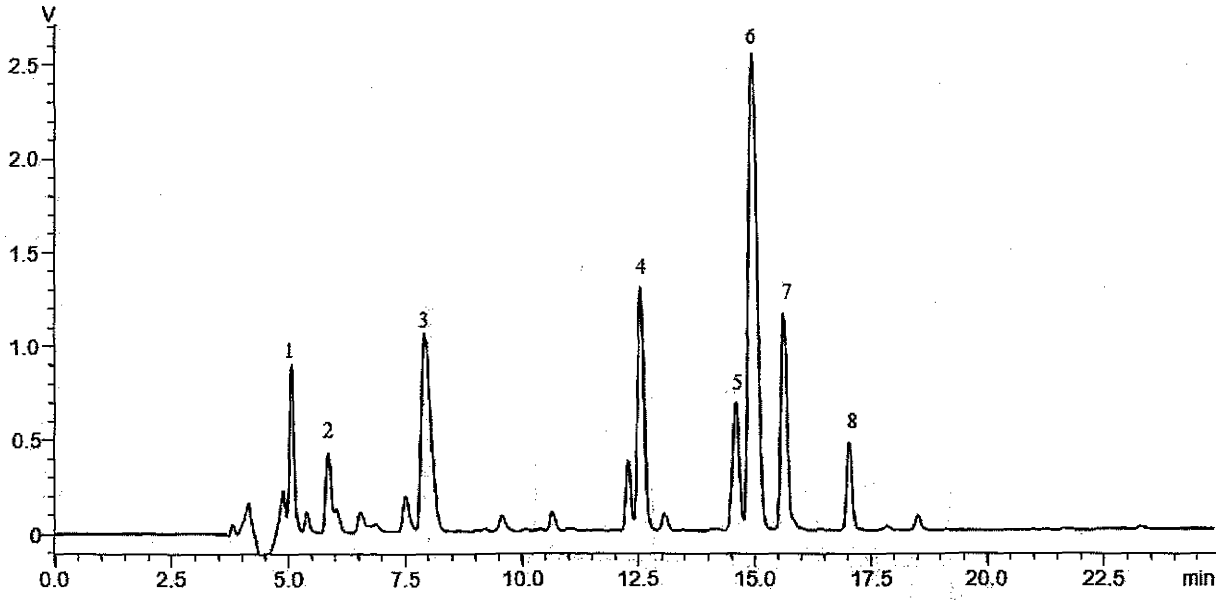

Fig.-1: Typical chromatogram of Trypsin digest of *teriparatide (1-34)*

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Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	96	4
6	96	4
20	45	55
25	0	100
25.1	96	4
35	96	4

Inject the reference solution. The test is not valid unless the chromatogram obtained with reference solution shows five prominent peaks as given in Fig.-2.

Inject the test solution. The profile of the chromatogram obtained with test solution corresponds to that of the chromatogram obtained with the reference solution showing five major fragments.

C. Determine by liquid chromatography (2.4.14) as described in test for assay. In the test for assay, the principle peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with the reference solution.

D. Determine by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12) under non-reducing conditions.

Gel dimensions. 1.5 mm thick. 10 cm x 10.5 cm.

Resolving gel. 20 per cent acrylamide. Mix the components in the order shown.

Solution components	Component volume (ml) per gel volume of 7.5 ml
Water	1.20
50 per cent acrylamide solution*	3.00
Tris-SDS solution (pH 8.45)	2.49
Glycerol	0.79
10 per cent APS**	0.025
TEMED***	0.007

*50 per cent acrylamide solution: 50 per cent acrylamide/bisacrylamide (50:1) solution.

**10 per cent APS: a 100 g per litre solution of ammonium persulphate. Prepare freshly.

***TEMED: tetramethylethylenediamine.

Stacking gel. 4 per cent acrylamide. Mix the components in the order shown.

Solution components	Component volume (ml) per gel volume of 5.0 ml
Water	3.05
30 per cent acrylamide solution*	0.65
Tris SDS solution (pH 8.45)	1.25
10 per cent APS**	0.05
TEMED***	0.01

*30 per cent acrylamide solution: 30 per cent acrylamide/bisacrylamide (29.2:0.8) solution.

**10 per cent APS: a 100 g per liter solution of ammonium persulphate. Prepare freshly.

***TEMED: tetramethylethylenediamine.

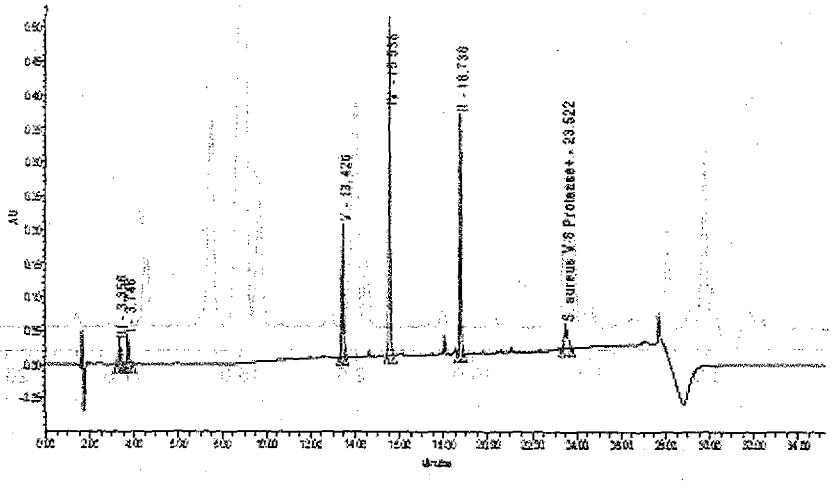

Fig.-2: Typical chromatogram of a S. aureus V-8 protease digest of teriparatide (1-34)

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Tris-SDS solution. Mix 0.3 per cent *sodium dodecyl sulphate* in *3.0 M tris-hydrochloride* buffer solution pH adjusted to 8.45.

Sample buffer. 0.2 M tris-hydrochloride buffer solution pH 6.8 containing 48 per cent *glycerol*, 2 per cent *sodium dodecyl sulphate* and 0.4 mg per ml coomassie G250.

Test solution. Dilute the preparation under examination with sample buffer to obtain a concentration of 8 µg per well.

Reference solution (a). A solution of protein molecular weight markers suitable for calibrating SDS-polyacrylamide gels.

Reference solution (b). Dilute the contents of *teriparatide IPRS* in *water* to obtain a concentration of 0.25 mg per ml. To 38.4 µl of this solution add 15 µl of sample buffer. Dilute to 60 µl with *water*.

Reference solution (c). Dilute the test solution in *water* to obtain a concentration of 8 µg per well.

Sample treatment. Boil for 2 minutes.

Application:

Well	Solution(s)	Volume (µl)
1	Reference solution (a)	50
2	Reference solution (b)	50
3	Test solution	50
4	Reference solution (c)	50

Cathode buffer. 0.1 M tris and *0.1 M tricine* and 0.1 per cent *sodium dodecyl sulphate*. Do not adjust pH.

Anode buffer. 0.2 M tris-hydrochloride buffer solution pH 8.9.

Detection. By silver staining.

The test is not valid unless: a band is seen in the electropherogram obtained with reference solution (c).

The electropherogram obtained with the test solution shows a single broad band corresponding in position and intensity to the single broad band obtained with the reference solution (b).

E. Determine by capillary electrophoresis (Capillary Isoelectric Focusing) (2.4.32).

Ampholyte gel solution pI range 3.0 to 10.0. Prepare and use according to the provided instructions.

Test solution. Dilute the preparation under examination with *water*, if required to a protein concentration of 1 mg per ml. To the ampholyte gel solution, add 5 µl of sample under examination. Centrifuge the mixture at 7000 g for 2 minutes.

Reference solution. To the ampholyte gel solution add pI marker(s) suitable for calibrating in pI range of 3 to 10 as per manufacturer's instructions. Centrifuge the mixture at 7000 g for 2 minutes.

Capillary system
- material: neutral capillary,
- size: effective length = 20 cm, internal diameter = 50 µm,
- spectrophotometer set at 214 nm,
- injection. Fill the capillary with reference solution / test solution for 1.5 minutes under pressure or vacuum.

Prewashing. Rinse the capillary with *10 mM phosphoric buffer* for 1 minute, with *water* under pressure or vacuum.

Prefocusing. Apply a voltage of 15 kV for 6 minutes with 0.17 minutes ramping time, using anolyte (containing cIEF gel and *91 mM phosphoric acid* as per provided instructions) and *20 mM sodium hydroxide* as catholyte in both buffer reservoirs.

Migration. Apply a voltage of 21 kV for 64 minutes at 0.5 psi using above electrolytes.

Rinse with water for 1 minute under pressure or vacuum.

The electropherogram obtained with the test solution corresponds to that obtained with the reference solution. The Isoelectric point of the test solution should appear between 8.2 and 8.4.

Tests

Related Impurities. Determine by liquid chromatography (2.4.14) using method A or method B.

Method A

Test solution. Dilute the preparation under examination with mobile phase A to obtain a protein concentration of 0.25 mg per ml.

Reference solution. To a volume of the test solution, add a suitable volume of 0.06 per cent *hydrogen peroxide* and *3 mM acetic acid* to obtain a final protein concentration of 0.88 mg per ml. Allow to stand at room temperature for 1 hour. Add about 30 mg of *L-methionine* per ml of solution and mix.

Chromatographic system
- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilyl silica gel (3 µm) with a pore size of 20 nm,
- mobile phase: A. 0.1 per cent v/v solution of *trifluoroacetic acid*,
  B. to 800 ml of *acetonitrile* add 1.0 ml of *trifluoroacetic acid* and 200 ml of *water* for chromatography,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 40 µl.

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Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	76	24
26	76	24
26.01	66.5	33.5
30	60	40
30.01	76	24
40	76	24

Inject the reference solution. The test is not valid unless in the chromatogram obtained with the reference solution relative retention time of three peaks corresponding to oxidized teriparatide with reference to principal peak are 0.79, 0.66 and 0.46.

Inject the test solution. The chromatogram obtained with the test solution corresponds to that obtained with the reference solution. In the chromatogram obtained with the test solution, the sum of the area of any peaks other than the principal peak is not greater than 3.0 per cent of the total area of the peaks. The sum of the area of peaks corresponding to the oxidized form is not greater than 2.0 per cent of the total area of the peaks.

Method B

Test solution. Dissolve the preparation under examination in mobile phase A to obtain a concentration of 0.7 mg per ml.

Reference solution. Use an appropriate solution containing about 0.8 per cent of the first post-main peak in mobile phase A or dissolve the content of *teriparatide IPRS* in *water* to obtain a concentration of 2 mg per ml. Adjust the pH to 3.0 with *hydrochloric acid.* Incubate at 50° for 9 days. The solution may be aliquoted and stored frozen. Dilute 1 volume of this solution with 1 volume of mobile phase A prior to injection. This gives approximately 0.8 per cent solution of first post-main peak. The first post-main peak is a degradation product resulting from this process and elutes immediately after teriparatide peak.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilyl silica gel (3.5 µm),
- mobile phase: A. a mixture of 10 volumes of *acetonitrile* and 90 volumes of *0.2 M sulphate buffer* prepared by dissolving 28.4 g of anhydrous *sodium sulphate* in 1000 ml *water,* pH adjusted to 2.3 with 85 per cent *orthophosphoric acid,*

  B. a mixture of 50 volumes of *acetonitrile* and 50 volumes of *0.2 M sulphate buffer pH 2.3,*
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
5	65	35
35	60	40
45	0	100
45.1	100	0
55	100	0

NOTE — The mobile phase composition at 5 minutes and 35 minutes may be adjusted in order to obtain the desired retention time of teriparatide peak. During adjustment of mobile phase composition same gradient slope should be maintained.

Inject the reference solution. The test is not valid unless (i) the ratio of the height of first post-main peak to the valley between the teriparatide peak and the first post peak is not less than 1.5 (ii) the tailing factor for the teriparatide peak is not more than 2.0.

Inject the test solution. The chromatogram obtained with the test solution corresponds to that obtained with the reference solution. The relative retention time of *Met+O(8)teriparatide* is about 0.48, *Met+O(18)teriparatide* is about 0.58 and *Met+O(8,18) teriparatide* is about 0.38 approximately with reference to teriparatide.

Calculate the percentage of methionyl sulfoxides of teriparatide in the portion of teriparatide taken using the following expression:

$$\frac{[(r_{Met+O(8)} + r_{Met+O(18)} + r_{Met+O(8,18)})}{r_T} \times 100$$

where,

$r_{Met+O(8)}$ = peak response of *Met+O(8) teriparatide* (oxidized on Met 8),

$r_{Met+O(18}$ = peak response of *Met+O(18) teriparatide* (oxidized on Met 18),

$r_{Met+O(8,18)}$ = peak response of *Met+O(8,18) teriparatide* (oxidized on Met 8 and Met 18),

r_T = sum of the responses for all of the peaks.

Calculate the percentage of the largest other related impurity of teriparatide in the portion of teriparatide taken using the following expression:

$$\frac{r_i}{r_T} \times 100$$

where, r_i = peak response of the largest other related impurity of teriparatide,

r_T = sum of the responses for all of the peaks.

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Calculate the total percentage of teriparatide related impurities in the portion of teriparatide taken using the following expression:

$$\frac{r_I}{r_T} \times 100$$

where, r_I = sum of peak response of the teriparatide related compound

The content of (i) Methionyl sulfoxides of teriparatide [consisting of *Met+O(8) teriparatide, Met+O(18) teriparatide* and *Met +O (8, 18)*] is not more than 0.5 per cent, (ii) largest other individual related impurities is not more than 0.5 per cent and (iii) the total related impurities is not more than 2.5 per cent.

Acetate. Determine by liquid chromatography (2.4.14) using the normalisation procedure.

Store the solutions at 2° to 8° and use them within 72 hours.

Test solution. Dissolve the preparation under examination in mobile phase to obtain a concentration of 5 mg per ml.

Reference solution. Dissolve separately 100 mg, 200 mg and 300 mg of *anhydrous sodium acetate* in mobile phase and dilute to 100 ml with mobile phase. Further dilute 1 ml of each solution to 10 ml with the mobile phase to prepare a standard curve with acetate concentrations in the range of 0.072-0.216 mg per ml.

Chromatographic system

- a stainless steel column 25 cm x 9.0 mm, packed with ion exclusion resin (7.5 µm),
- mobile phase: 0.5 per cent v/v solution of *dilute sulphuric acid*,
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 100 µl,
- run time: 1.5 times the retention time of acetate.

Inject each concentration of reference solutions in triplicate. The test is not valid unless (i) the area of the principal peak obtained with the repeatability of triplicate injections of 200 mg concentration reference solution has the relative standard deviation of not more than 1.25 per cent; (ii) the correlation coefficient (r) calculated for the standard curve is not less than 0.999.

Inject the test solution. The retention time of acetate is about 10 minutes. Plot peak areas versus injected acetate content and perform linear regression to create a standard curve. Calculate the acetate content using the standard curve and the area of the peak due to acetate in the chromatogram obtained with the test solution. The acetate content is not more than 5.0 per cent.

Chloride. Determine by liquid chromatography (2.4.14) using the normalisation procedure.

Test solution. Dissolve the preparation under examination in *water* to obtain a concentration of 1 mg per ml.

Reference solution (a). Dissolve 165.9 mg of *sodium chloride*, previously dried at 105° for 30 minutes, in *water* and dilute to 100 ml with the same solvent.

Reference solution (b). Dissolve 150 mg of *sodium nitrite* in *water* and dilute to 100 ml with the same solvent. Mix 1.0 ml of the solution and 2.5 ml of reference solution (a) and dilute to 100 ml with *water*.

Reference solutions. Dilute reference solution (a) with *water* to prepare a standard curve with at least 4 concentrations in the range of 10 to 40 µg per ml.

Chromatographic system

- a stainless steel pre-column 5 cm x 4.0 mm, packed with strongly basic anion-exchange resin (15 µm),
- a stainless steel column 25 cm x 4.0 mm, packed with strongly basic anion-exchange resin (15 µm),
- mobile phase: dissolve 285.7 mg of *sodium hydrogen carbonate* and 381.6 mg of *anhydrous sodium carbonate* in *water* and dilute to 2 liters with the same solvent,
- flow rate: 2 ml per minute,
- conductivity detector: use a self regenerating anion suppressor at 100 mA,
- injection volume: 50 µl,
- run time: 6 times the retention time of chloride.

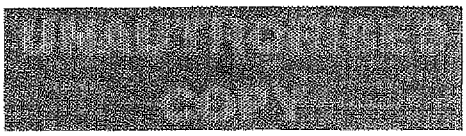
Inject reference solution (b). The test is not valid unless (i) the resolution between the peaks due to chloride and nitrite is not less than 1.5; (ii) the symmetry factor for the peaks due to chloride and nitrite is not more than 2.0 (iii) the area of the principal peak due to chloride and nitrite obtained with the repeatability of five injections has the relative standard deviation of not more than 2.0 per cent; (iv) the correlation coefficient (r) calculated for the standard curve is not less than 0.999.

Inject the test solution. The retention time of chloride is about 1.6 minutes and of nitrite is about 1.8 minutes. Plot peak areas versus injected chloride content and perform linear regression to create a standard curve. Calculate the chloride content using the standard curve and the area of the peak due to chloride in the chromatogram obtained with the test solution. The chloride content is not more than 4.0 per cent.

Water (2.3.43): Not more than 10 per cent.

Bacterial endotoxins (2.2.3). Not more than 50 EU in the volume containing 1 mg of protein.

Microbial enumeration tests. The total microbial count is not more than 100 cfu per g.



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Assay. Determine by liquid chromatography (2.4.14).

Solution (a). Dissolve 28.4 g of *anhydrous sodium sulphate* in 900 ml *water* and adjust to pH 2.3 with *orthophosphoric acid*. Dilute to 1000 ml with *water*.

Solution (b). Prepare a mixture of 25 volumes of *acetonitrile* and 75 volumes of solution (a).

Test solution. Dissolve the preparation under examination in solution (b) to obtain a concentration of 250 µg per ml. Test solution is stable for not more than 72 hours when stored at refrigerated temperature in sealed container.

(NOTE—Teriparatide should be equilibrated and weighed in a controlled humidity chamber of 25 ± 5 per cent relative humidity, then dissolved in diluent. Determine the water content within 24 hours of weighing).

Reference solution. Dissolve the content of *teriparatide IPRS* in solution (b) to obtain a concentration of 250 µg per ml. Reference solution is stable for not more than 72 hours when stored at refrigerated temperature in sealed container.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilyl silica gel (3.5 µm),
- mobile phase: A. a mixture of 90 volumes of *sulphate buffer* pH 2.3 and 10 volumes of *acetonitrile*,
  B. a mixture of 50 volumes of *sulphate buffer* pH 2.3 and 50 volumes of *acetonitrile*.
- a linear gradient programme using the conditions given below,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl,
- run time: 20 minutes.

Elute with a mixture of 63 volumes of mobile phase A and 37 volumes of mobile phase B.

Inject the reference solution in triplicate. The test is not valid unless (i) The relative standard deviation of teriparatide peak area is not more than 1.25 per cent; (ii) the tailing factor of the teriparatide peak is not more than 1.5.

Inject the test solution. The retention time of teriparatide is approximately 8 minutes. The estimated potency of teriparatide is not less than 90.0 per cent and not more than 110.0 per cent calculated on an anhydrous, acetic acid-free and chloride-free basis.

Storage. Store in sterile airtight container at or below the temperature of -10°.

Labelling. The label states (i) the teriparatide content in µg per ml; (ii) the name and the concentration of any other excipients; (iii) indication that the material has been produced by methods based on recombinant DNA technology

Teriparatide Concentrated Solution

Recombinant Human Parathyroid Hormone (rhPTH$^{1-34}$)

SVSEIQLMHN LGKHLNSMER VEWLRKKLQD VHNF

$C_{181}H_{291}N_{55}O_{51}S_2$ Mol. Wt. approx. 4117.8 Da

Teriparatide is a biologically active N-terminal fragment of endogenous (native) human parathyroid hormone produced by recombinant DNA technology in bacteria as host cells. It is produced under conditions designated to minimize microbial contamination of the product.

Category. Bone forming agent.

Description. A clear and colourless solution

Host cell-derived proteins. Not more than 100 ppm.

Host cell-derived DNA. Not more than 10 ng per dose.

Each batch of Teriparatide complies with the following requirements.

Identification

A. The biological activity of teriparatide is estimated to beequipotent to that *teriparatide IPRS* based on its comparativeability to stimulate the accumulation of CAMP in UMR-106 cells with respect to the appropriate reference standard of teriparatide.

Determination of the biological activity of teriparatide is based on the stimulation of adenylatecyclase activity in the rat osteosarcoma cell line UMR 106. Activation of the PTH receptor initiates a cascade event which culminates in an intracellular rise in cAMP concentration.

The method uses cAMP end-point measurement by ELISA. Incubate UMR-106 cells in tissue culture plate until an even monolayer is observed. Maintain the cells in serum free media for 18 to 26 hours in order to decrease the levels of endogenous cAMP. Suitably wash the cells and incubate with varying dilutions of test and reference preparations for up to 8 dilutions for the suitable time. Collect the cell lysate containing the cAMP and determine the cAMP accumulation by using a suitable ELISA or suitable detection assay.

Analyze the data by fitting a sigmoidal dose-response curve to the data obtained and by using a suitable statistical method, for example the 4-parameter or parallel line model.

The relative potency is not less than 60 per cent and not more than 120 per cent of the stated potency.

B. Determine by Peptide mapping (2.3.47).

Test solution. Dilute the preparation under examination in *0.05 M tris-hydrochloride buffer solution pH 7.0* to obtain a concentration of 1 mg per ml and transfer 500 µl of it to a clean

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sample container. Add 10 µl of 1 mg per ml *trypsin solution* (in *water*) and incubate for 24 hours at 37°. Stop the reaction by freezing at or below -20°.

Reference solution. Dissolve the content of *teriparatide IPRS* in *0.05 M tris-hydrochloride buffer solution pH 7.0* to obtain a concentration of 1 mg per ml and transfer 500 µl of it to a clean sample container. Add 10 µl of 1 mg per ml *trypsin solution* (in *water*) and incubate for 24 hours at 37°. Stop the reaction by freezing at or below -20°.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilyl silica gel (3 µm) with a pore size of 20 nm,
- mobile phase: A. 0.05 per cent v/v solution of *trifluoroacetic acid*,
  B. to 800 ml of *acetonitrile*, add 0.5 ml of *trifluoroacetic acid* and 200 ml of *acetonitrile*,
- a linear gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 50 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	95	5
30	45	55
30	0	100
35	0	100
35	95	5
45	95	5

Inject the reference solution. The test is not valid unless the chromatogram obtained with the reference solution shows eight prominent peaks as given in Fig.-1.

Inject the test solution. The profile of the chromatogram obtained with test solution corresponds to that of the chromatogram obtained with the reference solution.

C. Determine by liquid chromatography (2.4.14) as describedin test for assay. In the test for assay, the principle peak in thechromatogram obtained with the test solution correspondstothe peak in the chromatogram obtained with the referencesolution.

Tests

Related Impurities. Determine by liquid chromatography (2.4.14).

Test solution. Dilute the preparation under examination withmobile phase A to obtain a protein concentration of 0.25 mgper ml.

Reference solution. To a volume of the test solution, add asuitable volume of 0.06 per cent *hydrogen peroxide* and *3 mM acetic acid* to obtain a final protein concentration of 0.88 mgper ml. Allow to stand at room temperature for 1 hour. Addabout 30 mg of *L-methionine* per ml of solution and mix.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed withoctadecylsilyl silica gel (3 µm) with a pore size of 20 nm,
- mobile phase: A. 0.1 per cent v/v solution of *trifluoroacetic acid*,

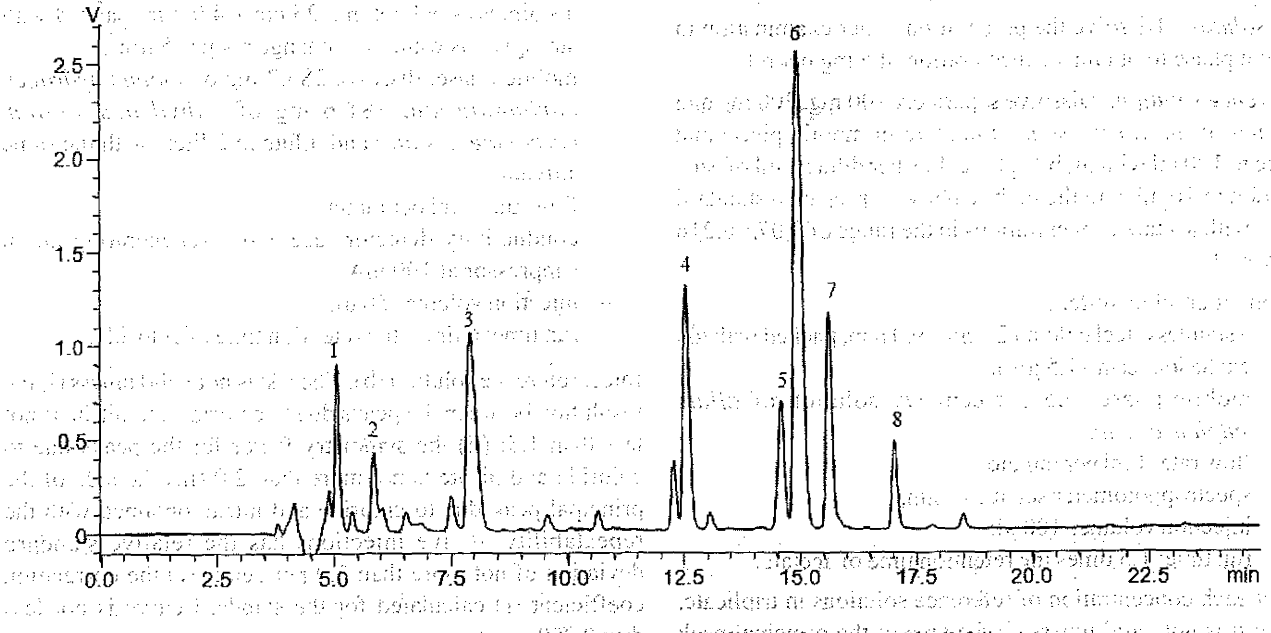

Fig.-1: Typical chromatogram of Trypsin digest of *teriparatide (1-34)*

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B, to 800 ml of acetonitrile and 200 ml of *water* for chromatography,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 40 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0.01	76	24
26	66.5	33.5
26.01	60	40
30	60	40
30.01	76	24
40	76	24

Inject the reference solution. The test is not valid unless in the chromatogram obtained with the reference solution relative retention time of three peaks corresponding to oxidized Teriparatide with reference to principal peak arc 0.79, 0.66 and 0.46.

Inject the test solution. The chromatogram obtained with the test solution corresponds to that obtained with the reference solution. In the chromatogram obtained with the test solution, the sum of the area of any peaks other than the principal peak is not greater than 3.0 per cent of the total area of the peaks. The sum of the area of peaks corresponding to the oxidized form is not greater than 2.0 per cent of the total area of the peaks.

Acetate. Determine by liquid chromatography (2.4.14) using the normalisation procedure.

Store the solutions at 2° to 8° and use them within 72 hours.

Test solution. Dissolve the preparation under examination in mobile phase to obtain a concentration of 5 mg per ml.

Reference solution. Dissolve separately 100 mg, 200 mg and 300 mg of *anhydrous sodium acetate* in mobile phase and dilute to 100 ml with mobile phase. Further dilute 1 ml of each solution to 10 ml with the mobile phase to prepare a standard curve with acetate concentrations in the range of 0.072-0.216 mg per ml.

Chromatographic system
- a stainless steel column 25 cm x 9.0 mm, packed with ion exclusion resin (7.5 µm),
- mobile phase: 0.5 per cent v/v solution of *dilute sulphuric acid,*
- flow rate: 1 ml per minute,
- spectrophotometer set at 210 nm,
- injection volume: 100 µl,
- run time: 1.5 times the retention time of acetate.

Inject each concentration of reference solutions in triplicate. The test is not valid unless (i) the area of the principal peak obtained with the repeatability of triplicate injections of 200 mg concentration reference solution has the relative standard deviation of not more than 1.25 per cent; (ii) the correlation coefficient (r) calculated for the standard curve is not less than 0.999.

Inject the test solution. The retention time of acetate is about 10 minutes. Plot peak areas versus injected acetate content and perform linear regression to create a standard curve. Calculate the acetate content using the standard curve and the area of the peak due to acetate in the chromatogram obtained with the test solution. The acetate content is not more than 5.0 per cent.

Chloride. Determine by liquid chromatography (2.4.14) using the normalisation procedure.

Test solution. Dissolve the preparation under examination in *water* to obtain a concentration of 1 mg per ml.

Reference solution (a). Dissolve 165.9 mg of *sodium chloride,* previously dried at 105° for 30 minutes, in *water* and dilute to 100 ml with the same solvent.

Reference solution (b). Dissolve 150 mg of *sodium nitrite* in *water* and dilute to 100 ml with the same solvent. Mix 1.0 ml of the solution and 2.5 ml of reference solution (a) and dilute to 100 ml with *water.*

Reference solutions. Dilute reference solution (a) with *water* to prepare a standard curve with at least 4 concentrations in the range of 10 to 40 µg per ml.

Chromatographic system
- a stainless steel pre-column 5 cm x 4.0 mm, packed with strongly basic anion-exchange resin (15 µm),
- a stainless steel column 25 cm x 4.0 mm, packed with strongly basic anion-exchange resin (15 µm),
- mobile phase: dissolve 285.7 mg of *sodium hydrogen carbonate* and 381.6 mg of *anhydrous sodium carbonate* in *water* and dilute to 2 liters with the same solvent,
- flow rate: 2 ml per minute,
- conductivity detector: use a self regenerating anion suppressor at 100 mA,
- injection volume: 50 µl,
- run time: 6 times the retention time of chloride.

Inject reference solution (b). The test is not valid unless (i) the resolution between the peaks due to chloride and nitrite is not less than 1.5; (ii) the symmetry factor for the peaks due to chloride and nitrite is not more than 2.0 (iii) the area of the principal peak due to chloride and nitrite obtained with the repeatability of five injections has the relative standard deviation of not more than 2.0 per cent; (iv) the correlation coefficient (r) calculated for the standard curve is not less than 0.999.

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Inject the test solution. The retention time of chloride is about 1.6 minutes and of nitrite is about 1.8 minutes. Plot peak areas versus injected chloride content and perform linear regression to create a standard curve. Calculate the chloride content using the standard curve and the area of the peak due to chloride in the chromatogram obtained with the test solution. The chloride content is not more than 4.0 per cent.

Bacterial endotoxins (2.2.3). Not more than 50 EU in the volume containing 1 mg of protein.

Microbial enumeration tests. The total microbial count is not more than 100 cfu per g.

Assay. Determine by liquid chromatography (2.4.14)

Mobile phase A. 0.05 per cent v/v *trifluoroacetic acid* in *water*.

Mobile phase B. 0.05 per cent v/v *trifluoroacetic acid* in *acetonitrile*.

Diluent. 0.05 per cent v/v *trifluoroacetic acid* in *water*.

Test solution. Dilute the test sample to 0.1 mg per ml with diluent.

Reference solution. Dilute the test sample to 0.1 mg per ml with diluent.

Chromatographic system

- a stainless steel C18 column 25 cm x 4.6 mm, packed with octadecylsilyl silica gel (3 µm) with a pore size of 120A°,
- temperature: 40°,
- detector: 225 nm,
- a linear gradient programme using the conditions given below,
- flow rate: 1.4 ml per minute,
- injection volume: 100 µl,
- run time: 55 minutes.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	90	10
10	76	24
40	66.5	33.5
45	66.5	33.5
45.1	90	10
55	90	10

The column efficiency for reference solution should not be less than 1500 theoretical plates.

The relative standard deviation for area and retention time of five replicates of standard injections should not be more than 2.0 per cent.

Calculation:

$$\text{Assay (mg per ml)} = \frac{AT}{AS} \times \frac{PF}{1} \times \frac{VL}{DS} \times \frac{DT}{VL1}$$

Where, AT = Peak area of unknown impurity in sample injection;

AS = Peak area of teriparatide in standard injection;

PF = Concentration of teriparatide in standard injection;

VL = Volume of standard taken in ml;

DS = Dilution of the standard solution;

VL1= Volume of sample taken in ml;

DT = Dilution of sample solution.

The estimated potency of Teriparatide is not less than 90.0 per cent and not more than 110.0 percentcalculated on an anhydrous, acetic acid-free and chloride-freebasis.

Storage. Store in sterile airtight container at or below the temperature of -20°.

Labelling.The label states (i) the name of product (ii) content in mg per ml (iii) the name and the concentration of any other excipients; (iv) indication that the material has been produced by methods based on recombinant DNA technology; (v) storage condition.

Teriparatide Injection

Recombinant Human Parathyroid Hormone ($rhPTH^{1-34}$) injection

Recombinant Human Parathyroid Hormone ($rhPTH^{1-34}$) injection, is a sterile solution for injection containing teriparatide ($rhPTH^{1-34}$) as an active ingredient with suitable buffering and preservative agents.

Teriparatide injection contains not less than 90.0 per cent and not more than 110.0 per cent of the labeled amount of teriparatide.

Usual Strength. Pre-filled cartridge / vial contains 250 µg per ml of teriparatide ($rhPTH^{1-34}$).

Description. Clear, colorless or slightly yellowish solution.

Identification

Test C may be omitted if tests A and B are carried out. Tests B may be omitted if tesis A and C are carried out.

A. The biological activity of teriparatide is estimated to be equipotent to that *teriparatide IPRS* based on its comparative ability to stimulate the accumulation of cAMP in UMR-106 cells with respect to the *teriparatide IPRS*.

Determination of the biological activity of teriparatide is based on the stimulation of adenylate cyclase activity in the rat osteosarcoma cell line UMR-106. Activation of the PTH receptor initiates a cascade event which culminates in an intracellular rise in cAMP concentration.

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The method uses cAMP end-point measurement by ELISA. Incubate UMR-106 cells in tissue culture plate until an even monolayer is observed. Maintain the cells in serum free media for 18 to 26 hours in order to decrease the levels of endogenous cAMP. Suitably wash the cells and incubate with varying dilutions of test and reference preparations for up to 8 dilutions for the suitable time. Collect the cell lysate containing the cAMP and determine the cAMP accumulation by using ELISA or suitable detection assay.

Analyze the data by fitting a sigmoidal dose-response curve to the data obtained and by using a suitable statistical method, for example the 4- parameter or parallel line model.

The relative potency is not less than 75 percent and not more than 125 percent of the stated potency.

B. Determine by Liquid chromatography (2.4.14) as described in assay. In assay, the retention time of the Teriparatide peak in the chromatogram obtained with the test solution corresponds to that of the peak in the chromatogram obtained with the reference solution.

C. Determine by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (2.4.12) under non-reducing conditions.

Gel dimensions. 1.5 mm thick, 10 cm × 10.5 cm.

Resolving gel. 20 per cent *acrylamide*. Mix the components in the order shown:

Solution components	Component volume (ml) per gel volume of 7.5 ml
Water	1.20
50 per cent acrylamide solution*	3.00
Tris-SDS solution (pH 8.45)	2.49
Glycerol	0.79
10 per cent APS**	0.025
TEMED***	0.007

*50 per cent acrylamide solution: 50 per cent acrylamide/bisacrylamide (50:1) solution.

**10 per cent APS: a 100 g per litre solution of ammonium persulphate. Prepare freshly.

***TEMED: tetramethylethylenediamine.

Stacking gel. 4 per cent acrylamide. Mix the components in the order shown.

Solution components	Component volume (ml) per gel volume of 5.0 ml
Water	3.05
30 per cent acrylamide solution*	0.65
Tris SDS solution (pH 8.45)	1.25
10 per cent APS**	0.05
TEMED***	0.01

*30 per cent acrylamide solution: 30 per cent acrylamide/bisacrylamide (29.2:0.8) solution.

**10 per cent APS: a 100 g per liter solution of ammonium persulphate. Prepare freshly.

***TEMED: tetramethylethylenediamine.

Tris-SDS solution. A mixture of 0.3 per cent *sodium dodecyl sulphate* in *3.0 M tris-hydrochloride buffer* solution, pH 8.45.

Sample buffer. A mixture of *0.2 M tris-hydrochloride buffer* solution pH 6.8 containing 48 per cent *glycerol*, 2 per cent *sodium dodecyl sulphate*, 0.4 mg per ml *coomassie G250*.

Test solution. Dilute the preparation under examination in sample buffer to obtain a concentration of 8 µg per well.

Reference solution (a). A solution of protein molecular weight markers suitable for calibrating SDS-polyacrylamide gels.

Reference solution (b). Dilute *teriparatide IPRS* in *water* to obtain a concentration of 0.25 mg per ml. To 38.4 µl of this solution add 15 µl of *sample buffer*. Dilute to 60 µl with *water*.

Reference solution (c). Dilute the test solution in sample buffer to obtain a concentration of 8 µg per well.

Sample treatment. Boil for 2 minutes.

Application:

Well	Solution(s)	Volume (µl)
1	Reference solution (a)	50
2	Reference solution (b)	50
3	Test solution	50
4	Reference solution (c)	50

Cathode buffer. 0.1 M tris and *0.1 M tricine* and 0.1 per cent *sodium dodecyl sulphate*. Do not adjust pH.

Anode buffer. 0.2 M tris-hydrochloride buffer solution pH 8.9.

Detection. By silver staining.

The test is not valid unless; a band is seen in the electropherogram obtained with reference solution (c)

The electropherogram obtained with the test solution shows a single broad band corresponding in position and intensity to the single broad band obtained with the reference solution (b).

Tests

pH (2.4.24). 3.8 to 4.5.

Related Impurities. Determine by Liquid chromatography (2.4.14) using normalization procedure. Use method A or B.

Method A

Test solution. Dilute the preparation under examination with mobile phase A to obtain a protein concentration of 0.25 mg per ml.

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Reference solution. To a volume of the test solution, add a suitable volume of 0.06 per cent *hydrogen peroxide* and *3 mM acetic acid* to obtain a final protein concentration of 0.88 mg per ml. Allow to stand at room temperature for 1 hour. Add approx. 30 mg of *L-methionine* per ml of solution and mix.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilyl silica gel (3 µm) with a pore size of 20 nm,
- mobile phase: A. 0.1 per cent v/v solution of *trifluoroacetic acid*,
  B. to 800 ml of *acetonitrile* add 1.0 ml of *trifluoroacetic acid* and 200 ml of *water* for chromatography,
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 40 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	76	24
26	76	24
26.01	66.5	33.5
30	60	40
30.01	76	24
40	76	24

Inject the reference solution. The test is not valid unless in the chromatogram obtained with the reference solution relative tetention time of three peaks corresponding to oxidized teriparatide with reference to principal peak are 0.79, 0.66 and 0.46.

Inject the test solution. The chromatogram obtained with the test solution corresponds to that obtained with the reference solution. In the chromatogram obtained with the test solution, the sum of the area of any peaks other than the principal peak is not greater than 3.0 per cent of the total area of the peaks. The sum of the area of peaks corresponding to the oxidized form is not greater than 2.0 per cent of the total area of the peaks.

Method B

Test solution. Dissolve the preparation under examination in mobile phase A to obtain a concentration of approximately 250 µg per ml.

Reference solution. Dissolve the content of *teriparatide IPRS* in *water* to obtain a concentration of 2 mg per ml. Adjust the pH to 3.0 with *hydrochloric acid.* Incubate at 50° for 9 days. The solution may be aliquoted and stored frozen. Dilute 1 volume of this solution with 3 volume of mobile phase A prior to injection. This gives approximately 0.8 per cent solution of first post-main peak. The first post-main peak is a degradation product resulting from this process and elutes immediately after teriparatide peak.

0.2 M sulphate buffer. 28.4 g per litre of *anhydrous sodium sulphate* in *water.* Adjust with 85 per cent *phosphoric acid* to a pH of 2.3.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilyl silica gel (3.5 µm),
- mobile phase: A. a mixture of 10 volumes of *acetonitrile* and 90 volumes of *0.2 M sulphate buffer pH 2.3*,
  B. a mixture of 50 volumes of *acetonitrile* and 50 volumes of *0.2 M sulphate buffer pH 2.3*,
- a linear gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 50 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	100	0
2	100	0
8	76	24
68	60	40
75	0	100
80	0	100

Inject the reference solution. The test is not valid unless (i) the ratio of the height of first post-main peak to the valley between the teriparatide peak and the first post peak is not less than 1.5 (ii) the tailing factor for the teriparatide peak is not more than 2.0.

Inject the test solution. The relative retention time of $rhPTH^{1-30}$ is about 0.77 to 0.78 and *teriparatide succinimide (30)* is about 0.98 to 0.99 with reference to teriparatide.

Calculate the percentage of a related impurity, the cleavage product of teriparatide, rhPTH (1-30), in the portion of injection taken using the following expression:

$$\frac{r_{rhPTH\,(1-30)}}{r_T} \times 100$$

where,

$r_{rhPTH(1-30)}$ = peak response of rhPTH (1-30) (a cleavage product of teriparatide at Asn 30),

r_T = sum of all the peak responses excluding peaks due to added preservatives or excipients.

Calculate the percentage of a related impurity, teriparatide succinimide (30) in the portion of injection taken using the following expression:

$$\frac{r_{Suc}}{r_T} \times 100$$

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where, r_{Suc} = peak response of teriparatide succinimide (30) (formation of succinimide on Asn 30),

r_T = sum of all the peak responses excluding peaks due to added preservatives or excipients.

Calculate the percentage of the largest other related impurity of teriparatide in the portion of teriparatide taken using the following expression:

$$\frac{r_i}{r_T} \times 100$$

where, r_i = peak response of the largest other related impurity of teriparatide,

r_T = sum of the responses for all of the peaks excluding peaks due to added preservatives or excipients.

Calculate the total percentage of teriparatide related impurities in the portion of teriparatide taken using the following expression:

$$\frac{r_I}{r_T} \times 100$$

where, r_I = sum of peak response of the teriparatide related compound,

r_T = sum of the responses for all of the peaks excluding peaks due to added preservatives or excipients.

The content of (i) $rhPTH^{1-30}$ is not more than 1.2 per cent; (ii) teriparatide succinimide (30) is not more than 1.2 per cent; (iii) largest other individual related impurity is not more than 1.0 per cent and (iv) the total related impurities is not more than 7.0 per cent.

Bacterial endotoxins (2.2.3). Not more than 100 EU per mg of teriparatide drug product.

Other tests. Complies with the tests stated under Parenteral Preparations (Injections).

Assay. Determine by liquid chromatography (2.4.14).

Solution (a). Dissolve 28.4 g of *anhydrous sodium sulphate* in 900 ml *water* and adjust to pH 2.3 with 85 per cent *phosphoric acid*. Dilute to 1000 ml with water to obtain a solution of 0.20 M.

Solution (b). Dissolve 38.8 g of *anhydrous sodium sulphate* in 900 ml *water* and adjust to pH 2.3 with 85 per cent *phosphoric acid*. Dilute to 1000 ml with water to obtain a solution of 0.27 M.

Solution (c). Mix 75 volumes of solution (a) and 25 volumes of *acetonitrile*.

Solution (d). Mix 69 volumes of solution (b) and 31 volumes of *acetonitrile*.

Test solution. Prepare the solution in duplicate. Dissolve the preparation under examination in solution (d) to obtain a concentration of approximately 50 to 100 µg per ml. Test solution is stable for not more than 48 hours when stored at refrigerated temperature in sealed container.

Reference solution. Prepare the solution in triplicate. Dissolve the content of *teriparatide IPRS* in solution (c) to obtain a concentration of 100 µg per ml. Reference solution is stable for not more than 48 hours when stored at refrigerated temperature in sealed container.

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with octadecylsilyl silica gel (3.5 µm),
- mobile phase: A. a mixture of 90 volumes of solution (a) and 10 volumes of *acetonitrile*,
  B. a mixture of 50 volumes of solution (a) and 50 volumes of *acetonitrile*.
- a gradient programme using the conditions given below,
- flow rate: 0.8 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 25 µl,
- Run time: 20 minutes.

NOTE—The Mobile phase composition may be adjusted to obtain the retention time of approximately 8 minutes for the teriparatide main peak.

Inject the reference solution in triplicate. The test is not valid unless (i) The relative standard deviation (*IPRSD*) of rh-PTH (1-34) peak area is not more than 1.25 per cent; (ii) the tailing factor of the teriparatide peak is not more than 1.5.

Inject the test solution. The estimated potency is not less than 90.0 per cent and not more than 110.0 per cent of the labeled amount of teriparatide.

Storage. Store in sterile airtight, tamper proof container at temperature of 2° to 8°. Do not freeze.

Labelling. The label states (1) the teriparatide content in µg per ml; (2) the name and the concentration of any other excipients; (3) indication that the material has been produced by methods based on recombinant DNA technology.

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ALLERGEN PRODUCTS

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Allergen Products

This monograph does not apply to: chemicals that are used solely for diagnosis of contact dermatitis; chemically synthesised products; allergens derived by recombinant DNA. It does not necessarily apply to allergen products for veterinary use.

Definition

Allergen products are pharmaceutical preparations derived from extracts of naturally occurring source materials containing allergens, which are substances that lead to and/ or provoke allergic reactions. The allergenic components are most often of a proteinaceous nature. Allergen products are intended for *in vivo* diagnosis or treatment of allergic diseases attributed to these allergens.

Allergen products are available as finished products and as finished products used as customized therapeutic preparations. Allergen products are generally presented as parenteral preparations, eye preparations, preparations for inhalation, preparations for oral use, sublingual preparations or preparations for skin tests.

For diagnostic use, allergen products are usually prepared as unmodified extracts in a 50 per cent V/V solution of glycerol for skin-prick testing. For intradermal diagnosis,or for provocation tests by nasal, ocular or bronchial administration, suitable dilutions of allergen products may be prepared by dilution of aqueous or glycerinated extracts, or by reconstitution of unmodified freeze-dried extracts.

For *specific immunotherapy*, allergen products are usually unmodified extracts.

Production

Source Materials

Source materials for the preparation of allergen products are products of animal or vegetable origin, mostly pollens, moulds, mites, animal epithelia and outgrowths (such as hair and feathers) and/or dander, hymenoptera venoms, insects.

The source materials are defined by their origin, nature, method of collection or production and pretreatment. Control methods and acceptance criteria relating to identity and purity are established. The acceptance criteria must ensure the consistency of the allergenic source material from qualitative and quantitative point of view. The source materials are stored under defined conditions justified by stability data.

The collection or production, as well as the handling of the source materials are such that uniform composition is ensured as far as possible from batch to batch.

Pollens. Potential chemical contaminants, such as pesticides, heavy metals, must be minimised. The content of foreign pollen must be limited to 1 per cent of total mixed pollens and 0.5 per cent of any individual pollen as determined by a microscopic particle count. Detectable mould spores must not exceed 1 per cent. The contamination with particles of plant origin other than pollen must be kept to a minimum. The maximum allowed contamination must be justified.

Moulds. Biologically active contaminants such as mycotoxins in moulds must be minimised and any presence justified. Appropriate measures, have to be implemented to avoid contamination by foreign mould strains. Care must be taken to minimize any allergenic constituents of the media used for the cultivation of moulds as source materials. Culture media that contain substances of human or animal origin must be justified and, when required, must be suitably treated to ensure the inactivation or elimination of possible transmissible agents of disease,

The production method is validated to demonstrate that allergen products obtained from moulds and intended for parenteral administration, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.2.1).

Mites. Appropriate measures have to be implemented to avoid contamination by foreign mite strains. Care must be taken to minimise any allergenic constituents of the media used for the cultivation of mites as source materials. Culture media that contain substances of human or animal origin must be justified and, when required, must be suitably treated to ensure the inactivation or elimination of possible transmissible agents of disease.

Animal epithelia and outgrowths and/or dander. They are obtained from healthy animals selected to avoid possible transmissible agents of disease.

Hymenoptera venoms. The species of hymenoptera from which the venom is extracted is identified and specified. The methods of insect collection and venom extraction are described and must ensure that the source material is of proper quality.

Allergen products are generally obtained by extraction, and may be purified, from the source materials using appropriate methods shown to preserve the allergenic properties of the components. Allergens for which there are not enough patients to determine the total allergenic activity in vivo or in vitro, the extraction ratio indicating the relative proportions (m/V) of allergenic source materials and solvents is a minimum requirement. Allergen products presented as parenteral preparations, eye preparations, preparations for inhalation and preparations for skin testing are manufactured under aseptic conditions.

In the manufacture, packaging, storage and distribution of allergen products intended for administration by other routes, suitable measures are taken to ensure their microbial quality;

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recommendations on this aspect are provided in chapter 2.2.9. Microbial contamination in non-sterile product.

All allergen preparations are manufactured under conditions designed to minimise exogenous and endogenous enzymatic degradation.

Any purification procedure is designed to minimise the content of any potential irritant low molecular mass components and non-allergenic components.

Allergen products may contain suitable antimicrobial preservative, the nature and concentration of which have to be justified.

The manufacturing process comprises various stages:

— source material;

— active substance: it is generally a unmodified allergen extract; where applicable it is stored under conditions ensuring its stability, for example freeze-dried;

— finished product;

— **In-House Reference Preparation**. An appropriate representative preparation is selected as the In-House Reference Preparation (IHRP), characterised and used to verify batch-to-batch consistency. The IHRP is stored in suitably sized aliquots under conditions ensuring its stability, for example freeze-dried.

— **Characterisation of the In-House Reference Preparation**.The extent of characterisation of the IHRP depends on the nature of the allergenic source material, knowledge of the allergenic components and availability of suitable reagents, as well as the intended use. The characterised IHRP is used as reference in the batch control of active allergen substances or intermediate and, if possible, in the batch control of finished products.

The In-House Reference Preparation (IHRP) is characterised by the protein content determination and a protein profile using appropriate methods (such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Allergenic components may be detected by appropriate methods (for example, immunoblotting or crossed radio- immunoelectrophoresis). Characterisation of the allergenic components may include identification of relevant allergens based on serological or other techniques using a pooled or individual sera from allergic patients, or allergen-specific polyclonal or monoclonal antibodies.

Determination of the content of relevant allergens is performed wherever possible. This determination may be made using individual allergen specific reference standards, when available. The choice of the relevant allergen components subjected to the determination must be justified. Individual allergens are identified and named according to internationally established nomenclature whenever possible.

The biological potency of the first IHRP is determined in patients by in-vivo techniques such as skin testing, and expressed in units of biological activity except when not enough patients are available. In this case, the potency of the first IHRP is determined by an in-vitro method. Subsequently, the biological activity of future IHRPs is demonstrated by in-vitro methods by comparison with the results obtained with the first IHRP. The in-vitro potency may be measured by a suitable immunoassay (for example, an essay based on the inhibition of the binding capacity of specific immunoglobulin E antibodies).

Identification

The tests for identification are performed as late as possible in the manufacturing process. In the case of products used on a named patient basis, the control is performed on the active substance and/ or at the intermediate stage between the active substance and the finished product.

Identity is confirmed by comparison with the IHRP using protein profiling by appropriate methods (for example, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (2.4.12).

In exceptional cases if no IHRP is available, a representative batch may be used to confirm identity.

Identity may also be confirmed by comparison with individual allergen specific reference standards, when available.

Tests

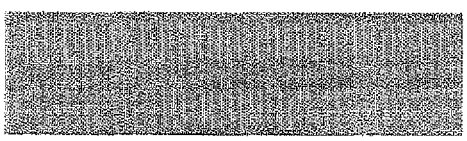
The tests are performed as late as possible in the manufacturing process. In the case of products used on a named patient basis, the control is performed on the active substance and/ or at the intermediate stage between the active substance and the finished product.

Water (2.3.43). Not more than 5 per cent for freeze-driedproducts.In the case of oral lyophilizates the water content may be higher than 5 per cent where, justified and authorised.

Sterility (2.2.11). Allergen products presented as parenteral preparations, eye preparations, preparations for inhalation or preparations for skin testing comply with the test for sterility.

Protein content. 80 per cent to 120 per cent of the statedcontent, unless otherwise justified and authorised.

Modified Lowry's procedure. The procedure is based on Peterson's modification of the Lowry method and utilizes sodium dodecyl sulphate, to facilitate the dissolution of relatively insoluble lipoproteins. For many proteins, the Lowry réaction can be run directly on the protein solution. However, interference in the direct Lowry procedure is commonly caused by other chemicals in the protein solution, such as tris, ammonium sulfate, EDTA, sucrose, citrate, amino acid and



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peptide buffers, and phenols. The procedure with protein precipitation, which uses DOC (deoxycholate) and TCA (trichloroacetic acid), eliminates all these interferences with the exception of phenols. However, the amount of various proteins recovered through the precipitation step may vary depending on the particular proteins assayed.

The procedure is based on two chemical reactions. The first is the biuret reaction, in which the alkaline cupric tartrate reagent complexes with the peptidé bonds of the protein resulting in formation of tetradentate Copper-protein complexes. This is followed by the reduction of the Folin and Ciocalteu's phenol reagent, producing a'water-soluble product which yields a purple color. Absorbance of the colored solution is read at 750 nm. The protein concentration is determined from a calibration curve.

Prepare all the following reagents/buffers for protein estimation.

Phosphate Buffered Saline . Dissolve 8.0 g of *sodiun chloride*, 0.2 g of *potassium chloride*, 0.2 g of *potassium dihydrogen phosphate*, 1.15 g of *disodium hydrogen phosphate*, 2.0 mg of *sodium azide* in 1000 ml of *water*, adjusted to pH 7.2.

Solution A. Dissolve 50 mg of *copper sulphate*, 100 mg of *potassium sodium tartrate* in 25 ml of *water*.

NOTE — Solution A prepares freshly for every use.

Solution B. Dissolve 20 g of *sodium carbonate* in 100 ml of *water*.

Solution C. Mix 25 ml of solution A and 25 ml of solution B to yield 50 ml of solution C.

10 per cent sodium dodecyl sulphate. Dissolve 10 g of *sodium dodecyl sulphate* in 100 ml of *water*.

0.8 N sodium hydroxide. Dissolve 3.2 g of *sodium hydroxide* in 100 ml of *water*.

Folin-ciocalieau (FC) reagent. Mix 10 ml of *folin- ciocalteau reagent* with 50 ml of *water*.

Alkaline copper sulphate solution. Mix 10 ml of solution C, 10 ml of *0.8 N sodium hydroxide*, 10 ml of 10 per cent *sodium dodecyl sulphate* and 10 ml of *water*.

Prepare the stock concentration of protein standard (Bovine Serum Albumin) containing 1 mg per 2 ml of the phosphate buffered saline and test products (1 mg per ml) in the phosphate buffer saline. Prepare different dilutions of bovine serum albumin standard (0 µg per ml as Blank) (duplicates), 10 µg per ml, 20 µg per ml, 30 µg per ml, 40 µg per ml and 60 µg per ml) make the final volume upto 1 ml using *water*. Add 0.1 ml of 0.15 per cent w/v solution of *sodium deoxycholate* to each test tube including blank, standards and test products (sample). Mix well. After 10 minutes, add 0.1 ml of 72 per cent w/v solution of *tri-cholro acetic acid* to each sample, mix immediately and incubate at room temperature for 10 minutes. Centrifuge the test tubes at 3000 rpm for 15 minutes. Discard the supernatant and dissolve the pellets in 1 ml of *water*. Add 1 ml of alkaline copper sulphate solution in each test tube. Mix them well and incubate at room temperature for 10 minutes. Add 0.5 ml of folin-ciocalteau reagent in each test tube, mix them well and incubate at room temperature for 30 minutes. Read in spectrophotometer at wavelength of 750 nm within 30 minutes. Estimate the amount of protein present in the given sample from the standard curve.

Bradford method. It is an alternate method for allergen extracts containing phenol. It is based on the absorption shift from 470 nm to 595 nm observed when the Coomassie brilliant blue G250 dye binds to protein. The Coomassie brilliant blue G250 dye binds most readily to arginine and lysine residues in the protein which can lead to variation in the response of the assay to different proteins. The protein used as reference substance must therefore be the same as the protein to be determined. There are relatively few interfering substances, but it is preferable to avoid detergents and ampholytes in the test sample. Highly alkaline samples may interfere with the acidic reagent.

Use *distilled water* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the standard curve.

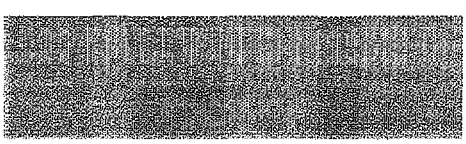
Reference solutions. Dissolve the reference substance for the protein to be determined in the prescribed buffer (e.g. phosphate buffer saline with 0.4 per cent *phenol*). Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 0.1 mg per ml and 1 mg per ml.

Blank. Use the buffer used to prepare the test solution and the reference solutions.

Bradford reagent. Dissolve 0.10 g of Coomassie brilliant blue G250 in 50 ml of ethanol. Add 100 ml of *phosphoric acid*, dilute to 1000 ml with *water* and mix. Filter the solution and store in an amber-bottle at room temperature. Slow precipitation of the dye occurs during storage. Filter the reagent before using.

Test tube procedure. Add 5 ml of bradford reagent to 0.100 ml of each reference solution, of the test solution and of the blank. Mix by inversion. Avoid foaming, which will lead to poor reproducibility. Determine the absorbances of the standard solutions and of the test solution at 595 nm, using the blank as compensation liquid.

NOTE — Do not use quartz (silica) spectrophotometer cells because the dye binds to this material.



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Microplate procedure. Add 0.25 ml of Bradford reagent to 0.010 ml of each reference solution in triplicates, of the test solution and of the blank to the designated wells in the microplate. Mix for 10 seconds on shaker at 500 rpm. Avoid foaming, which will lead to poor reproducibility. Incubate for 10 minutes at room temperature. Determine the absorbances of the standard solutions and of the test solution at 595 nm, using the blank as compensation liquid.

The assay method described here can also be performed using kits from commercial sources.

Calculations. The relationship of absorbance to protein concentration is non -linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

Protein profile. The band pattern in the protein profile of the Allergen product is to be determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (2.4.12). The protein profile determined by suitable methods corresponds to that of the IHRP.

Storage. Unless otherwise prescribed, store in a sterile, airtight, tamper-proof container, protected from light, at a temperature of 2° to 8°.

Labelling. The label states:

- Name of the allergen product;
- the protein content (µg/ml or mg/ml) and/or biological potency and/or the extraction concentration;
- the route of administration and the intended use;
- the storage conditions;
- where applicable, the name and amount of added antimicrobial preservative;
- where applicable, for freeze-dried preparations:
  - the name, composition and volume of the reconstituting liquid to be added
  - the period of time within which the preparation is to be used after reconstitution;
- where applicable, that the preparation is sterile;
- Caution: It is dangerous to take this prescription except under medical supervision of professional trained in Allergy and Immunotherapy.

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RADIOPHARMACEUTICAL PREPARATIONS

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Radiopharmaceutical Preparations

1. General Requirements

1.1 Introduction

Radiopharmaceuticals, as the name suggests, are pharmaceutical formulations consisting of radioactive substances (radioisotopes and molecules labeled with radioisotopes), which are intended for use either in diagnosis or therapy.

Radiopharmaceuticals are essential components of nuclear medicine practice. A modality where radiopharmaceuticals are administered to patients for diagnosing, managing and treating a large number of diseases.

In imaging, the unique properties of γ-rays emitted from the radioactive isotopes allow the radiopharmaceutical to be traced or imaged non-invasively, thus providing functional information of the target tissue or organ. In therapy, the β-ray energy from the radioisotope is delivered to the target tissue to partially or completely destroy the diseased tissues .

Radiopharmaceuticals are unlike conventional pharmaceuticals in many respects: the most striking feature is the property of the radionuclide, which disintegrates or decays with time, often resulting in a limited shelf life of the product. The physical half-life of the radionuclides used in radiopharmaceuticals are generally short, and hence the final preparation needs to be carried out before administration to the patient. Hence, the concept of "Hospital Radiopharmacy" unit to prepare radiopharmaceuticals has become a practice in Nuclear Medicine departments in hospitals. At the hospital radiopharmacy, a trained radiopharmacist prepares the various radiopharmaceutical formulations, tests each formulation for its quality (quality control). The formulations are then provided to nuclear medicine physician for administration into the patient for investigation or for therapy .

Radiopharmaceuticals are either ready to use (available from suppliers), or prepared in-house from 'cold kits' and radioisotopes from generators, or synthesised from radioisotopes and suitable precursors as with cyclotron produced radioisotopes. Some centres synthesize the necessary ligands at their hospital radiopharmacy and formulate the radiopharamceuticals. All are subjected to the required quality control tests, to ensure the formulations fulfill radiological, pharamceutical safety and efficacy in accordance with the specifications laid-down.

The use of radioactive material necessitates careful and safe handling of these products by trained and authorized personnel, in approved/authorized laboratory facility as per the guide lines of Atomic Energy Regulatory Board (AERB) in India.

1.2 Definitions and Terminology

Radiopharmaceutical refers to any medicinal or pharmaceutical product, which when ready for use contains one or more radionuclides (radioactive isotopes) intended for human use either for diagnosis or therapy.

Nuclide is an elemental species characterized by (a) its mass number 'A', (the sum of the number of protons and neutrons in its nucleus), (b) its atomic number 'Z' (number of protons which is also same as number of electrons in a neutral atom) and also by (c) its nuclear energy state.

Isotopes of an element are nuclides with the same atomic number 'Z' but different mass numbers 'A'. They occupy the same place in the periodic table and have similar chemical properties.

Radionuclide. Nuclides containing an unstable arrangement of protons and neutrons that transform spontaneously to either a stable or another unstable combination of protons and neutrons with a constant statistical probability by emission of radiation. These are said to be radioactive and are called radionuclides. The initial unstable nuclide is referred to as the 'parent radionuclide' and the nuclide after transformation as the 'daughter nuclide'. Such a transformation is also known as 'Radioactive transmutation' or 'radioactive disintegration' or 'radioactive decay'.

Radioactivity. The phenomenon of emission of radiation owing to the spontaneous transformation or disintegration of a radionuclide is known as 'Radioactivity'. However, the term radioactivity is also used to express the physical quantity (activity or strength) of this phenomenon. The radioactivity of a preparation is the number of nuclear disintegrations or transformations per unit time.

Units of Radioactivity. In the International System (SI), the unit of radioactivity is one nuclear transmutation per second and is expressed in Becquerel (Bq), named after the scientist Henri Bequerel. The old unit of radioactivity was Curie (Ci), named after the scientists Madame Marie Curie and Pierre Curie, the pioneers who studied the phenomenon of radioactivity. One Ci is the number of disintegrations emanating from 1 g of Radium-226, and is equal to 3.7×10^{10} Bq. Absolute radioactivity measurements require a specialized laboratory, but identification and measurement of radiation can be carried out relatively by comparing with standardized preparations provided by Reference Laboratories recognized by the International or National Authorities. (With all statements involving radioactivity, it is necessary to include a reference date of measurement in case of radionuclides with half-life less than 30 days. The time of standardization should be expressed to the nearest hour. For radionuclides with a half-life period of less than one day, a more precise statement of reference time should be given.)

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Half-Life Period. The time in which a given quantity of a radionuclide decays to half its initial value is termed as half-life ($T_{1/2}$).

Radionuclide generator. Any system or device incorporating a fixed parent radionuclide from which is produced a daughter radionuclide by elution or by any other method and used in radiopharmaceutical preparation. For e.g., the most widely used radionuclide generator in radiopharmacy is the $^{99}Mo \rightarrow {}^{99m}Tc$ generator.

Radionuclidic purity. The ratio, expressed as a percentage, of the radioactivity of the radionuclide of interest to the total radioactivity of the radioactive preparation is referred to as 'Radionuclidic Purity'. In the context of radiopharmaceuticals, 'Radionuclidic purity' is an important quality parameter and it is mandatory that the radionuclidic impurities to be within the stipulated limits. Such radionuclidic impurities, arise during the radionuclide production and are, hence, dependent on the production route. In the context of radiopharmaceuticals, the acceptable limits for the possible radionuclides are listed in the individual monographs.

Isotopic carrier is a stable isotope of the element either present or added to the radioactive isotope of the same element. Often, the radionuclides contain isotopic carriers and their content depends on the route/method followed for the production of the radionuclide.

Radiochemical purity. The ratio, expressed as a percentage, of the radioactivity of the radionuclide of interest in a stated chemical form, to the total radioactivity of that radionuclide present in the preparation, is referred to as 'Radiochemical Purity'. In the context of radiopharmaceuticals, radiochemical purity is an important quality parameter, which needs to be within the stipulated limits. The relevant radiochemical impurities are listed with their limits in the individual monographs for each Radiopharmaceutical.

Chemical purity. Chemical purity of a chemical substance is the percentage of the chemical of interest in the specified chemical form. In the monographs on radiopharmaceutical preparations, chemical purity of the active ingredient is indicated and controlled by specifying limits on chemical impurities.

Specific radioactivity. The radioactivity of a radionuclide per unit mass of the element or of the chemical form of the radioactive preparation is referred to as the 'Specific Radioactivity'; sometimes also referred as 'specific activity'.

Radioactive concentration. This refers to the radioactivity of a radionuclide per unit volume of the radioactive preparation.

Total radioactivity. The radioactivity of the radionuclide per unit of the dispensed formulation (vial, capsule, ampoule, generator, etc) is the total radioactivity, which is an important parameter in dispensing and administration of the radioactive material to the patient as well as from the regulatory requirement for safe handling of the radioactive materials in a facility.

Kit for radiopharmaceutical preparation. It is a set of non-radioactive reagents to be reconstituted and/or combined with radionuclides following the protocol suggested by the manufacturer for preparing the final radiopharmaceutical formulations, prior to its administration. Such kits are also often referred to as 'cold kits', as they are devoid of radioactivity.

Radiopharmaceutical precursor. It is a chemical compound or ligand used in the synthesis of the radiopharmaceutical preparation. It could either be an inactive chemical compound or a radiolabeled intermediate produced for the preparation of radiopharmaceutical formulation, prior to administration.

Period of validity or Shelf life of the Radiopharmaceutical. The time during which specifications described in the monograph are complied with, by the radiopharmaceutical denotes the shelf life of the radiopharmaceutical preparation. Any radiopharmaceutical preparations including the cold kits have limited shelf life, which needs to be clearly stated on the label as the expiry date and, if necessary, the time.

1.3 Phenomenon of Radioactive decay and the Radiations

The radioactive decay or transformation, as described earlier, involves transformation of the unstable radioactive nucleus to attain a more stable configuration. As the nucleus contains protons and neutrons, such transformations involve reactions of these sub-atomic particles. In a simplified manner, it could be stated that the stability of the nucleus predominantly depends on the total number of nucleons (protons and neutrons) as well the ratio of the protons (p) to the neutrons (n). While nearly all the isotopes beyond element Bismuth (Atomic number 83) are radioactive as the total number of nucleons become too large for stability, the lower atomic number elements have stable as well as radioactive isotopes, depending on the p/n ratio and certain other properties of the nuclide. Generally, a proton rich (or neutron deficient) nuclide would transform to reduce the proton content; while a neutron rich nuclide would transform vice versa. A very heavy radionuclide may attain stability by shedding some nucleons. Such transformations may involve the emission of charged particles, capture of electron from the extra-nuclear orbits by the nucleus, also known as electron capture (EC) or isomeric transition (IT). The charged particles emitted from the nucleus may be alpha (α) particles (helium nucleus of mass number 4) or beta (β) particles. Beta particles may be either, negatively charged β^-, also known as negatrons, generally equivalent to electrons or positively charged β^+, and also known as positrons. The emission of charged particles from the nucleus may be accompanied by the emission of gamma (γ) rays, which

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are energetic photons of electromagnetic radiation and do not have any charge or mass. Gamma rays are also emitted in a process called isomeric transition (IT), where an excited state of a radionuclide decays to the de-excited state by gamma-ray emission, with no changes in atomic, mass or neutron number. The emissions of gamma rays may be partly replaced by the ejection of electrons known as internal conversion (IC) electrons, due to the interaction of the gamma ray with the extranuclear orbital electrons. This phenomenon, like the process of electron capture, causes a secondary emission of X-rays (due to the vacancies created in the electronic orbits which are then filled by re-organization of the electrons from the outer orbits in the atom). This secondary emission of X-rays may itself be partly replaced by the ejection of outer electrons known as Auger electrons, due to the interaction of the X-rays with the outer electrons.

The decay of a radionuclide is governed by the laws of probability with a characteristic decay constant (λ) and follows an exponential law. The time in which a given quantity of a radionuclide decays to half its initial value is termed the half-life ($T_{1/2}$).

Each radionuclide is characterized by an invariable $T_{1/2}$, expressed in units of time and by the nature and energy of its radiation or radiations. The energy is expressed in electron volts (eV), kilo-electron volts (keV) or mega-electron volts (MeV).

The penetrating power of each radiation varies considerably according to its nature and its energy. Alpha particles, which are the heaviest among the radiations, have the minimum penetration, followed by the beta particles and gamma rays have the most penetrating power. Alpha particles can be stopped within a thickness of a few micrometers to few tens of micrometers of matter, while beta particles require several millimeters to several centimeters of matter for complete attenuation. Gamma rays, on the other hand are the most penetrating and are attenuated in an exponential manner in matter. High density materials such as lead are used to stop the gamma rays and a tenfold reduction of energetic gamma rays may require several centimeters of lead. The denser the material used, the higher the attenuation of radiations.

Modes of Radioactive Decay

Alpha decay (α). Radioactive nuclei having too many nucleons (n and p) often undergo alpha decay, in order to achieve nuclear stability. Alpha particle has a mass of 4 units and a charge of +2 units, and is therefore, equivalent to helium+2 ion. Alpha particles from radionuclides have energy ranging from 1.8 to 11.7 MeV. But, artificially, rays (He ions) can be accelerated to energies reaching several Giga-electron volts (GeV).

Negatron decay (β^-). Radioactive nuclei having neutrons in excess than what is needed for a stable configuration, mostly undergo negatron or β^- decay, in order to achieve nuclear stability. Negatrons have the same mass and electrical charge of orbital electrons, but they originate from nucleus at the very instant of decay, when a neutron transforms to a proton. Such a transformation results in increase in atomic number by 1, while the mass does not change significantly. The β^- decay phenomenon could be expressed as the following nuclear reaction:

$$n \rightarrow p + \beta^- + \upsilon^*$$

υ^* represents 'anti-neutrino' a sub-atomic entity which does not have any mass or charge, but can posses energy. The β^- decay equation has to be balanced with respect to mass, charge, energy, momentum as well as spin. The υ^* is important for accounting for the conservation of momentum, spin and energy. Thus, unlike particles which are emitted with a single energy from a nuclide, β^- particles from a certain radionuclide could have varied energies, accompanied by the υ^* carrying complementary amount of energy, with the total energy being same. β^- particles have energies in the range from a few KeV to 14 MeV. For a given transition, negatrons have a continuum spectrum of energies.

An example of beta decay is

$$^{32}P \rightarrow {}^{32}S + \beta^- + \upsilon^*$$

Positron decay (β^+). Radioactive nuclei having neutrons lesser than what is needed for a stable configuration undergo positron β^+ decay in order to achieve stability, if adequate energy is available from the nucleus for transformation of a proton to a neutron. Such a transformation results in decrease in atomic number by 1, while the mass does not change significantly. The β^+ decay phenomenon could be expressed as the following nuclear reaction:

$$p \rightarrow n + \beta^+ + \upsilon$$

As in the case of β^- decay, in order to conserve momentum, spin and energy, a sub-atomic entity which does not have any mass or charge known as 'neutrino' represented by υ is also emitted, which carries some energy with it. Thus, like β^- particles, β^+ particles also have varying energies. However, unlike β^- particles, in the case of β^+ particle emission, the proton which is lighter is transformed to the heavier particle neutron, along with a positron, resulting in the generation of mass equivalent to 2 electrons (1 positron and another the difference between a neutron and a proton). This cannot be possible, unless energy is available for conversion into the mass equivalent to 2 electrons, which is 1.02 MeV. Hence, unlike β^- decay, β^+ decay can occur only when at least 1.02 MeV of energy is available. During transmutation, due to the changes in nuclear energy levels, certain nuclides have

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energy more than 1.03 MeV, in which case, β^+ decay can occur. The energy in excess of 1.02 MeV is shared by the β^+ and υ. An example of β^+ decay is

$$^{32}F \rightarrow {}^{32}O + \beta^+ + \upsilon$$

While the β^- particles or negatrons, which are equivalent to electrons are found all around in matter as these are constituents of atoms, β^+ particles or positrons are not naturally present in matter. These are 'anti-matter' particles, which when meet with the corresponding 'matter' will annihilate each other, resulting in conversion of matter into energy as per Einstein's mass-energy equation ($E=mc^2$). In the case of β^+ particles, once they come in contact with the electrons, they will be annihilated. When a positron is emitted, initially it spends all its energy as it travels through matter, comes across an electron, and both undergo annihilation, resulting in two photons of 511 KeV each traveling in opposite directions. It is noteworthy that for β^+ emission energy of 1.02 MeV (2 times 511 keV) is necessary, which later appears as 2 photons of 511 keV each. In order to have conservation of mass, energy and momentum, the 2 photons are emitted in exactly opposite directions.

It may be noted that "Positron Emission Tomography", a nuclear medicine imaging technique employing radiopharmaceuticals labeled with positron emitting radionuclide(s), is a highly sensitive imaging technique based on the coincidence counting of the 2 photons emitted at 180°.

Electron Capture (EC). Radioactive nuclei having neutrons lesser than what is needed for a stable configuration and which do not have adequate energy available to undergo positron β^+ decay, decay by another route named "Electron Capture', in order to achieve stability. In this mode of decay, an orbital electron is captured and taken into the nucleus, thus facilitating conversion of a proton to a neutron, resulting in a nuclide with decrease in atomic number by 1. An electron capture reaction can be written as

$$^{+}p + e^{-} \rightarrow n + \upsilon$$

Since there are several orbital electrons (except in the case of elements with very low Z), EC process is a statistical phenomenon, where varied probabilities for EC arise for the K-shell (inner most shell) electrons, L-shell electrons and so on. EC phenomenon results in the depletion of electron in one of the inner shells of the atom, which in turn is a vacancy that is filled by one of the outer shell electrons accompanied by emission of characteristic X rays. Often, EC mode of decay is accompanied by γ rays and characteristic X rays as well as Auger electrons that arise due to the interaction of the γ rays and X rays with the outer orbit electrons.

An example of EC is

$$^{125}I \rightarrow {}^{125}Te + 35\,KeV\ \gamma\ ray\,(7\ per\ cent) + 27 - 32\,KeV\ Te\ X\text{-}rays\,(136\ per\ cent) + \sim 19\ Auger\ electrons$$

While positron emission can occur only if, at least 1.02 MeV of energy is available from the decay reaction. EC does not need such energy and both modes of decay result in nuclides with atomic number lower by one. However, when energy is available for β^+ emission, EC may also occur in some cases, while the vice-versa is not possible. One example is of ^{64}Cu, which decays by β^- emission, β^+ emission as well as EC. It is noteworthy that several factors that influence nuclear stability are responsible in determining the modes of decay and their probabilities.

Gamma decay (γ). Gamma rays are electromagnetic rays coming out of nucleus as a result of the difference in nuclear energy levels of the excited and the ground states of the daughter nuclide when a nuclear transmutation takes place. Most radioactive decays are accompanied by γ rays, although it is not essential. Since γ rays carry the energy arising out of the difference in nuclear energy levels, these are often highly energetic, with energy greater than those of X-rays.

Isomeric Transition (IT). When an excited nucleus de-excites by emission of a delayed gamma ray, the daughter nucleus is a nuclear isomer of the parent and the process is called isomeric transition.

As mentioned earlier, gamma rays are emitted owing to the energy difference in the nuclear states of the excited and the ground states of the daughter nuclide after a transmutation or decay. Such γ ray emissions are very quick and happen within nanoseconds. However, if the de-excitation of the daughter nuclide from the higher state to ground state does not occur easily (due to rules that govern such transitions – nuclear physics), then such transitions become slow and the excited state of the nuclide is referred to as 'metastable' state, indicated by the symbol 'm' after the atomic number (e.g. ^{99m}Tc). Nuclear isomers have same number of protons and same number of neutrons, only that they are arranged in a more stable configuration in the daughter nucleus.

1.4 Radiation exposure and the units of radiation dose

Exposure

Sum of all electrical charges of one sign produced by photons in a given mass of air. The unit is the Roentgen (R) which is equal to 2.58×10^{-4} Coulomb of electric charge produced in 1 kg of dry air at standard temperature and pressure (STP). This definition applies to X-rays and γ rays under 3 MeV of energy. The intensity of gamma radiation field is measured in terms of exposure rate at some distance from the source and is expressed as Roentgen per hour (R/h).

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a) Acute Exposure: A high dose of radiation is delivered within a short time. This type of exposure results in nonstochastic effects, which means that the severity of the effect increases with the dose given.

b) Chronic Exposure: A low dose of radiation is delivered over a long time. Chronic exposure results in stochastic effects, which means that the probability of observing the effect increases with the dose given. Back ground radiation and occupational exposure of radiation workers are examples of chronic exposure. To prevent unnecessary low-level exposures of radiation workers, the principle of As Low As Reasonable Achievable (ALARA) must be practiced at all times.

Roentgen (R), a unit of exposure of X- or γ-radiation equal to 2.58×10^{-4} coulomb per Kg in air is superseded by the SI unit of exposure, the coulomb per Kg ($C\,Kg^{-1}$). $1\,C\,Kg^{-1} = 3.876 \times 10^{3}$ R.

Absorbed Dose

Energy transferred to and absorbed by a unit mass of a material. The special unit is the radiation absorbed dose (rad), which is equal to 0.01 Joule (J) of energy absorbed per Kg (10^{-2} J per Kg) of any material. In the MKS system, *1J = force of one Newton acting over a distance of 1 m*, and *1N = force which gives a mass of 1 Kg an acceleration of 1m per s each second.* The SI unit of absorbed dose is Gray defined as 1 J per Kg and supersedes the rad as a unit of absorbed dose. *1 Gray = 100 rads.*

The Roentgen and the rad in soft tissue are approximately equivalent in magnitude for the moderate energies.

Critical Organ. The organ that is functionally essential for the body and receives the highest radiation dose after administration of radiopharmaceutical.

Quality Factor (QF). The relative effectiveness of the radiation in producing biological response.

Dose Equivalent (DE)

Absorbed dose multiplied by Quality Factor (QF). Quality factor are values, based on linear energy transfer that permit estimates of radiation energy caused by various types of radiation. They are based on degree of ionization produced in water. Radiation that produces 100 ion pairs in 1 micron of water, spends 3.5 KeV of energy per micron receives a Quality factor = 1. Those that produce 100 – 200 ion pairs gets Quality factor = 2 and so on.

The unit of DE is Rontgen-equivalent-man (rem). A rem is numerically equal to the absorbed dose in rads multiplied by the appropriate Quality factor defining the biological effect and by any other modifying factors. The SI unit of DE is Sievert (Sv) and supersedes rem as the unit of dose equivalent. The units for Sievert is joule per kg ($J\,Kg^{-1}$) equal to 100 rem. Sievert (Sv), is numerically equal to the absorbed dose in Gray multiplied by the appropriate Quality factor defining the biological effect and by any other modifying factors expressed in J per Kg.

Annual Limit of Intake (ALI)

In order to simplify the comparison of the committed effective doses from intakes with equivalent dose limits, it is convenient to define the secondary dose limit called the ALI. It normally corresponds to a committed effective dose from an intake of a given radionuclide equal to the appropriate equivalent dose limits for workers. Restrictions of intake in each year to less than ALI, therefore, ensures that the maximum annual equivalent dose from that radionuclide will always be less than the equivalent dose even if intake occurred every year for 50 years.

1.5 Production of Radionuclides

A Radiopharmaceutical preparation monograph describes as precisely as possible, the method of production of the radionuclide. A radiopharmaceutical preparation contains its radionuclide as an element in atomic or molecular form, e.g. [^{133}Xe], [^{15}O]O_2; as an ion, e.g. [^{131}I]iodide, [^{99m}Tc]pertechnetate; included in or attached to organic molecules by chelation, e.g. [^{111}In]oxine or by covalent bonding, e.g. 2-[^{18}F]fluoro-2-deoxy-D-glucose.

The practical ways of producing radionuclides for use in, or as radiopharmaceutical preparations are by (a) neutron bombardment of target materials (generally in nuclear reactors); (b) charged particles bombardment of target materials (in accelerators such as cyclotrons); (c) nuclear fission of heavy nuclides of target materials (generally after neutron or particle bombardment); and (d) from a radionuclide generator.

Neutron or Charged particle bombardment

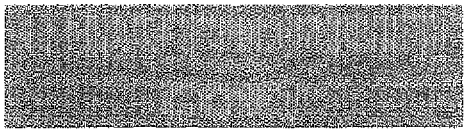
The nuclear reaction and the probability of its occurrence in unit time are dependent on the nature and physical properties of the target material and the nature, energy and quantity of the incident particles.

The nuclear transformation occurring through particle bombardment may be written in the form:

target nucleus (bombarding particle, emitted particle or radiation) produced nucleus

Examples: $^{58}Fe\,(n,\gamma)^{59}Fe$; $^{18}O(p,n)^{18}F$

In addition to the desired nuclear reaction, adventitious transformations may occur. These will be influenced by the energy of the incident particle and the purity of the target material. Such adventitious transformations may give rise to radionuclidic impurities.



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Nuclear fission

A small number of nuclides with a high atomic number are fissionable and the most frequently used reaction is the fission of uranium-235 by neutrons in a nuclear reactor. Iodine-131, molybdenum-99 and xenon-133 may be produced by nuclear fission of uranium-235. Their extraction of the desired radioisotope from a mixture of more than 200 other radionuclides must be carefully controlled in order to minimize the radionuclide impurities, which have to be within permissible levels.

Radionuclide generators

Radionuclide generator systems use a relatively long-lived parent radionuclide which decays to a daughter radionuclide, usually with a shorter half-life. The parent radionuclide and the daughter radionuclide exist in transient or secular equilibria depending on the ratio of the $T_{1/2}$ of parent and daughter radionuclides. The daughter radionuclide is separated from the parent radionuclide using a chemical or physical process. It is possible to use the daughter at a considerable distance from the production site of the generators despite its short half-life. The duration for which the generator can be used will depend on the $T_{1/2}$ of the parent radionuclide.

Target materials

The isotopic composition and purity of the target material determines the relative percentages of the principal radionuclide and radionuclidic impurities. The use of isotopically enriched target material in which the abundance of the required target nuclide has been artificially increased, can improve the production yield and the purity of the desired radionuclide.

The chemical form, the purity, the physical state and the chemical additives, as well as the bombardment conditions and the direct physical and chemical environment will determine the chemical state and chemical purity of the radionuclides which are produced.

In the production of radionuclides and particularly of short-lived radionuclides, it may not be possible to determine any of these quality criteria before further processing and manufacture of radiopharmaceutical preparations. Therefore, each batch of target material must be tested in test production runs before its use in routine radionuclide production and manufacture of the radiopharmaceutical preparations, to ensure that under specified conditions, the target yields the radionuclide in the desired quantity and quality specified.

The target material is contained in a holder in gaseous, liquid or solid state, in order to be irradiated by a beam of particles. For neutron bombardment, the target material is commonly contained in quartz ampoules or high purity aluminum or titanium containers. It is necessary to ascertain that no interaction can occur between the container and its contents under the irradiation conditions (temperature, pressure, time).

For charged particle bombardment, the holder for target material is usually built of aluminum or another appropriate metal, with a low cross section for the irradiating particles and also having a good thermal conductivity to remove the heat generated. The target will have inlet and outlet ports, a surrounding cooling system and usually a thin metal foil target window. The nature and thickness of the target window have a particular influence on the yield of the nuclear reaction and may also affect the radionuclidic purity.

The production procedure shall clearly describe the target material; construction of the holder for target material; loading of target material into the irradiation system; method of irradiation (bombardment); separation of the desired radionuclide, and evaluates all effects on the efficiency of the production in terms of quality and quantity of the produced radionuclide.

The chemical state of the isolated radionuclide may play a major role in all further processing.

Precursors for synthesis

Generally, these precursors are not produced on a large scale. Some precursors are synthesized by the radiopharmaceutical production laboratory; others are supplied by specialized producers or laboratories.

Tests for identity, for chemical purity and assay must be performed by validated procedures.

When batches of precursors are accepted based on the data from the certificates of analysis, suitable evidence has to be established to demonstrate the consistent reliability of the analyses by suppliers and at least one identity test must be conducted. It is recommended to test precursor materials in production runs before their use for the manufacture of radiopharmaceutical preparations, to ensure that under specified production conditions, the precursor yields the radiopharmaceutical preparation in the desired quantity and quality specified.

Performance of the production system

All operations, from the preparation of the target to the dispensing of the final radiopharmaceutical preparation, must be clearly documented including their impact on the purity of the final product and the efficiency of the procedure. Where possible, in-process controls are performed and the results recorded at each production step to identify at which level a possible discrepancy from the normal production procedure may have occurred.

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a) The production of radiopharmaceutical preparations may make use of mechanical and automated processes that are used in the pharmaceutical industry, subject to adapting these for use with radioactive material and to the requirements of radioprotection.

b) For radiopharmaceutical preparations containing short-lived radionuclides, such as certain positron emitters, remotely controlled production and automated radiosynthesis are generally used. For radionuclides with a very short half-life (less than 20 min), the control of the performance of the production system is an important measure to assure the quality of the radiopharmaceutical preparation before its release.

c) Any production procedure must be validated in test/trial production runs before its use in routine manufacture of radiopharmaceutical preparations, to ensure that under specified production conditions, the production system yields the radiopharmaceutical preparation in the desired quantity and specified quality.

d) The preparation of the dosage form of the final radiopharmaceutical preparation in the practice of nuclear medicine generally involves limited radioactivity starting from ready-to-use radiopharmaceutical preparations, generators, kits and radioactive precursors. All conditions which may affect the quality of the product (e.g. radiochemical purity, sterility etc) must be clearly defined and must include appropriate measures for radiation protection.

1.6 Identification

Radioactive decay

Radioactivity decays at an exponential rate with a particular decay constant and is a characteristic of each radionuclide.

The exponential decay (decay curve) is described by the equation:

$$A_t = A_o e^{-\lambda t}$$

Where, A_t = the radioactivity at time t,
A_o = the radioactivity at time t = 0,
λ = the decay constant characteristic of each radionuclide,
e = the base of Napierian logarithms.

The half-life ($T_{1/2}$) is related to the decay constant (λ) by the equation:

$$T_{1/2} = 0.693/\lambda$$

The radionuclide is generally identified by its half-life or by the nature and energy of its radiation or radiations emitted or by both, as prescribed in the monograph.

Measurement of $T_{1/2}$

The $T_{1/2}$ is measured with a suitable radiation detector such as an ionization chamber, a Geiger-Müller counter, a scintillation counter (solid crystal or liquid) or a semiconductor detector. The radiopharmaceutical preparation to be tested is used as such or diluted or dried in a capsule after appropriate dilution. The radioactivity chosen, having regard to experimental conditions, must be of a sufficiently high level to allow detection during several estimated $T_{1/2}$, but not too high to minimize count rate losses, for example due to dead time.

The radioactive source is prepared in a manner that will avoid loss of material during handling. If it is a liquid (solution), it is contained in bottles or sealed tubes. If it is a solid (residue from drying in a capsule), it is protected by a cover consisting of a sheet of adhesive cellulose acetate or of some other material.

The same source is measured in the same geometry and at intervals usually corresponding to half of the estimated half-life throughout a time equal to about three half-lives. The performance of the apparatus is checked using a source of long $T_{1/2}$ and, if necessary, corrections for any changes in the count rate have to be applied (see Measurement of Radioactivity).

A graph can be drawn with time as the abscissa and the logarithm of the relative instrument reading (e.g. count rate) as the ordinate. The calculated $T_{1/2}$ should not differ by more than 5 per cent from the expected $T_{1/2}$, unless otherwise stated in the Pharmacopoeia.

Determination of the nature and energy of the radiation

The nature and energy of the radiation emitted may be determined by several procedures including the construction of an attenuation curve and the use of spectrometry. The attenuation curve can be used for analysis of β radiation. Spectrometry is mostly used for identification of γ rays and detectable X-rays.

The attenuation curve is drawn for pure electron emitters when no spectrometer for beta rays is available or for beta/gamma emitters when no spectrometer for gamma rays is available. This method of estimating the maximum energy of beta radiation gives only an approximate value. The source, suitably mounted to at fixed geometry, is placed in front of the thin window of a Geiger-Muller counter or a proportional counter. The source is protected as described above. The count rate of the source is then measured. Between the source and the counter are placed, in succession, at least six aluminum screens of increasing mass per unit area. Within such limits that with a pure beta emitter this count rate is not affected by the addition of further screens. The screens are inserted in such a manner that constant geometrical conditions are maintained. A graph

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is drawn showing, the mass per unit area of the screen expressed in milligrams per square centimeter as the abscissa and, the logarithm of the count rate as the ordinate for each screen examined. A graph is drawn in the same manner for a standardized preparation. The mass attenuation coefficients are calculated from the median parts of the curves, which are practically rectilinear.

The mass attenuation coefficient μ_m, expressed in square centimeters per milligram, depends on the energy spectrum of the beta radiation and on the nature and the physical properties of the screen. It therefore allows beta emitters to be identified. It is calculated using the equation:

$$\mu_m = \frac{2.303 (\log A_1 - \log A_2)}{m_1 + m_2}$$

Where, m_1 = mass per unit area of the lightest screen,

m_2 = mass per unit area of the heaviest screen, m_1 and m_2 being within the rectilinear part of the curve,

A_1 = count rate for mass per unit area m_1,

A_2 = count rate for mass per unit area m_2.

The mass attenuation coefficient μ_m, thus calculated, does not differ by more than 10 per cent from the coefficient obtained under identical conditions using a standardized preparation of the same radionuclide.

The range of β^- particles is an additional parameter which can be used for the determination of the β^- energy. It is obtained from the graph described above as the mass per unit area corresponding to the intersection of the extrapolations of the descending rectilinear part of the attenuation curve and the horizontal line of background radioactivity.

Liquid scintillation counting may be used to obtain spectra of α and β^- emitters (see measurement of radioactivity).

Gamma spectrometry is used to identify radionuclides by the energy and intensity of their γ rays and X-rays.

The preferred detector for γ and X-ray spectrometry is a germanium semiconductor detector. A thallium-activated sodium iodide scintillation detector is also used but this has a much lower energy resolution.

The gamma detector has to be calibrated using standard sources because the detection efficiency is a function of the energy of the γ and X-rays as well as the form of the source and the source-to-detector distance. The detection efficiency may be measured using a calibrated source of the radionuclide to be measured or, for more general work, a graph of efficiency against γ and X-ray energy may be constructed from a series of calibrated sources of various radionuclides.

The γ and X-ray spectrum of a radionuclide which emits γ and X-rays is unique to that nuclide and is characterized by the energies and the number of photons of particular energies emitted per transformation from one energy level to another energy level. This property contributes to the identification of radionuclides present in a source and to their quantification. It allows the estimation of the degree of radionuclidic impurity by detecting peaks other than those expected.

It is possible to establish the rate of the decay of radioactivity using γ -spectrometry since the peaks diminish in amplitude as a function of the $T_{1/2}$. If, in such a source, a radioactive impurity with a different $T_{1/2}$ is present, it is possible to detect the latter by identification of the characteristic peak or peaks whose amplitudes decrease at a different rate from that expected for the particular radionuclide. A determination of the $T_{1/2}$ of the additional peaks by repeated measurements of the sample will help to identify the impurity.

The Table of physical characteristics of radionuclides summarizes the most commonly accepted physical characteristics of radionuclides used in radiopharmaceutical preparations. The Table also states the physical characteristics of the main potential impurities of the radionuclides.

By "transition probability" is meant the probability of the transformation of a nucleus in a given energy state, via the transition concerned. Instead of "probability" the terms "intensity" and "abundance" are frequently used.

By "emission probability" is meant the probability of an atom of a radionuclide giving rise to the emission of the particles or radiation concerned.

Irrespective of whether the one or the other meaning is intended, probability is usually measured in terms of 100 disintegrations.

1.7 Measurement of radioactivity

The radioactivity of a radiopharmaceutical preparation is stated at a given date and, if necessary, time. The absolute measurement of the radioactivity of a given sample may be carried out if the decay scheme of the radionuclide is known, but in practice many corrections are required to obtain accurate results. For this reason, it is common to carry out the measurement with the aid of a primary standard source. Primary standards may not be available for short-lived radionuclides e.g. β^+ emitters. Measuring instruments are calibrated using suitable standards for the particular radionuclides. Standards are available from the laboratories recognized by the competent authority. Ionization chambers and Geiger-Müller counters may be used to measure β^- and β^-/γ emitters; scintillation or semiconductor counters or ionization chambers may be used for measuring gamma emitters; low-energy β^- emitters require a liquid-scintillation counter. For the detection and measurement of α emitters, specialized equipment and techniques are required. For an accurate comparison of

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radioactive sources, it is essential for samples and standards to be measured under similar conditions.

Low-energy β⁻ emitters may be measured by liquid-scintillation counting. The sample is dissolved in a solution containing one or more often two organic fluorescent substances (primary and secondary scintillators), which convert part of the energy of disintegration into photons of light, which are detected by a photomultiplier and converted into electrical impulses. When using a liquid-scintillation counter, comparative measurements are corrected for light-quenching effects. Direct measurements are made, wherever possible, under similar conditions, (e.g. volumes and type of solutions) for the source to be examined and for the standard source.

All measurements of radioactivity must be corrected by subtracting the background due to radioactivity in the environment and due to spurious signals generated in the equipment itself.

With some equipment, when measurements are made at high levels of radioactivity, it may be necessary to correct for the loss by coincidence due to the finite resolving time of the detector and its associated electronic equipment. For a counting system with a fixed dead time τ following each count, the correction is:

$$N = \frac{N_{obs}}{1 - N_{obs} \times \tau}$$

Where, N = the true count rate per second,
N_{obs} = the observed count rate per second,
τ = the dead time, in seconds.

With some equipment this correction is made automatically. Corrections for loss by coincidence must be made before the correction for background radiation.

If the time of an individual measurement, t_m is not negligible short compared with the half-life, $t_{1/2}$, the decay during this measurement time must be taken into account. After having corrected the instrument reading (count rate, ionization current, etc.) for background and, if necessary, for losses due to electronic effects, the decay correction during measurement time is:

$$R_{corr} = \frac{R \frac{t_m \ln 2}{T_{1/2}}}{1 - \exp\left(\frac{-t_m \ln 2}{T_{1/2}}\right)}$$

Where, R_{corr} = instrument reading corrected to the beginning of the individual measurement,
R = instrument reading before decay correction, but already corrected for background, etc.

The results of radioactivity determination show variation, which mainly are derived from the random nature of nuclear transformation. A sufficient number of counts must be registered in order to compensate for variations in the number of transformations per unit of time. The standard deviation is the square root of the counts, so at least 10000 counts are necessary to obtain a relative standard deviation of not more than 1 per cent (confidence interval: 1 sigma).

All statements of radioactive content should be accompanied by a statement of the date and, if necessary, the time at which the measurement was made. This statement of the radioactive content must be made with reference to a time zone (GMT, CET). The radioactivity at other times can be calculated from the exponential equation or from tables.

The radioactivity of a solution is expressed per unit volume to indicate the radioactive concentration.

Radionuclidic purity

In most of the cases, to state the radionuclidic purity of a radiopharmaceutical preparation, the identity of every radionuclide present and their radioactivity must be known. The most generally useful method for the examination of radionuclidic purity is by gamma spectrometry. It is not a completely reliable method because alpha- and beta-emitting impurities are not usually easily detectable and, when sodium iodide detectors are employed, the peaks due to gamma emitting impurities are often obscured by the gamma spectrum of the principal radionuclide.

The individual monographs prescribe the radionuclidic purity required (for example, the γ-ray spectrum does not significantly differ from that of a standardized preparation) and may set limits for specific radionuclidic impurities (for example, cobalt-60 in cobalt-57). While these requirements are necessary, they are not in themselves sufficient to ensure that the radionuclidic purity of a preparation is sufficient for human use. The manufacturer must examine the product in detail and especially, must examine preparations of radionuclides of short half-life for impurities of long half-life after a suitable period of decay. In this way, information on the suitability of the manufacturing processes and the adequacy of the testing procedures may be obtained. In cases where two or more β⁺ emitting radionuclides need to be identified and/or differentiated, as e.g. ^{18}F-impurities in ^{13}N-preparations, half-life determinations need to be carried out in addition to gamma spectrometry.

Due to differences in the half-lives of the different radionuclides present in a radiopharmaceutical preparation, the radionuclidic purity changes with time. The requirement of the radionuclidic purity must be fulfilled throughout the period of validity. It is sometimes difficult to carry out these tests before authorizing the release for use of the batch when

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the half-life of the radionuclide in the preparation is short. The test then constitutes a control of the quality of production.

Radiochemical purity

The determination of radiochemical purity requires separation of the different chemical substances containing the radionuclide and estimating the percentage of radioactivity associated with the declared chemical substance. Radiochemical impurities may originate from radionuclide production; subsequent chemical processing; incomplete preparative separation and chemical changes during storage.

The requirement of the radiochemical purity must be fulfilled throughout the period of validity.

In principle, any method of analytical separation may be used in the determination of radiochemical purity. For example, the monographs for radiopharmaceutical products may include paper chromatography (2.4.15), thin-layer chromatography (2.4.17), instant thin-layer chromatography (ITLC), electrophoresis (2.4.12), size-exclusion chromatography (2.4.16), gas chromatography (2.4.13) and liquid chromatography (2.4.14). The technical description of these analytical methods is set out in the monographs. Moreover, certain precautions special to radioactivity must also be taken for radiation protection.

Instant thin-layer chromatography (ITLC) is a rapid, miniaturized thin layer assay method developed to determine the labeling efficiency of radiopharmaceuticals. The assay uses specific cellulose backed silica gel chromatography strips as solid phase. ILTC method offers advantages such as easy to use, rapid and can be incorporated easily in a routine quality control program.

In a hospital environment, thin-layer and paper chromatography are mostly used. In paper and thin-layer chromatography, a volume equal to that described in the monograph is deposited on the starting-line as prescribed in the general methods for chromatography. It is preferable not to dilute the preparation to be examined, but it is important to avoid depositing such a quantity of radioactivity that counting losses by coincidence occur during measurement of the radioactivity. On account of the very small quantities of the radioactive material applied, a carrier may be added when specified in a particular monograph. After development of the chromatogram, the support is dried and the positions of the radioactive areas are detected by autoradiography or by measurement of radioactivity over the length of the chromatogram using suitable collimated counters or by cutting the strips and counting each portion. The positions of the spots or areas permit chemical identification by comparison with solutions of the same chemical substances (non-radioactive) using a suitable detection method.

Radioactivity may be measured by integration using an automatic-plotting instrument or a digital counter. The ratios of the areas under the peaks give the ratios of the radioactive concentration of the chemical substances. When the strips are cut into portions, the ratios of the quantities of radioactivity measured give the ratio of concentrations of the radioactive chemical species.

Specific radioactivity

Specific radioactivity is usually calculated taking into account the radioactive concentration (radioactivity per unit volume) and the concentration of the chemical substance being studied, after verification that the radioactivity is attributable only to the radionuclide (radionuclidic purity) and the chemical species (radiochemical purity) concerned.

Specific radioactivity changes with time. The statement of the specific radioactivity therefore includes reference to a date and if necessary, time. The requirement of the specific radioactivity must be fulfilled throughout the period of validity.

Chemical purity

The determination of chemical purity requires quantification of the individual chemical impurities specified in the monograph.

Enantiomeric purity

Where appropriate, the stereoisomeric purity has to be verified.

1.8 Physiological distribution

A physiological distribution test is prescribed, if necessary, for certain radiopharmaceutical preparations. The distribution pattern of radioactivity observed in specified organs, tissues or other body compartments of an appropriate animal species (usually rats or mice) can be a reliable indication of the expected distribution in humans and thus of the suitability for the intended purpose.

The individual monograph prescribes the details concerning the performance of the test and the physiological distribution requirements which must be met for the radiopharmaceutical preparation. A physiological distribution conforming to the requirements will assure appropriate distribution of the radioactive compounds to the intended biological target in humans and limits its distribution to non-target areas.

In general, the test is performed as follows.

A minimum of three animals are used per test and each animal is injected intravenously with the preparation to be tested. If relevant, the species, sex, strain and weight and/or age of the animals is specified in the monograph. The test injection is the radiopharmaceutical preparation as it is intended for human use. Where applicable, products are reconstituted according

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to the manufacturer's instructions. In some cases, dilution immediately before injection may be necessary.

The administration will normally be made via the intravenous route for which purpose the caudal vein is used. Other veins such as saphenous, femoral, jugular or penile veins may be used in special cases. Animals showing evidence of extravasation of the injection (observed at the time of injection or revealed by subsequent assay of tissue radioactivity) are rejected from the test.

Immediately after injection each animal is placed in a separate cage which will allow collection of excreta and prevent contamination of the body surface of the animal.

At the specified time after injection, the animals are euthanised by an appropriate method and dissected. Selected organs and tissues are assayed for their radioactivity using a suitable instrument as described elsewhere in this monograph. The physiological distribution is then calculated and expressed in terms of percentage of the radioactivity which is found in each of the selected organs or tissues. For this purpose, the radioactivity in an organ may be related to the injected radioactivity calculated from the radioactive content of the syringe measured before and after injection. For some radiopharmaceutical preparations, it may be appropriate to determine the ratio of the radioactivity in weighed samples of selected tissues (radioactivity/mass).

For a preparation to meet the requirements of the test, the distribution of radioactivity in at least two of the three animals must comply with all the specified criteria. The preparation may be released for use before completion of the test.

1.9 Sterility

Radiopharmaceutical preparations for parenteral administration must be prepared using precautions designed to exclude microbial contamination and to ensure sterility. The test for sterility is carried out as described in the general method for sterility (2.2.11). Special difficulties arise with radiopharmaceutical preparations because of the short half-life of some radionuclides, small size batches and the radiation hazards. It is not always possible to wait for the results of the test for sterility before authorization of the release of the radiopharmaceutical product for patients' use. Parametric release of the product manufactured by a fully validated process is the method of choice in such cases. When aseptic manufacturing is used, the test for sterility has to be executed as a control of the quality of production.

When the size of a batch of the radiopharmaceutical preparation is limited to one or a few samples (e.g. therapeutic or very short-lived radiopharmaceutical preparation), sampling the batch for sterility testing may not be applicable. If the radiopharmaceutical preparation is sterilised by filtration and/or aseptically processed, process validation is critical.

When the half-life of the radionuclide is very short (e.g. less than 20 min), the administration of the radiopharmaceutical preparation to the patient is generally on-line with a validated production system.

For safety reasons (high level of radioactivity), it is not possible to use the quantity of the radiopharmaceutical preparations as required in the test for sterility (2.2.11). The method by membrane filtration is to be preferred to limit radiation exposure to personnel.

Notwithstanding the requirements concerning the use of antimicrobial preservatives in parenteral preparations, their addition to radiopharmaceutical preparations in multidose containers is not obligatory, unless prescribed in the monograph. The preparation may be released for use before completion of the test.

1.10 Bacterial Endotoxins - Pyrogens

For certain radiopharmaceutical preparations a test for bacterial endotoxins is prescribed. The test is carried out as described in the general method (2.2.3), taking the necessary precautions to limit radiation exposure to the personnel carrying out the test. For Radiopharmaceuticals made with short-lived radioisotopes, endotoxin testing after product release is permitted. However, with the introduction of the kinetic (photometric) LAL test, which can be completed in 20 minutes, it is possible to complete the test for bacterial endotoxins before releasing the radiopharmaceuticals with $T_{1/2}$ of > 30 minutes.

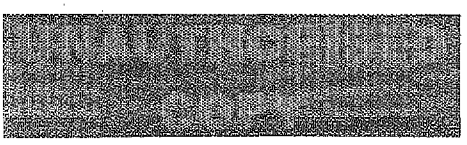
The limit for bacterial endotoxins is indicated in the individual monograph.

When the nature of the radiopharmaceutical preparation results in an interference by inhibition or activation and it is not possible to eliminate the interfering factor(s), the test for pyrogens (2.2.8) may be specifically prescribed. This happens with many of the ^{99m}Tc-radiopharmaceuticals since their formulation uses reducing agents and metal chelating agents, which will give false negative or false positive results with the LAL test.

It is sometimes difficult to carry out these tests before releasing the batch for use when the half-life of the radionuclide in the preparation is short. The test then constitutes a control of the quality of production.

1.11 pH

The pH of radiopharmaceuticals like all pharmaceutical preparations is important and is determined as a part of Quality Control testing. However, due to the small volumes involved and the radioactivity present, the conventional use of a glass pH electrode is not practical or necessary. The sample available for all the Quality Control testing (excluding sterility) is very



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small and the pH range permitted is sufficiently large. Hence, the use of narrow range pH strips is adequate and is preferred as the pH can be tested with a few microlitres of the sample. Further, as they are radioactive after use, they can be easily and safely disposed. pH electrodes, even miniature electrodes, require much cleaning after use and generate radioactive washings that have to be stored and disposed.

1.12 Storage

Store in an airtight container in a place that is sufficiently shielded to protect personnel from exposure to primary or secondary emissions and that complies with national and international regulations concerning the storage of radioactive substances. During storage, containers may darken due to irradiation. Such darkening does not necessarily involve deterioration of the preparations.

Radiopharmaceutical preparations are intended for use within a short time and the end of the period of validity must be clearly stated.

Radiopharmaceuticals intended for parentral use should be stored in such a manner so that pharmaceutical purity of the product is maintained.

1.13 Labelling

The labelling of radiopharmaceutical preparations complies with the relevant national legislation and complies with the labelling requirements as per Good Manufacturing Practice.

Apart from the general labeling requirements, the label on the direct container should state (1) notification that the product is radioactive in nature, (2) the name of the preparation and/or its reference, (3) the name of the manufacturer, (4) an identification number, (5) for liquid and gaseous preparations: the total radioactivity in the container, or the radioactive concentration per milliliter at a stated date and stated time, and the volume of liquid in the container; (6) for solid preparations (such as freeze-dried preparations): the total radioactivity at a stated date and stated time. After reconstitution with the appropriate solution, the preparation is considered as a liquid preparation, (7) for capsules: the radioactivity per capsule at a stated date and time and the number of capsules in the container, and (8) route of administration.

The labeling can be adapted in certain cases (e.g. radio-pharmaceutical preparations containing short-lived radionuclides).

The label on the outer package states, in addition to those on the direct container: (1) the route of administration (2) the period of validity or the expiry date (3) the name and concentration of any added antimicrobial preservative and (4) where applicable, any special storage conditions.

2.0 Techno Legal Regime in the use of Radiopharmaceuticals in the Indian Scenario

Widespread utilization of ionising radiation and radioactive substances and Radioactive Substances for multifarious applications in medicine, industry, agriculture, research etc. has brought in its wake the need for exercising regulatory controls to ensure safety of users, members of the public and the environment. The Atomic Energy Regulatory Board (AERB), constituted under the Atomic Energy Act, 1962 by the Government of India, is entrusted with the responsibility of developing and implementing appropriate regulatory measures aimed at ensuring radiation safety in all applications involving ionizing radiation and radioactive substances. One of the ways to meet these responsibilities is to develop and enforce specific safety codes and standards dealing with radiation safety aspects of various applications of ionizing radiation and radioactive substances to cover the entire spectrum of operations, starting from design of radiation equipment, their installation and use to ultimate decommissioning and safe disposal.

Specifies mandatory requirements published as AERB Codes and Guides for a nuclear medicine facility, covering the entire spectrum of operations ranging from the site approval, laboratory design and setting up of a facility to its ultimate decommissioning, including procedures to be followed during an emergency situation. The Code also stipulates requirements of qualified personnel's and their responsibilities.

AERB SAFETY CODE NO. AERB/RF-MED/SC-2 (Rev. 2), NUCLEAR MEDICINE FACILITIES, March 2011. This code can be found at:

http://www.aerb.gov.in/T/PUBLICATIONS/CODESGUIDES/SC-MED-02R.pdf

There is also an AERB code that elaborates on regulatory requirements and control of use, possession, transport of Radioactive Substances and Radiation sources.

AERB SAFETY GUIDE NO. AERB/NRF-TS/SG-10, SECURITY OF RADIOACTIVE MATERIAL DURING TRANSPORT, January 2008. This code can be found at :

(http://www.aerb.gov.in/T/PUBLICATIONS/CODESGUIDES/sg-10.pdf)

3.0 The Dose Limits for Exposures from Ionizing Radiations for workers and the members of the public from Aerb Safety Code No. Aerb/Rf-med/Sc-2 (Rev. 2)

AERB Directive No. 01/2011[Under Rule 15 of the Atomic Energy (Radiation Protection) Rules 2004]Ref. No.CH/AERB/ITSD/125/2011/1507 dated April 27, 2011

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Subject: The Dose Limits for Exposures from Ionising Radiations for workers and the members of the public

In exercise of rule 15 of the Atomic Energy (Radiation Protection) Rules, 2004, the Chairman, Atomic Energy Regulatory Board, being the Competent Authority under the said rules, hereby issues an order prescribing the dose limits for exposures from ionising radiations for workers and the members of the public, which shall be adhered to.

Dose Limits

General

i) The limits on effective dose apply to the sum of effective doses from external as well as internal sources. The limits exclude the exposures due to natural background radiation and medical exposures.

ii) Calendar year shall be used for all prescribed dose limits.

3.1 Occupational Dose Limits

3.1.1 Occupational Workers

The occupational exposures of any worker shall be so controlled that the following limits are not exceeded:

a. an effective dose of 20 mSv per year averaged over five consecutive years (calculated on a sliding scale of five years);
b. an effective dose of 30 mSv in any year;
c. an equivalent dose to the lens of the eye of 150 mSv in a year;
d. an equivalent dose to the extremities (hands and feet) of 500 mSv in a year and
e. an equivalent dose to the skin of 500 mSv in a year;
f. Limits given above apply to female workers also. However, once pregnancy is declared the equivalent dose limit to embryo/fetus shall be 1 mSv for the remainder of the pregnancy.

3.1.2 Apprentices and Trainees

The occupational exposure of apprentices and trainees between 16 and 18 years of age shall be so controlled that the following limits are not exceeded:

a. an effective dose of 6 mSv in a year;
b. an equivalent dose to the lens of the eye of 50 mSv in a year;
c. an equivalent dose to the extremities (hands and feet) of 150 mSv in a year and
d. an equivalent dose to the skin of 150 mSv in a year.

3.2 Dose Limits for Members of the Public

The estimated average doses to the relevant members of the public shall not exceed the following limits:

a. an effective dose of 1 mSv in a year;
b. an equivalent dose to the lens of the eye of 15 mSv in a year; and
c. an equivalent dose to the skin of 50 mSv in a year.

Table: Annual Limit for Intake (ALI) of important Radionuclides

Radionuclide	ALI (Ingestion) (Ci)	ALI (Inhalation) (Ci)	Derived Air Conc. (DAC) (Inhalation) (Ci per ml)
Tc-99m	8×10^{4}	2×10^{5}	1×10^{-4}
F-18	5×10^{4}	7×10^{4}	3×10^{-5}
I-125	4×10^{1}	6×10^{1}	3×10^{-6}
P-32	6×10^{3}	3×10^{3}	1×10^{-6}
I-131	9×10^{1}	5×10^{1}	2×10^{-8}

4.0 Safety Considerations

Radiopharmaceuticals owing to their detrimental effects on the cellular structures, needs to be monitored for their harmful effects on the personnel involve in handling them. The critical operations include manufacture, storage, transport, compounding, testing, dispensing, and administration to the patients.

Complying as per ALARA (As Low As Reasonable Achievable) principle, in order to minimize the harmful effects of radiopharmaceuticals, there should be specialized techniques for controlling the risk. A major consideration of the ALARA principle includes exposure time, distance and radiation shielding. These factors should be carefully considered while designing the safety protocols for radiopharmaceuticals.

Radiation Sign

Restricted area	< 2 mR/h and < 100 mR/year
Caution, Radiation Area	may exceed 5 mR per hour at 30 cm from source
Caution, High Radioactive Area	may exceed 100 mR per hour at 30 cm from source
Danger, Very High Radiation Area	may reach 500 R per hour at 1 m from source (Not in NM)
Caution Radioactive Material	100 Ci for Cs-137 and Sr-89 1 mCi for Mo-99, I-123 and Co-57 10 mCi for Tc-99m, Ga-67 and Xe-133.

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Radiation safety Instruments	
GM Survey meters	Laboratory survey
Portable Ion Chambers	High level exposure rate monitoring
Pocket Dosimeter	Personnel exposure monitoring
Wipe Test Counters	GM Counters to detect low level activity
Film Badges	To detect and measure personnel exposure
Thermoluminescent Dosimeters	More sensitive than film badges

Scintillation Detectors

- Portable scintillation survey detectors for gamma survey of lab surfaces
- Well type single channel analyzers for measurement of wipe tests
- Multiwell gamma ray spectrometer to identify and quantify gamma contamination.

5.0 Physical Characteristics of Radionuclides

The values are obtained from the database of the National Nuclear Data Center (NNDC) at Brookhaven National Laboratory, Upton, N.Y., USA, directly accessible via Internet at the address: 'http://www.nndc.bnl.gov/nndc/nudat/radform.html'.

In case another source of information is preferred (more recent values), this source is explicitly mentioned.

Other data sources:

* DAMRI (Departement des Applications et de la Métrologie des Rayonnements Ionisants, CEA Gif-sur-Yvette, France),

** PTB (Physikalisch-Technische Bundesanstalt, Braunschweig, Germany),

***NPL (National Physical Laboratory, Teddington, Middlesex, UK).

The uncertainty of the half-lives is given in parentheses. In principle the digits in parentheses are the standard uncertainty of the corresponding last digits of the indicated numerical value ('Guide to the Expression of Uncertainty in Measurement', International Organisation for Standardisation (ISO), 1993, ISBN 92-67-10188-9).

The following abbreviations are used:

e_A = Auger electrons,
ce = conversion electrons,
β^- = electrons,
β^+ = positrons,
γ = gamma rays,
X = X-rays.

Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Tritium (^{3}H)	*12.33 (6) years	*β^-	*0.006$^{(I)}$ (max: 0.019)	*100			
Carbon-11 (^{11}C)	20.385 (20) min	β^+	0.386$^{(I)}$ (max: 0.960)	99.8	γ	0.511	199.5$^{(II)}$
Nitrogen-13 (^{13}N)	9.965 (4) min	β^+	0.492$^{(I)}$ (max: 1.198)	99.8	γ	0.511	199.6$^{(II)}$
Oxygen-15 (^{15}O)	122.24 (16) s	β^+	0.735$^{(I)}$ (max: 1.732)	99.9	γ	0.511	199.8$^{(II)}$
Fluorine-18 (^{18}F)	109.77 (5) min	β^+	0.250$^{(I)}$ (max: 0.633)	96.7	γ	0.511	193.5$^{(II)}$
Phosphorus-32 (^{32}P)	14.26 (4) days	β^-	0.695$^{(I)}$ (max: 1.71)	100			
Phosphorus-33 (^{33}P)	25.34 (12) days	β^-	0.076$^{(I)}$ (max: 0.249)	100			
Sulphur-35 (^{35}S)	87.51 (12) days	β^-	0.049$^{(I)}$ (max: 0.167)	100			
Chromium-51 (^{51}Cr)	27.7025 (24) days	e_A	0.004	67	X	0.005	22.3
					γ	0.320	9.9

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Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Cobalt-57 (^{57}Co)	271.79 (9) days	e_A+ce	0.006-0.007	177.4	X	0.006-0.007	57
		ce	0.014	7.4	γ	0.014	9.2
			0.115	1.8		0.122	85.6
			0.129	1.3		0.136	10.7
						0.692	0.15
Cobalt-58 (^{58}Co)	70.86 (7) days	e_A	0.006	49.4	X	0.006-0.007	26.3
		β^+	0.201[(I)]	14.9	γ	0.511	29.9[(II)]
						0.811	99.4
						0.864	0.7
						1.675	0.5
Iron-59 (^{59}Fe)	44.495 days	β^-	0.1492 (max: 0.4657)	53.1	γ	1.0992	56.5
			0.081 (max: 0.2734)	45.3		1.2916	43.2
Cobalt-60 (^{60}Co)	5.2714 (5) years	β^-	0.096[(I)] (max: 0.318)	99.9	γ	1.173	100.0
						1.333	100.0
Copper-64[(III)] (^{64}Cu)[(III)]	12.7 hours	β^-	0.1907 (max: 0.5794)	39			
		β^+	0.278 (max: 0.653)	17.86	γ	0.511	35.2*
		EC		43.08		1.34577	0.475
Gallium-66 (^{66}Ga)	9.49 (7) hours	e_A	0.008	21	X	0.009-0.010	19.1
		β^+	0.157[(I)]	1	γ	0.511	112[(II)]
			0.331[(I)]	0.7		0.834	5.9
			0.397[(I)]	3.8		1.039	37
			0.782[(I)]	0.3		1.333	1.2
			1.90[(I)]	50		1.919	2.1
						2.190	5.6
						2.423	1.9
						2.752	23.4
						3.229	1.5
						3.381	1.5
						3.792	1.1
						4.086	1.3
						4.295	4.1
						4.807	1.8
Copper-67 (^{67}Cu)	61.83 hours	β^-	0.121 (max: 0.377)	57			
			0.154 (max: 0.4684)	22	γ	0.185	48.7
			0.189 (max: 0.5617)	20		0.093	16.1
Gallium-67 (^{67}Ga)	3.2612 (6) days	e_A	0.008	62	X	0.008-0.010	57
		ce	0.082-0.084	30.4	γ	0.091-0.093	42.4
			0.090-0.092	3.6		0.185	21.2
			0.175	0.3		0.209	2.4
						0.300	16.8
						0.394	4.7
						0.888	0.15

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Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Germanium-68 (^{68}Ge)	270.82 (27) days	e_A	0.008	42.4	X	0.009-0.010	44.1
in equilibrium with	(^{68}Ga: 67.629	β^+	0.353[(1)]	1.2	γ	0.511	178.3
Gallium-68 (^{68}Ga)	(24) min)		0.836[(1)]	88.0		1.077	3.0
Gallium-68 (^{68}Ga)	67.629 (24) min	e_A	0.008	5.1	X	0.009-0.010	4.7
		β^+	0.353[(1)]	1.2	γ	0.511	178.3
			0.836[(1)]	88.0		1.077	3.0
Krypton-81m (^{81m}Kr)	13.10 (3) s	ce	0.176	26.4	X	0.012-0.014	17.0
			0.189	4.6			
					γ	0.190	67.6
Rubidium-81 (^{81}Rb)	4.576 (5) hours	e_A	0.011	31.3	X	0.013-0.014	57.2
in equilibrium with		ce	0.176	25.0	γ	0.190	64
Krypton-81m (^{81m}Kr)			0.188	4.3		0.446	23.2
						0.457	3.0
		β^+	0.253[(1)]	1.8		0.510	5.3
			0.447[(1)]	25.0		0.511	54.2
	(^{81m}Kr: 13.10 (3) s)					0.538	2.2
Rubidium-82 (^{82}Rb)	1.2575 min.	β^+	1.5346 (max: 3.378)	81.76	γ	0.511	190.9*
			1.1676 (max: 2.601)	13.13			
Strontium-82 (^{82}Sr)	25.34 days	Auger L	0.00168	105.7	X	0.01334	16.9
		Auger K	0.0114	28		0.01339	32.7
Strontium-89 (^{89}Sr)	50.53 (7) days	β^-	0.583[(1)] (max: 1.492)	99.99	γ	0.909	0.01
in equilibrium with	(^{89m}Y: 16.06 (4) s)						
Yttrium-89 (^{89m}Y)							
Strontium-90 (^{90}Sr)	28.74 (4) days	β^-	0.196[(1)] (max: 0.546)	100			
in equilibrium with	(^{90}Y: 64.10						
Yttrium-90 (^{90}Y)	(8) hours)						
Yttrium-90 (^{90}Y)	64.10 (8) hours	β^-	0.934[(1)] (max: 2.280)	100			
Molybdenum-99	65.94 (1) hours	β^-	0.133[(1)]	16.4	X	0.018-0.021	3.6
(^{99}Mo) in equilibrium			0.290[(1)]	1.1			
with Technetium-99m			0.443[(1)]	82.4	γ	0.041	1.1
(^{99m}Tc)						0.141	4.5
						0.181	6
						0.366	1.2
	(^{99m}Tc: 6.01 (1)					0.740	12.1
	hours)					0.778	4.3

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Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Technetium-99m (^{99m}Tc)	6.01 (1) hours	ce	0.002	74	X	0.018-0.021	7.3
		e_A	0.015	2.1	γ	0.141	89.1
		ce	0.120	9.4			
			0.137-0.140	1.3			
Technetium-99 (^{99}Tc)	2.11 x 10^5 years	β^-	0.085[(1)] (max: 0.294)	100			
Ruthenium-103 (^{103}Ru) in equilibrium with Rhodium-103m (^{103m}Rh)	39.26 (2) days (^{103m}Rh: 56.114(20) min)	e_A+ce	0.017	12	X	0.020-0.023	9.0
		ce	0.030-0.039	88.3	γ	0.497	91
						0.610	5.8
		β^-	0.031[(1)]	6.6			
			0.064[(2)]	92.2			
Indium-111 (^{111}In)	2.8047 (5) days	e_A	0.019	15.6	X	0.003	6.9
						0.023-0.26	82.3
		ce	0.145	7.8			
			0.167-0.171	1.3	γ	0.171	90.2
			0.219	4.9		0.245	94.0
			0.241-0.245	1.0			
Iodine-123 (^{123}I)	13.27 (8) hours	e_A	0.023	12.3	X	0.004	9.3
						0.027-0.031	86.6
		ce	0.127	13.6			
			0.154	1.8	γ	0.159	83.3
			0.158	0.4		0.346	0.1
						0.440	0.4
						0.505	0.3
						0.529	1.4
						0.538	0.4
Iodine-124 (^{124}I)	4.18 days	β^+	0.6870 (max: 1.5349)	11.7			
					γ	0.511	45*
			0.9747 (max: 2.1376)	10.7		0.6027	62.9
Iodine-125 (^{125}I)	59.402 (14) days	e_A+ce	0.004	80	X	0.004	15.5
			0.023-0.035	33		0.027	114
						0.031	26
					γ	0.035	6.7
Iodine-126 (^{126}I)	12.93 (5) days	ce	Range of energies	51.62	X	0.027-0.031	42.2
		β^-	0.111[(1)] (0.378)	3.6	γ	0.388	34
			0.290[(1)] (0.869)	33.4		0.491	2.9
			0.459[(1)] (1.258)	10.3		0.666	33
						0.754	4.2
						0.880	0.8
						1.420	0.3
		β^+	0.469[(1)] (1.133)	1.08		0.511	2.3[(11)]

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Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Iodine-129 (^{129}I)	1.57×10^7 years	β^-	0.041 (max: 0.154)	100	X	0.02978	36.7
						0.02946	19.9
Iodine-131 (^{131}I)	8.02070 (11) days	ce	0.46	3.5	X	0.029-0.030	3.9
			0.330	1.6			
					γ	0.080	2.6
		β^-	0.069[(1)]	2.1		0.284	6.1
			0.097[(1)]	7.3		0.365	81.7
			0.192[(1)]	89.9		0.637	7.2
						0.723	1.8
Xenon-131m (^{131m}Xe)	11.84 (7) days	e_A	0.025	6.8	X	0.004	8.3
						0.030	44.0
		ce	0.129	61		0.034	10.2
			0.159	28.5			
			0.163	8.3	γ	0.164	2.0
Xenon-133 (^{133}Xe)	5.243 (1) days	e_A	0.026	5.8	X	0.004	6.3
						0.031	40.3
		ce	0.045	55.1		0.035	9.4
			0.075-0.080	9.9			
					γ	0.080	38.3
		β^-	0.101[(1)]	99.0			
Xenon-133m (^{133m}Xe) (decays to radioactive Xenon-133)	2.19 (1) days	e_A	0.025	7	X	0.004	7.8
						0.030	45.9
		ce	0.199	64.0		0.034	10.6
			0.228	20.7			
			0.232	4.6	γ	0.233	10.0
Caesium-137 (^{137}Cs) in equilibrium with Barium-137m (^{137m}Ba)	30.04 (3) years	e_A	0.026	0.8	X	0.005	1
						0.032-0.036	7
		ce	0.624	8.0			
			0.656	1.4	γ	0.662	85.1
		β^-	0.174[(1)]	94.4			
	(^{137m}Ba: 2.552 (1) min)		0.416[(1)]	5.6			
Samarium-153 (^{153}Sm)	46.28 hours	β^-	0.2253 (max: 0.7044)	49.4			
			0.1995 (max: 0.6347)	31.3	γ	0.103	29.25
			0.2643 (max: 0.8076)	18.4			
Lutetium-177 (^{177}Lu)	6.647 days	β^-	0.1493 (max: 0.498)	79.4	γ	0.208	10.36
						0.1129	6.17

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Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Lead-201 (^{201}Pb)	9.33 (3) hours	e_A	0.055	3	X	0.070-0.073	69
(decays to radioactive						0.083	19
Thallium-201)		ce	0.246	8.5			
			0.276	2	γ	0.331	79
			0.316	2.3		0.361	9.9
						0.406	2.0
						0.585	3.6
						0.692	4.3
						0.767	3.2
						0.826	2.4
						0.908	5.7
						0.946	7.9
						1.099	1.8
						1.277	1.6
Thallium-201 (^{201}Tl)	72.912 (17) hours	ce	0.016-0.017	17.7	X	0.010	46.0
			0.027-0.029	4.1		0.069-0.071	73.7
			0.052	7.2		0.080	20.4
			0.084	15.4			
			0.153	2.6	γ	0.135	2.6
						0.167	10.0
Thallium-202 (^{202}Tl)	12.23 (2) days	e_A	0.054	2.8	X	0.010	31.0
						0.069-0.071	61.6
		ce	0.357	2.4		0.080	17.1
					γ	0.440	91.4
Lead-203 (^{203}Pb)	51.873 (9) hours	e_A	0.055	3.0	X	0.010	37.0
						0.071-0.073	69.6
		ce	0.194	13.3		0.083	19.4
					γ	0.279	80.8
						0.401	3.4
Astatine-211(^{211}At)	7.214 hours	α	5.869	41.80	γ	0.0792	20.7
Bismuth-213 (^{213}Bi)	45.59 min.		5.869	1.94			
		α	0.4922 (max: 1.423)	65.8	γ	0.440	25.94
		β^-	0.3204 (max: 0.983)	30.74			
Actinium-225 (^{225}Ac)	10 days	α	5.830	50.7	γ	0.150	0.6
			5.792	18.1		0.188	0.45

(I)Mean energy of the β spectrum.

(II)Maximum emission probability corresponding to a total annihilation in the source per 100 disintegrations.

(III)Monographie BIPM-5, Table of radionuclides (2004 and updated 2006).

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6.0 Positron Emission Tomography Radiopharmaceuticals (PET-RPs) and Associated Radiopharmacy Practices

6.1 Introduction

Most of the radionuclides used in Positron Emission Tomography Radiopharmaceuticals (PET-RPs) are ones with short physical half-lives, $T_{1/2}$ (e.g., $T_{1/2}$ of ^{18}F = 109.8 min, ^{68}Ga = 67.7 min, ^{11}C = 20.4 min, ^{13}N = 9.96 min, ^{15}O = 2.03 min and ^{82}Rb = 70 s). As a result, these radionuclides usually are produced using dedicated cyclotrons (particle accelerators, that produce a proton or deuteron beam) or from generators (similar in principle to the $^{99}Mo/^{99m}Tc$ generators), and then are processed into the final PET-RPs for use in PET-investigations. Due to their short $T_{1/2}$ the PET-RPs have to be produced close to the nuclear medicine centres where they are used. Further, the short $T_{1/2}$ poses several constraints in the formulation and testing of PET-RPs. This chapter on PET-RPs is an annexure to the main General Chapter on Radiopharmaceuticals to emphasize the differences that exist vis-a-vis the production and use of ^{99m}Tc-RPs, the work horse of SPECT (Single Photon Emission Computed Tomography) and Planar-imaging, and how the production and testing are designed to ensure that the PET-RPs are safe for clinical use.

Table 1. Differences and similarities between ^{99m}Tc- And PET-Radiopharmacy[1]

^{99m}Tc Radiopharmacy	PET-Radiopharmacy
T½ of ^{99m}Tc = 6 h	T½: ^{18}F = 109.8 min, ^{68}Ga = 67.7 min, ^{11}C = 20.4 min, ^{13}N = 9.96 min, ^{15}O = 2.03 min and ^{82}Rb = 70 s
Sterile $^{99m}TcO_4^-$ produced on site from Generator.	^{18}F, ^{11}C, ^{13}N and ^{15}O produced on site from Cyclotron, and ^{68}Ga and ^{82}Rb from generators
^{99m}Tc RPs are complexes of ^{99m}Tc and a chelating agent with required properties and are, hence, easy to prepare – no chemical synthesis.	PET RPs are 'true analogs' of metabolites and have to be synthesized with the PET isotope at a specific site in the molecule.
'Cold' sterile reagents available in a vial – just add sterile $^{99m}TcO_4^-$ and mix well (no further processing or minimal without opening the vial).	Rapid chemical synthesis of the PET-RPs is necessary using special precursors (to reduce synthesis time) for ^{18}F and ^{11}C labeled RPs. Only simple molecules possible with ^{13}N and ^{15}O.
Low risk Radiopharmacy: Quality Control of radiopharmaceutical is minimal since the generator produced sterile a and endotoxin free $^{99m}TcO_4^-$ is aseptically added to the sterile and endotoxin free 'cold kit' for making the desired ^{99m}Tc-RP.	Moderate to high risk radiopharmacy except in case of ^{82}Rb, which is low risk: Quality Control is more elaborate, since the PET- RP is synthesized from the cyclotron or generator produced radionuclide, except in the case of ^{82}Rb.
'Parametric release' permitted if quality assurance is validated.	'Parametric release' permitted if quality assurance is validated.
The prepared radiopharmaceutical can be used throughout the day; atleast 8 hours.	^{18}F- and ^{68}Ga-RPs Should be used within a few hours – two or more synthesis required per day. In case of ^{11}C, ^{13}N and ^{15}O, one production run is usually sufficient for one patients, hence high cost per patient. Several elutions of ^{82}Rb would be required for one patient.
γ = 140 keV, 20 mm lead shielding is adequate.	β^+ emitter, γ = 2 x 511 keV, 70 mm lead shielding required.
Multi-patient dose in a single vial possible. Upto 13GBq (~350 mCi) of ^{99m}Tc-RP	Multi-patient dose in a single vial possible.Upto 1.85GBq (50 mCi) maximum[1], sufficient for a few patients depending on the PET-isotope and radiopharmaceutical. Preferred in syringes, ready to inject in patients.
Should be Sterile	Should be Sterile
Endotoxin levels below 175 per V[a] (IU)[b]	Endotoxin levels below 175 per V[a] (IU)[b]

[a]V: Maximum recommended dose. [b]IU: International units.

[1]13GBq of ^{99m}Tc and 1.85GBq of ^{18}F point sources would have a comparable radiation field at 1 cm. Hence, dose to the personnel handling them could be comparable (i.e., administering them to patients etc) provided they follow the ALARA principle of minimizing exposure to radiation.

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The production of PET-RPs has the following constraints:

i. It is not possible to complete all the quality control tests before releasing the PET-RPs for use.

ii. The entire production batch or sub-batch of a PET-RP may be in a single vial. Samples withdrawn for quality control) testing are representative of the entire batch or sub-batch.

iii. One production batch of a PET-RP may be administered to a single patient.

iv. The mass of the active pharmaceutical ingredient in a PET-RP usually ranges from picogram to microgram quantities.

v. PET-RPs are made in-house and used or supplied to nearby licensed users and are not sold or distributed to unknown users.

vi. Medical Cyclotron facilities are usually small centres dedicated to the production of one or a very limited number of PET-RPs with limited staff and resources, and, hence, require permission for:

   a. multiple operations in one area with adequate controls; and

   b. producing and testing of more than one PET-RPs using shared equipment.

   In addition, the facility will also require complying with requirements for:

   a. aseptic operations;

   b. systems and equipment for production of PET-RPs;

   c. for quality control of components, materials, and supplies.

   The limited scope of the facility and staff also requires that to be viable there should be permission for:

   a. Self-verification of significant steps in radionuclide production, PET-RP production, or compounding and testing;

   b. Single-person oversight of

   – production and compounding;

   – review of batch records; and

   – release authorization for in-house use.

This annexure to the General Chapter on Radiopharmaceuticals describes the production and formulation of PET-RPs for clinical use according to national regulations controlling the practice of medicine and pharmacy controlling the use of PET-RPs for clinical and research use.

6.2 Definitions

The following definitions apply to words and phrases used in this section.

Active Pharmaceutical Ingredient. A substance that is incorporated into a finished PET-RP and is intended to furnish direct effect in the diagnosis or monitoring of a disease or a manifestation of a disease in humans, or treatment of disease for therapeutic procedures (e.g., tumor therapy).

Batch. A quantity of PET-RP that is intended to have uniform character and quality, within specified limits, and that is made in a single operational cycle.

Conditional Final Release. A final release for patient administration before completion of required tests because of a malfunction of analytical equipment.

Lot. A quantity of materials (e.g., reagents, solvents, gases, purification columns, and other auxiliary materials) that have uniform character and quality within specified limits and are used to make PET-RPs.

Parametric Release. Parametric release is a system of release that provides assurance of quality based on information collected during the process of manufacture and on compliance with the total quality system. Parametric release is useful if based on existing batch and validation data, derived by internationally agreed analytical principles.

PET-Radiopharmaceutical (PET-RPs). A finished form of a radioactive drug that exhibits spontaneous disintegration of unstable nuclei by the emission of positrons and is intended for human administration in diagnosis or therapy.

PET-Radiopharmaceutical Product. A finished dosage form that contains a PET drug.

Compounding. the process of synthesis or formulation of a PET-RPs for use under the practice of pharmacy and medicine.

Production.the process of synthesis or formulation of a PET-RPs for investigational or research uses.

Quality Assurance (QA). A planned and systematic program to ensure that a PET-RPs possesses defined identity, strength, quality, and purity required for its intended purpose.

Quality Control (QC). A system for testing the quality of components, materials, supplies, and PET-RPs by procedures, tests, analytical methods, and acceptance criteria.

Specific Activity. The radioactivity of a radionuclide per unit mass of the element or compound.

The unit of specific activity is radioactivity per mass expressed on a gram or mole basis (e.g., mCi per mg [MBq per mg], Ci per mmol [GBq per mmol]).

Strength. The radioactivity concentration of the active pharmaceutical ingredient on a volume basis at the time of calibration. The unit of strength is the amount of radioactivity per volume at the time of calibration (e.g., mCi per ml [MBq per ml]).

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Sub-batch. A quantity of PET-RPs having uniform character and quality, within specified limits, that is produced during one succession of multiple irradiations using a given synthesis or purification operation.

A group of sub-batches collectively form a batch that is intended to have uniform character and quality, within specified limits. Sub-batches are required for PET-RPs with very short-lived radionuclides (e.g., ^{13}N and ^{15}O) because quality control tests cannot be completed before use.

Validation. Establishment of documented evidence that a method, process, or system meets its intended requirements.

Verification. Confirmation that an established method, process, or system meets predetermined acceptance criteria.

6.3 Personnel and Resources

Personnel with the appropriate qualification and training are needed for the preparation and quality control testing of PET-RPs. Since this is a new field of expertise in India, personnel with experience may be hard to find. The personnel should include chemists, technical assistants and health physicists. The number employed will depend on the size and complexity of the facility. Personnel should be trained so that they get sufficient hands-on experience and suitably examined before they begin to make and test PET-RPs. Training should address radionuclide production techniques, synthetic and purification methods, materials, components, reagents, stock solutions, automated and manual apparatus used to make PET-RPs, and quality control methods, including equipment, software, and documentation.

Personnel Requirements for Aseptic Operations. Training should address aseptic manipulations as well as the techniques and equipment used to achieve and maintain Class A (Class 100) environmental conditions. Training also should address all aseptic operations, including the assembly of sterile components, compounding, and filtration. Manipulations of sterile solutions should be performed by operators who are qualified to use aseptic techniques.

Personnel involved in aseptic operations should be evaluated periodically and if he/she do not clear the evaluation, they should be immediately re-instructed and re-evaluated to ensure correction of aseptic practice deficiencies.

6.4 Quality Control, Quality Assurance and Oversight

Quality Assurance and Quality Control are important elements in the process of making PET-RPs. Quality Assurance is a broad concept that covers all matters that influence identity, strength, quality, and purity of a PET-RPs. Quality Control is a subset of Quality Assurance that deals with testing of materials and PET-RPs to determine if they meet acceptance criteria. The Quality Assurance function typically consists of oversight activities, and the Quality Control function consists of execution activities.

i. Quality Control functions include the following:
   a. Evaluation of each lot of incoming material and certifying that it meets established specifications before use in the formulation/testing of PET-RPs.
   b. Evaluate each batch of a PET-RPs produced for the various required specifications before authorizing the final release or rejection of the batch.

ii. The Quality Assurance functions include the following:
   a. Review completed batch records for accuracy and completeness.
   b. Ensure that PET-RPs have adequately defined identity, strength, quality, and purity.
   c. Investigate errors and ensure that appropriate corrective action is taken to prevent their recurrence.
   d. Handle complaints.
   e. Ensure that the PET-RPs are distributed according to the established procedures and practices for PET-RPs.

iii. The oversight functions should carry out the following functions:
   a. Validate and approve procedures, specifications, processes, and methods.
   b. Ensure that personnel are properly trained and qualified, as appropriate.
   c. Conduct periodic audits to monitor compliance with established procedures and practices for PET-RPs.

It may be noted that due to the small size of PET-RPs facilities, the personnel at the facility may perform bothQuality Assurance and Quality Control functions.

6.5 Facilities and Equipment

Facilities should have adequate area that is physically or appropriately segregated for the production, compounding, and Quality Control of PET-RPs so as to prevent human errors like cross-contamination etc., especially in areas used for making more than one PET-RPs. Work areas should be periodically cleaned, sanitized and fumigated to prevent build-up of microbial contamination on equipment, materials, components, or PET-RPs by personnel or environmental conditions that could badly affect PET-RPs quality. These requirements should be described in written procedures, and their routine execution should be documented.

Since the sterility test results for parenteral PET-RPs are obtained after release, the process used should ensure that the facilities and equipment used will ensure sterile PET-RPs.

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Aseptic Work-area—The primary environmental control has to be designed for aseptic operations, which is Class A (Class 100). This can be achieved with aseptic work-stations (e.g., laminar airflow work-area with high efficiency particulate air filters (HEPA), aseptic isolator, biological safety cabinet, or other suitable device). The aseptic work-area should be protected from sources of microbial contamination by limiting personnel traffic, avoiding storage of materials that shed particulate matter. The proper operation of the aseptic work-area must be certified by measurement of airborne particles and HEPA filter integrity testing. Certification should be performed at the inception of operation and at least annually thereafter or after repair or replacement of the HEPA filter. The inside of the aseptic work-area should be clean and permit easy cleaning and disinfection. The internal surfaces should be cleaned and disinfected with appropriate approved disinfectants.

Microbiological Testing—Microbiological testing of the environment where the work-area is located is periodically necessary to assess air quality and surface disinfection. Non-viable particle counts may be determined once a month or quarterly depending on the number of personnel working and the number of batches of PET-RPs made. More the personnel working and batches made, more the frequency of testing. Alert and action limits should be established based on results of the tests and typically, alert levels are set at less than three colony-forming units (CFU) per plate. More than three CFU require appropriate corrective actions. The results of microbiological testing should be kept in mind while investigating positive sterility tests.

Equipment for the production/formulation of PET-RPs

Equipment used to make and test PET-RPs should be of a design such that the PET-RPs can be produced as per specifications consistently. They should be installed, cleaned, and maintained in an appropriate manner.

Newly installed equipment should be qualified before use either by the manufacturer, in the form of type-approval certificate from an authorized certifying agency in the country of manufacture or evaluated locally to make PET-RPs. All qualification activities should be properly documented, including the date and the name of the person who performed the qualification.

Equipment calibration is an important criteria and should be performed before use. Subsequently there should be in place a schedule for the recalibration to ensure accurate results. Calibration activities should be properly documented, including the date and the name of the person who performed the calibration.

Preventive maintenance plays an important role in minimizing equipment downtime. A schedule should be developed for PET-radiochemistry synthesis and dispensing modules and Quality Control-testing equipment, including gas chromatographs, HPLCs and others. Preventative maintenance is usually practiced as annual maintenance contracts (AMCs) with the equipment suupliers. Activities should be properly documented, including the date of such performance and the name of the person who performed them.

Cleaning Equipment and Components. Equipment used in production or compounding of PET-RPs includes automated, computer-controlled devices, as well as manually operated apparatus. Before it is used in making PET-RPs, equipment should be properly cleaned to ensure that the resulting PET-RPs meet established specifications for identity, strength, quality, and purity (see *Controls and Acceptance Criteria for Finished PET-RPs*, below). Once cleaned, equipment should be maintained in a state of cleanliness before use. Equipment may be used to make multiple batches of one or more PET-RPs. Documented studies should demonstrate the effectiveness of the cleaning process between batches. All impurities should be controlled at levels that conform to established specifications for identity, strength, quality, and purity.

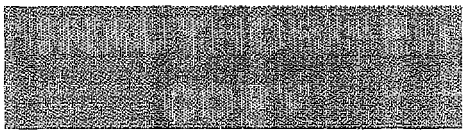
Daily Checks-lists are necessary to ensure that the personnel clean the equipment prior to use, the right chemicals/reagents/precursors are used, that they are used beyond their shelf life, the right computer programme is selected for the synthesis and the synthesis parameters are the correct ones. The day check-lists will also include that the right quantities of the radiopharmaceuticals are dispensed and distributed. The day check-lists play a valuable role in audit trails to identify and trouble shoot complaints and other problems.

Quality Control Equipment checks are necessary to that the PET-RPs produced and supplied are as per the specifications. This implies that system suitability tests are necessary for all the Quality Control equipment used, which include TLC, radio-TLC scanners, dose calibrators, gamma spectrometers, HPLC, GLC etc.

Components, materials, reagents and supplies that are used in the preparation of PET-RPs should be controlled to avoid contamination, mix-ups, and errors. Their procurement, quality-acceptance, storage, expiry date and record of use should be recorded and maintained as they are useful in the audit trail for trouble shooting and handling complaints. These records should be maintained for one year after their expiration or for one year after batch release, whichever is longer.

The following activities should be established and performed:

1. Establish written specifications for the identity, strength, quality, and purity of ingredients, reagents, target materials, and gases.
2. Establish written specifications for the identity and quality of sterile empty vials, transfer lines, sterile



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stopcocks, sterile needles, sterile membrane filters, and other components used in the PET-RPs vial assembly.

3. Establish written specifications for the identity, strength, quality, and purity of analytical supplies (e.g., solvents, chromatography columns, and authentic standards), sterility test media, and endotoxin test reagents used in the testing of PET-RPs.
4. Establish appropriate storage conditions (based on heat, light, humidity, and other factors) for components, materials, and supplies used to make and test PET-RPs.
5. Store components, materials, and supplies in a controlled-access area according to established storage conditions. Segregate components, materials, and supplies as appropriate to avoid mix-ups and errors.
6. Log each lot of shipment of components, materials, and supplies, and record the date of receipt, quantity received, manufacturer, manufacturer's lot number, and expiration date. If no expiration date is designated by the manufacturer, assign one based on knowledge of its physical and chemical properties and previous experience with its use. For organic substrates and reagents that are potentially susceptible to degradation or to a change in composition, the expiration date should be based on the material's documented evidence of stability.
7. Determine that each lot of components, materials, and supplies complies with established written specifications. Compliance with specifications can be demonstrated by inspection of the labeling or inspection of the COA (Certificate of Analysis) provided by the manufacturer. The identity of each lot of components, materials, and supplies should be verified by defined procedures, tests, or documented COAs, as appropriate.

   Perform an identity test for precursors (e.g., melting point determination or other appropriate tests). Alternatively, the COA can be used as the only acceptance criterion for a precursor if final testing of the PET-RPs ensures that the correct precursor has been used. Other components can be accepted on the basis of a COA only.
8. Membrane filters used with parenteral PET-RPs should have a certificate of conformance (COC) from the manufacturer. Examine the COC for each lot to ensure compliance with written specifications.
9. Media used in the sterility testing of PET-RPs should be commercially available. To ensure the viability of media, perform growth-promotion testing. A simplified growth-promotion test that employs a single organism is adequate to demonstrate the suitability of media. Alternatively, a positive control can be performed during the execution of each sterility test inoculation.

6.6 Process and Operational Controls

Process Controls. The following process controls should be established and summarized in a master formula for the PET-RPs. A designated person should be responsible for ensuring that these activities are carried out and completed properly.

1. Written acceptance criteria for the identity, strength, quality, and purity of each PET-RPs should be established. For PET-RPs intended for parenteral administration, specifications should include sterility and bacterial endotoxins. If a IP monograph exists or there are Quality Control specifications approved by the concerned Authority, then these standards should be applied as the minimum acceptance criteria.
2. Written procedures for the preparation of each PET-RPs should:
   i. Include sterile membrane filtration (0.22 µm) for RPs intended for parenteral administration and particulate filtration (0.45 µm) for RPs intended for inhalation;
   ii. Describe routine cleaning procedures for equipment and facilities;
   iii. Describe components, materials, and supplies used to make PET-RPs, including precursors, standards, reagents, stock solutions, and related items;
   iv. Describe the process and the steps used to make the PET-RPs;
   v. Describe the formulation process, including the use of stabilizers, buffers, and other agents;
   vi. Describe calculations used to determine key parameters associated with making and Quality Control testing,
   vii. including radiochemical yield, radiochemical purity, and so on;
   viii. Describe Quality Control tests for the final PET-RPs (see *Controls and Acceptance Criteria for Finished PET-RPs*, below), including a schedule that defines whether or not each test should be performed on each batch and that states if the test results should be complete at the time of release.
3. Documented studies should ensure that the processes and steps described in the master formula yield a PET-RPs that meets established acceptance criteria. Such studies should:
   i. Demonstrate a consistent process that is suitable for the intended use of the PET-RPs.
   ii. Be completed on three batches made according to the master formula, and all three batches should meet all acceptance criteria.

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iii. Include evaluation of radiochemical identity and purity, radionuclidic identity and purity, specific activity, sterility (for parenteral PET-RPs), bacterial endotoxins (for parenteral PET-RPs), pH, appearance, stereochemical purity (for applicable compounds), residual solvents, other toxic chemicals that may have been used during the synthesis or purification procedure, effective concentration of a stabilizer (if any), chemical purity of the PET-RPs, and equivalence of initial and final sub-batches.

iv. Be repeated if the process and steps described in the master formula have been altered in a way that could change the identity, strength, quality, or purity of the PET-RPs.

4. The processes and steps described in the master formula should be updated as needed and should be reviewed annually to ensure they are current. Appropriate controls of computer-controlled equipment should ensure that process changes are instituted only by authorized personnel and that such changes are documented and verified. Production, compounding, and test methods should be backed up and controlled to avoid accidental use of outdated methods. In the case of processes or test methods from a vendor that are used without alteration, it is acceptable to rely on vendor certification for software verification and proper operation.

Operational Controls. The following operational controls should be established and summarized in a batch record that is a subset of the master formula for the PET-RPs. The batch record should adequately document the routine process for making the PET-RPs. A designated person should be responsible for ensuring that these activities are carried out and completed properly. Maintain completed batch records and associated documentation for one year after batch release.

1. Inspect areas used to make and test PET-RPs, and inspect all equipment for cleanliness and suitability before use. Remove extraneous materials and labels from these areas and equipment.

2. Ensure the correct identity, strength, quality, and purity of components, materials, and supplies used in the preparation of the PET-RPs. Label components as appropriate for identity and traceability purposes.

3. Execute routine cleaning procedures for equipment and facilities.

4. Prepare the PET-RPs according to the current master formula, and for each batch maintain a batch record. Batch records may consist of paper documents, electronic records, or combinations thereof. Spreadsheets and other electronic record-keeping tools should be verified to ensure traceability, data integrity, accuracy of results for calculations, and so on. The batch record should include:

i. Lot numbers or other unique identifiers for all components, materials, and supplies used to make the PET-RPs product;

ii. A description of the individual procedures that were followed;

iii. The initials, signature, or other identifier of the responsible individual indicating that critical steps and processes used to make and test the PET-RPs were completed;

iv. The percent yield calculated on the basis of the known or expected amount of the starting radionuclide that is synthetically incorporated into the PET-RPs;

v. Raw analytical data on each batch of the PET-RPs;

vi. Labeling for the PET-RPs (see *Labeling and Packaging*, below);

vii. Calculations for key parameters defined in the master formula;

viii. Results obtained from Quality Control tests of the PET-RPs, including chromatograms, print-outs, and other test data;

ix. The initials of the analyst who performed each Quality Control test;

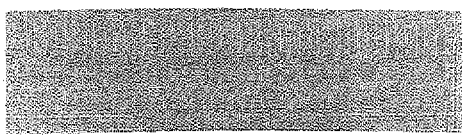
x. A notation of the result for each Quality Control test and whether or not the result meets the acceptance criteria;

xi. The date and time of release and the signature of the individual who assumes overall responsibility for, and adherence to, the procedures used to make the batch and authorizes the release of the batch for human administration; and

xii. Documentation on the batch record of process deviations, when applicable.

xiii. Entries in batch records should be made immediately after the activity is performed and should include the initials, signature, or other identifier for the person making the entry. Corrections to paper entries should be dated and initialed, signed, or noted with an identifier of the person making the corrections but leaving the original entry still readable.

Aseptic Operations for Parenteral PET-RPs. Because the sterility test results for parenteral PET-RPs are obtained after release for human administration, aseptic operations and procedures should adequately ensure a sterile PET-RPs. All aseptically prepared PET-RPs for parenteral administration



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should be filtered through a sterile membrane filter of 0.22 µm or finer pore size into a closed sterile vial or container. Although the chemical synthesis of a parenteral PET-RPs may take place in an open or closed apparatus, the membrane filtration of the PET-RPs should take place in a closed apparatus that is aseptically assembled from pre-sterilized, commercially available components.

Components. The sterile components used in the aseptically assembled apparatus typically consist of an empty vial, needles, membrane filters, vent needles, syringes, tubing, stopcocks, and perhaps others. All components should be single-use, commercially available, pre-sterilized items. If components in the aseptically assembled apparatus are sterilized by the PET-RPs facility, the sterilization processes should be verified. The exact configuration of the PET-RPs vial assembly is process dependent. A typical example is a sterile, empty vial with a membrane filter of 0.22 µm pore size attached to a needle that is inserted through the vial septum for filtration, a membrane filter of 0.22 µm pore size attached to a needle that is inserted through the vial septum for venting the vial during filtration, and a syringe with needle inserted through the vial septum for removal of the Quality Control sample after filtration is complete.

PET-RPs Vial Assembly. Aseptic techniques should be used in the preparation of the PET-RPs vial assembly, especially the assembly of all components downstream from the membrane sterilizing filter. These operations should be performed in an ISO Class 5 environment (see *Facilities and Equipment*, above)

Following the creation of the PET-RPs vial assembly in the ISO Class 5 environment, the assembly can be removed to another location for filtration. The location can be a non-controlled environment as long as the integrity of the PET-RPs vial assembly is not compromised during the process. Any PET-RPs vial assembly that is compromised during this process should be discarded.

Aseptic Techniques. Any sterile component downstream from the membrane filter that contacts the PET-RPs should be handled using suitable aseptic techniques inside the aseptic work-area. During aseptic operations, operators should wear proper attire, including a clean laboratory jacket, forearm sleeves, hair cover, sanitized gloves that cover the wrist, and beard/moustache covers (as appropriate). Multiple PET-RPs vial assemblies can be prepared in a single aseptic operational cycle. The storage time for assembled vials should be based on data from aseptic media fills.

Sterility Test Inoculations. Sterility tests should be performed to assess the quality of PET-RPs intended for parenteral administration. The inoculation of sterility test media should be performed in a manner that is consistent with personnel radiation exposure requirements but that also minimizes the risk of false positives caused by adventitious contamination during the inoculation process. For media tubes with a screw-cap opening, the inoculation should be performed in the aseptic work-area.

Media tubes with a septum cap can be inoculated in a shielded area that does not contain a HEPA filter.

6.7 Stability

The PET-RPs have an expiry time that is based on the $T_{1/2}$ of the radioactive isotope and also radiolytic damage that occurs with radioactive compounds. Hence, the expiration time should be based on the results of stability testing (and specific activity requirements, as appropriate). Stability testing of the PET-RPs should be performed at the highest strength of the PET-RPs and in the intended final vial or container. At least three batches of the PET-RPs should be stored according to proposed conditions and should be examined after a time period equal to the proposed shelf life. In addition, the PET-RPs should meet acceptance criteria for radiochemical purity, appearance, pH, and stabilizer or preservative effectiveness (as appropriate) at expiry. Analytical methods should be reliable, meaningful, and specific. Stability studies should be repeated if there is a change in strength, stabilizer (or preservative) content that has the potential to affect the stability, the final vial or container, storage conditions, or expiration time. The results of stability testing should be documented.

6.8 Controls and Acceptance Criteria for Finished PET-RPs

Written specifications for identity, strength, quality, and purity should be established for each PET-RPs. For PET-RPs intended for parenteral administration, specifications should be included for sterility and bacterial endotoxins

Written procedures should be developed for Quality Control tests. Quality Control and documentation requirements should be established for each batch or sub-batch of a PET-RPs. All Quality Control tests should be executed by qualified and trained personnel according to written procedures. The short half-life of PET radionuclides frequently precludes the completion of all Quality Control tests before shipment of the PET-RPs. This effectively creates two levels of release, one for distribution and the other for human administration.

This is acceptable as long as the Quality Control tests required for release of the PET-RPs for human administration are completed before administration. The controls used in the release for distribution should be previously established in writing and should be documented in routine practice. It is not necessary to retain reserve samples of PET-RPs but keeping a batch sample for repeating post-facto Quality Control tests may be desirable.

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If an IP compendial test procedure is employed, the procedure should be verified to demonstrate that the test works under the conditions of actual use. Noncompendial test procedures employed in the testing of PET-RPs should be reliable and specific. Supporting data for use of all analytical methods should be documented.

Data derived from process studies or from in-process controls can be used as a basis for the omission of some Quality Control tests. An example of this approach is the chlorodeoxyglucose determination in the testing of [^{18}F]fludeoxyglucose.

Supporting data from process studies or in process controls should be documented.

The following Quality Control tests should be performed on each batch before release for administration:

1. Measure the pH of parenteral dosage forms.
2. Determine the radiochemical purity and identity of all dosage forms.

In addition, the following Quality Control tests should be considered on each batch before release for administration:

1. Visually inspect parenteral dosage forms.
2. Determine the radionuclidic identity of all dosage forms by half-life measurement.
3. Determine the strength.
4. Determine the specific activity of PET-RPs that have mass-dependent localization or toxicity concerns.
5. Determine the residual solvents used in the synthesis or purification processes.
6. Determine the chemical purity and residual compounds used in the synthesis or purification processes (e.g., cryptand[2.2.2]).

For PET-RPs with very short-lived radionuclides, prepare an initial Quality Control sub-batch that is representative of successive sub-batches prepared in a defined operational cycle. The Quality Control tests described in the previous paragraph should be considered for the Quality Control sub-batch before release of subsequent sub-batches for human administration.

For subsequent sub-batches of parenteral and inhaled dosage forms, visual inspection should be performed before human administration. In certain cases, limited testing of each sub-batch before administration may be appropriate (e.g., for pH determination of [^{13}N] ammonia produced by Devarda's alloy).

For all PET-RPs, periodically measure the radionuclidic purity of decayed samples of the PET-RPs to assess the presence of long-lived radionuclides that are produced in targetry associated with the particle accelerator. For PET-RPs labeled with certain radionuclides (e.g., ^{94m}Tc, ^{124}I, ^{64}Cu, ^{76}Br, and others), consider the measurement of radionuclidic purity by gamma spectrometry.

Periodic Quality Control tests for PET-RPs also include low-level nontoxic impurities and Class 3 residual solvents. The periodic Quality Control testing should be performed at predetermined intervals rather than on a batch-to-batch basis.

For PET-RPs intended for parenteral administration, perform the following Quality Control tests in addition to those described previously:

1. Determine the integrity of the membrane filter. Filter units used to sterilize PET-RPs should be subjected to manufacturers' recommended integrity tests such as the bubble point test. Although it is not strictly a Quality Control test on the final PET-RPs, this test is important to ensure the preparation of a sterile solution. Perform the filter integrity test after completion of filtration and before release of the PET-RPs for human administration. In the case of PET-RPs with $T_{1/2}$ <10 min, the PET-RPs can be released for human administration before completion of the filter integrity test. In this case, the test should be completed as soon as possible after release.
2. Perform a test for bacterial endotoxins (2.2.8) on each batch or Quality Control sub-batch of a PET-RPs. The test can be performed using recognized procedures in IP. Regardless of which test is used, it should be initiated before release of each batch for human administration. For PET-RPs with very short-lived radionuclides, complete the test on the Quality Control sub-batch before the release of subsequent sub-batches for human administration. After a record of successful bacterial endotoxin tests is established for a particular PET-RPs, it is necessary only to test the first batch prepared each day for that PET-RPs.
3. Perform a test for sterility on each batch or Quality Control sub batch the sterility test consists of the inoculation and incubation of a sample into each of two media: tryptic soy broth and fluid thioglycollate. The inoculated volume may be adjusted to avoid excessive losses because of sterility testing (e.g., 0.1 ml inoculated into 10 ml of media). The incubation period for sterility tests should begin within 30 hours of the membrane filtration. The samples can be inoculated immediately after completion of the membrane filtration, or they can be allowed to decay in a shielded area for as long as 30 hours before inoculation. It is acceptable to exceed the 30-hour period because of weekends or holidays provided it is shown that the extended period does not significantly reduce the viability of a IP indicator organism (e.g., *E. coli*) in the sample. The sterility test (2.2.11) may be performed using other recognized procedures in IP. Samples should be tested individually and may not be pooled. After a record of

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successful sterility tests is established for a particular PET-RPs, it is only necessary to test the first batch prepared each day for that PET-RPs.

When a required Quality Control test for a PET-RPs cannot be completed because of a malfunction of testing equipment, it may be appropriate to conditionally release the batch. PET-RPs may not be released without determination of radiochemical identity and purity. The batch may be released if the following conditions are met:

1. Review historical Quality Control data to assess the frequency of out-of-specification (OOS) results or failures associated with the Quality Control test. A conditional release is appropriate only if the historical data reveal a record of successful completion of the Quality Control test.
2. Confirm that the acceptance criteria are met for all other Quality Control tests for the batch.
3. Retain a sample of the conditionally released batch, and complete the omitted Quality Control test on the sample as soon as possible after the malfunction has been corrected. This is not necessary if the omitted Quality Control result is meaningless after decay of the PET-RPs.
4. If the sample fails the omitted Quality Control test, notify the physician or receiving facility that ordered the PET-RPs.
5. Document all actions regarding the conditional release of the PET-RPs, including the justification for the release, results of completed testing, and any notifications and corrective actions resulting from the incident.
6. Promptly correct any malfunction of the testing equipment.

In addition to the finished Quality Control testing, other appropriate laboratory determinations could involve in-process testing of an attribute that is equivalent to finished-product testing of that attribute; continuous statistical process monitoring; or some combination of these approaches with finished testing of each PET-RPs.

6.9 If a PET-RPs Does Not Conform to Specifications

When the result of a Quality Control test for a PET-RPs does not meet established acceptance criteria, the result is out of specification. An OOS result does not necessarily mean that the final PET-RPs is a failure and should be rejected. Instead, an OOS investigation should be performed to determine if the OOS result indicates a true failure or an analytical error.

If an OOS investigation concludes that the OOS result was caused by an analytical error, invalidate the original test. If a printout is associated with this test, mark the printout invalid, retain it for the batch record, and repeat the test.

If an OOS investigation concludes that the OOS result was a true failure, the batch should be rejected and cannot be released for human administration. Segregate the batch to avoid its potential use. Investigate all failures and document the results according to written procedures. The investigation should include, but is not limited to, the examination of processes, operations, and records from previous batches, as well as complaints and other relevant sources of information. If possible, assign an actual or probable cause to the failure, and document corrective actions undertaken as a result of the investigation.

Depending on the nature of the failure, the PET-RPs may be reprocessed according to pre-established written procedures. When a sterility test for a PET-RPs shows signs of microbial growth, the test result is OOS and should be investigated. If the outcome of the investigation concludes that the OOS result was a true failure, notify the physician or receiving facility that ordered the PET-RPs. In addition, identify and correct the source of the contamination. The process should be revalidated and operators should be retrained as necessary to demonstrate the effectiveness of the corrective actions.

6.10 Further Purification and Processing

If a PET-RPs is rejected as a true failure, the batch may be reprocessed according to established procedures. It is not possible to describe all possible reprocessing operations, but some examples could include:

- pH adjustment;
- A second passage through a membrane filter in the event of a failed filter integrity test; and
- A second passage through a purification column to remove an impurity.

If a PET-RPs is reprocessed, the reprocessed batch should be tested to ensure it meets the established acceptance criteria for the PET-RPs before release for human administration.

6.11 Labelling and Packaging

The following information should appear on the label attached to the final PET-RPs container:

i. The name of the PET-RPs;
ii. The assigned batch number; and
iii. Any required warning statements or symbols (e.g.,radioactive).

The following information should appear on the shielding for the PET-RPs:

i. The name of the PET-RPs;
ii. The assigned batch number;

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iii. The date and time of calibration;

iv. Any required warning statements or symbols (e.g.radioactive);

v. As appropriate, the total radioactivity in MBq (or mCi) or the strength in MBq per ml (or mCi per ml) at time of calibration;

vi. Expiration time and date;

vii. Added substance(s) (e.g., stabilizer or preservative) if any;

viii. Other applicable warning statement(s) (e.g., "Do not use if cloudy or if it contains particulate matter" or investigational use labeling); and

ix. Other pertinent information (if required), such as storage condition(s), half-life, and name and place of business where the PET-RPs was made or distributed.

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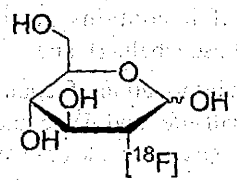
Fluorodeoxyglucose (^{18}F) Injection

Fludeoxyglucose (^{18}F) injection, ^{18}F-FDG injection.



Category. Diagnostic.

Description. A clear, colourless or a very slightly yellow solution. Fluorodeoxyglucose (^{18}F) Injection is a sterile solution consisting of 2-[^{18}F] Fluoro-2-deoxy-D-glucopyranose (2-[^{18}F] Fluoro-2-deoxy-D-glucose) and may contain sodium chloride and buffer salts. Fluorodeoxyglucose (^{18}F) Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of Fluorine-18 radioactivity at the date and time stated on the label. Fluorine-18 is not less than 99.0 percent of the total radioactivity. Not less than 90 per cent of the total fluorine-18 radioactivity is present as 2-[^{18}F]Fluoro-2-deoxy-D-glucose and 2-chloro-2-deoxy-D-glucose is not more than 0.5 mg per maximum recommended dose, in milliliters.

Radionuclide Production. The radionuclide Fluorine-18 is most commonly produced by proton irradiation of *water* enriched in oxygen-18.

Radiochemical Synthesis. 2-[^{18}F]Fluoro-2-deoxy-D-glucose is mostly prepared by phase transfer catalyzed nucleophilic substitution of 1, 3, 4,6-tetra-*O*-acetyl-2-*O*-triFluoromethane-sulphonyl-β-D-mannopyranose (tetraacetylmannose triflate) with [^{18}F]fluoride. Generally, [^{18}F]fluoride is adsorbed on an anion-exchange resin and eluted with a solution of *potassium carbonate* and evaporated to dryness. Addition of a phase-transfer catalyst, such as an aminopolyether in dry *acetonitrile*, may be used to enhance the nucleophilicity of the [^{18}F]fluoride so that it reacts easily with the *tetraacetylmannose triflate* (precursor used for preparing [^{18}F]FDG at elevated temperature. Alternately tetra-alkyl ammonium bicarbonate salt solution is used to elute the [^{18}F]fluoride from the anion-exchange column and the tetra-alkyl ammonium-[^{18}F]fluoride also serves to enhance the nucleophilicity of [^{18}F]fluoride in non-aqueous phase. A variation of the method uses solid-phase-catalysed nucleophilic substitution on derivatised anion-exchange resin, for example, derivatised with 4-(4-methylpiperidin-1-yl) pyridine.

Hydrolysis under either alkaline or acidic conditions yields 2-[^{18}F]Fluoro-2-deoxy-D-glucose. Depending on the conditions of hydrolysis, variable amounts of 2-chloro-2-deoxy-D-glucose and/or 2-[^{18}F]Fluoro-2-deoxy-D-mannose may be formed as a by-product.

The preparation can be purified by serial chromatography on combinations of ion-retardation resin, ion-exchange resin, alumina and octadecylsilyl derivatised silica gel.

Half-life and nature of radiation of Fluorine-18 complies with the table of physical characteristics of radionuclides stated under Radiopharmaceutical Preparations.

Identification

A. Free from particulate matter and colourless or slightly yellow solution when observed visually behind leaded glass under adequate light. The test must be completed before release of the drug product.

B. Complies with test A and test C or test B and test C given under Radionuclidic purity. Test A or test B should be completed before release of the product. Test C to be done periodically, once a month.

C. Radionuclide identity: The half life is between 105 min and 115 min as determined with a gamma ray counter, by not less than three measurements of the radioactivity of a sample in the same geometrical condition within a suitable period of decay time for example, three readings at 10 min intervals over 30 min. The test must be completed before release of the product.

D. In the method A for Radiochemical purity, the principal peak in the chromatogram obtained with the test solution corresponds to the peak in the chromatogram obtained with reference solution (a).

Radiochemical Identity. Determine by thin layer chromatography (2.4.17), using silica gel G coated plates.

Mobile phase. A mixture of 95 volumes of *acetonitrile* and 5 volumes of *water*.

Test solution. The injection under examination.

Reference solution: A solution of *Fluorodeoxyglucose (^{19}F) IPRS* in *water*.

The retention factor (R_f) value of test solution corresponds (e.g., < ±10 per cent) to the R_f value (about 0.4) of the reference solution when both are chromatographed together side by side on the same thin layer chromatography silica gel strip and scanned using a radiochromatographic TLC scanner. The R_f of the reference Fluorodeoxyglucose (^{19}F) is identified by treatment with an appropriate reagent. The test must be completed before release of the drug product.

Tests

pH (2.4.24). 4.5 to 8.5.

Chemical purity

NOTE — Particular tests for chemical impurities may be omitted if the substances mentioned are not used or cannot be formed in the production process.

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Fluorodeoxyglucose impurity A or 2-Chloro-2-deoxy-D-glucose. Determine by liquid chromatography (2.4.14).

Test solution. The injection under examination.

Reference solution (a). Dissolve 10.0 mg of *2-[^{19}F]Fluoro-2-deoxy-D-glucose IPRS* in *water* and dilute to 2.0 ml with *water.* Dilute 1.0 ml of the solution to a volume equivalent to 10 mg in maximum recommended dose in milliliters (V) with *water.*

Reference solution (b). Dissolve 1.0 mg of *2-chloro-2-deoxy-D-glucose IPRS (Fluorodeoxyglucose impurity A IPRS)* in *water* and dilute to 2.0 ml with *water.* Dilute an aliquot of this solution to give a volume equivalent to 0.5 mg per ml, the maximum dose in milliliters with *water.*

Reference solution (c). Dissolve 1.0 mg of *2-[^{19}F]Fluoro-2-deoxy-D-mannose IPRS* in *water* and dilute to 2.0 ml with *water.* Mix 0.5 ml of this solution with 0.5 ml of reference solution (a).

Chromatographic system
- a stainless steel column 25 cm × 4.0 mm, packed with strong anion exchange resin (10 µm),
- column temperature: 20° to 25°,
- mobile phase: 0.4 per cent w/v solution of *sodium hydroxide* in carbon dioxide-free water,
- flow rate: 1 ml per minute,
- pulse amperometric detector (carbohydrate detector) and radioactivity detector connected in series,
- injection volume: 20 µl.

Inject the test solution, reference solution (a) and (b) sequentially. Run the chromatogram for twice the retention time of the principal peak. In the chromatogram obtained with the test solution using carbohydrate detector the area due to 2-Fluoro-2-deoxy-D-glucose is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (10 mg per *V*) and the area due to Fluorodeoxyglucose impurity A is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 mg per *V*).

The test is not valid unless the resolution between the peaks due to 2-Fluoro-2-deoxy-D-glucose and Fluorodeoxyglucose impurity A (2-Chloro-2-deoxy-D-glucose) is not less than 1.5 and signal to noise ratio for the peak due to 2-Fluoro-2-deoxy-D-glucose is not less than 10. The relative retention time, with reference to 2-Fluoro-2-deoxy-D-glucose (retention time about 12 min), for Fluorodeoxyglucose impurity A is about 1.1.

Fluorodeoxyglucose impurity B or aminopolyether. Determine by thin-layer chromatography (2.4.17), silica gel G coated plates.

Mobile phase. A mixture of 90 volumes of *methanol* and 10 volumes of 30 per cent *ammonium hydroxide.*

Test solution. The injection under examination.

Reference solution (a). *water.*

Reference solution (b). A 0.22 per cent w/v solution of *aminopolyether IPRS (Fluorodeoxyglucose impurity B IPRS)* in *water.* Dilute 1 ml of the solution to appropriate volume equivalent to the maximum recommended dose in milliliters (V) with *water.*

Apply to the plate 2.5 µl of each solution and 2.5 µl of the test solution and reference solution (b) at the same place. Visually compare the spots 1 minute after application. The spots due to the successive application of the test solution and reference solution (b) is similar in appearance to the spot due to reference solution (b), characterised by a number of concentric circles; the darker innermost circle (of intensity proportional to the concentration of Fluorodeoxyglucose impurity B) may be surrounded by a bluish-black ring, outside of which is a lighter circle surrounded by a peripheral dark edge. The spot due to reference solution (a) has a more diffuse inner circle, which is brownish-pink and without a distinct margin between it and the surrounding lighter zone. The spot due to reference solution (b) is clearly different from the spot due to reference solution (a).

The central portion of the spot due to the test solution is less intense than that of the spot due to reference solution (b) (2.2 mg per *V*). The test must be completed before release of drug product.

Fluorodeoxyglucose impurity C or tetrabutylammonium hydroxide. Determine by liquid chromatography (2.4.14).

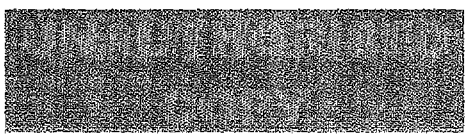
Test solution. The injection under examination.

Reference solution (a). A 0.27 per cent w/v solution of *tetrabutylammonium hydroxide (Fluorodeoxyglucose impurity C)* in *water.* Dilute 1.0 ml of this solution to appropriate volume equivalent to the maximum recommended dose in milliliters (V) with *water.*

Reference solution (b). A 0.26 per cent w/v solution of *tetrabutylammonium hydroxide* in *water.* Dilute 1 ml of this solution to 25 ml with *water.*

Chromatographic system
- a stainless steel column 10 cm × 4.6 mm, packed with octadecylsilane bonded to porous silica (3 µm),
- column temperature: 20° to 25°,
- mobile phase: a mixture of 25 volumes of a 0.095 per cent w/v solution of *toluenesulphonic acid* and 75 volumes of *acetonitrile,*
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 254 nm,
- injection volume: 20 µl.

Inject reference solution (b). The test is not valid unless the signal-to-noise ratio for the principle peak is not less than 10 and the symmetry factor is not more than 1.8.



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Inject the test solution and reference solution (a). Run the chromatogram for twice the retention time of tetrabutylammonium ions. In the chromatogram obtained with the test solution, the area of the peak due to Fluorodeoxyglucose impurity C is not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (2.75 mg per *V*).

Fluorodeoxyglucose Impurity D or 4-(4-methylpiperidin-1-yl) pyridine. Measure the absorbance of the injection under examination and the reference solution, [prepared by dissolving 20.0 mg of *4-(4-methylpiperidin-1-yl)pyridine (Fluorodeoxyglucose impurity D)*, dissolve in sufficient *water* to produce 100.0 ml. Dilute 0.1 ml of the solution to a volume equivalent to maximum recommended dose in milliliters with *water*] at the maximum of about 263 nm (2.4.7).

The absorbance of the injection under examination is not more than that of the reference solution (0.02 mg per *V*).

Other tests. Complies with the tests stated under Radiopharmaceuticals Preparation. The preparation may be released for use before completion of the tests.

Radioactivity assay. Use of suitably calibrated system to determine the total radioactivity of the Fluorodeoxyglucose (^{18}F) Injection in megabecquerals (or millicuries) and the radioactivity concentration in MBq per ml or mCi per ml. The test must be completed before release of drug product.

Radionuclidic purity. Either tests A and C or tests B and C may be applied. The test A or test B must be completed before release of the preparation. Test C is to be done on samples, after the decay of fluorine-18, periodically, once a month after the initial validation.

A. Determine by gamma-ray spectrometry using Sodium Iodide (Thallium) detector or Semiconductor detector. No less than 90per cent of the observed gamma-ray spectrum is concordant with that of gamma photons with energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed. The test must be completed before release of drug product.

B. Determine by gamma-ray counter. The half-life is between 105 min and 115 min determined with a gamma ray counter, by not less than three measurements of the radioactivity of a sample in the same geometrical conditions within a suitable period of decay time (for example, three readings at 10 minute intervals over 30 minutes) is between 105 and 115 minutes. The test must be completed before release of drug product.

C. The amount of Fluorine-18 and Radionuclidic impurities with a half-life longer than 2 hours. For the detection and quantification of impurities, retain the preparation under examination for minimum 24 hour to allow the Fluorine-18 to decay to a level that permits the detection of impurities. The total radioactivity due to Radionuclidic impurities is not more than 0.1 per cent. The injection may be released for use before completion of the test.

Radiochemical purity. Determine by Method A or by Method B. The test must be completed before release of the product.

Method A. Determine by liquid chromatography (2.4.14) as described in the test for 2-Fluoro-2-deoxy-D-glucose and Fluorodeoxyglucose impurity A.

If necessary, dilute the test solution with *water* to obtain a radioactivity concentration suitable for the radioactivity detector.

Inject the test solution and reference solutions (a) and (c) sequentially. Partially and fully acetylated derivatives of both compounds hydrolyse under the chromatographic conditions and therefore elute as 2-[^{18}F] Fluoro-2-deoxy-D-glucose and 2-[^{18}F] Fluoro-2-deoxy-D-mannose. Locate the peaks due to 2-[^{18}F] Fluoro-2-deoxy-D-glucose and 2-[^{18}F] Fluoro-2-deoxy-D-mannose using the chromatograms obtained with the carbohydrate detector and reference solutions (a) and (c). Fluorine-18 in the form of 2-[^{18}F] Fluoro-2-deoxy-D-glucose and 2-[^{18}F] Fluoro-2-deoxy-D-mannose not less than 95.0 per cent of the total radioactivity; 2-[^{18}F]Fluoro-2-deoxy-D-mannose is not more than 10 per cent of the total radioactivity due to 2-[^{18}F]Fluoro-2-deoxy-D-glucose and 2-[^{18}F]Fluoro-2-deoxy-D-mannose.

The test is not valid unless the resolution between the peaks due to 2-Fluoro-2-deoxy-D-mannose and 2-Fluoro-2-deoxy-D-glucose (see with reference solution (c)) is not less than 1.5 and signal to noise ratio for the peak due to 2-Fluoro-2-deoxy-D-glucose is not less than 10. The relative retention time, with reference to 2-Fluoro-2-deoxy-D-glucose (retention time about 12 min), for 2-Fluoro-2-deoxy-D-mannose is about 0.9.

Method B. Determine by thin-layer chromatography (2.4.17), using silica gel as stationary phase.

Mobile phase. A fresh mixture of 5 volumes of *water* and 95 volumes of *acetonitrile.*

Test solution. The injection under examination.

Reference solution. Dissolve, with gentle heating, 30 mg of *1, 2, 3, 4-tetra-O-acetyl-β-D-glucopyranose IPRS* and 20 mg of *glucose* in 1 ml of *water.*

Apply to the plate 2 µl of test solution, appropriately diluted if necessary to give optimum count rate. Develop over a path of 8 cm using the above mobile phase in a suitable chamber pre-saturated with the mobile phase. Allow the plate to dry in air for 1-2 min. Determine the radioactivity distribution in the chromatogram using a suitable method or a radio chromatographic TLC scanner. In this system 2-[^{18}F]fluoro-deoxy-glucose has an R_f of 0.45 ± 10 per cent, which will depend on the saturation in the chromatographic chamber,

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ambient temperature etc. The R_f of [^{18}F] fluoride is 0. Not less than 90 per cent of the total radioactivity is in the spot corresponding to 2-[^{18}F]fluoro-deoxy-glucose.

Retardation factors for [^{18}F] Fluoride is about 0; for 2-[^{18}F]Fluoro-2-deoxy-D-glucose and 2-[^{18}F]Fluoro-2-deoxy- D-mannose is about 0.45 ±10 per cent; for partially or fully acetylated derivatives of 2-[^{18}F]Fluoro-2-deoxy-D-glucose and 2-[^{18}F]Fluoro-2-deoxy-D-mannose have a R_f of about 0.8 to 0.95.

Apply to the plate reference solution. The test is not valid unless the chromatogram shows 2 clearly separated spots.

Fluorine-18 in the form of 2-[^{18}F]Fluoro-2-deoxy-D-glucose and 2-[^{18}F]Fluoro-2-deoxy-D-mannose not less than 95 per cent of the total radioactivity, Fluorine-18 in the form of Fluoride and partially or fully acetylated derivatives of 2-[^{18}F]Fluoro-2-deoxy-D-glucose and 2-[^{18}F]Fluoro-2-deoxy-D-mannose not more than 5 per cent of the total radioactivity.

Validation to be done periodically with every batch of silica gel TLC plates that is procured for the purpose, or once in three months if following the TLC procedure (Test B) for Radiochemical purity.

Apply to the plate 2 µl of each reference solution (a) and test solution. Develop over a path of 8 cm using a mixture of 5 volumes of *water* and 95 volumes of *acetonitrile*. Allow the plate to dry in air for 15 min. Immerse the plate in a 7.5 per cent w/v solution of *sulphuric acid* in *methanol* and dry with a hot air gun or at 150° until the appearance of dark spots in the chromatogram obtained with the reference solution. The R_f of the reference solution and the test solution should be similar.

Residual solvents. Estimate by gas chromatography (2.4.13). Not more than 0.04 per cent acetonitrile, 0.5 per cent dehydrated ethanol, 0.5 per cent ether and 0.5 per cent acetone. The reference standards for comparison are made with these solvents in water at the percentages specified. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). By LAL Test. Not more than 175 per V EU per ml, V being the maximum recommended dose in milliliters. Parametric release of the product can be made before the test. However, there is a kinetic LAL test, which can be completed within 20 minutes before the release of the product. The test must be completed before release of the preparation.

Sterility (2.2.11). Complies with the test for sterility. The test must be initiated within 24 hours however, the preparation may be released for use before completion of test.

Storage. Store in sterile and endotoxins free containers either as single dose or multiple doses. The containers should be adequately shielded for radiation safety.

Labelling. The label states, in addition to the information specified under injections (1) the amount of ^{18}FDG expressed in megabecquerels (microcuries or millicuries) per ml at the time of calibration; (2) the date of calibration; (3) the maximum recommended dose in milliliters; (4) the expiration date and time; (5) the statement "Caution—Radioactive Material" and "do not use if cloudy or if it contains particulate matter"; (6) in making dosage calculations, correction is to be made for radioactive decay; (7) radioactive half-life of ^{18}F is 109.7 minutes.

Gallium (^{68}Ga) Chloride Solution for Radiolabelling

Category. Diagnostic.

Description. A clear, colourless solution. The gallium (^{68}Ga) chloride solution for radiolabelling containing gallium-68 in the form of gallium chloride in dilute *hydrochloric acid* and it may contain *acetone*. The solution contains not less than 90 per cent and not more than 110 per cent of the stated gallium-68 radioactivity at the date and time stated on the label. Use the gallium (^{68}Ga) chloride solution for radiolabelling within 2 hour from date and time of preparation.

Identification

A. Determine by gamma-ray spectrometry as described in general chapter on Radiopharmaceutical Preparations. The most prominent gamma photon of Gallium chloride has energy 0.511 MeV and 1.077 MeV and sum peak of energy 1.022 MeV.

B. Determine the approximate half-life by no fewer than 3 measurements of the activity of a sample in the same geometrical conditions within a suitable period of time. (62 to 74 minutes).

These above test are not mandatory if the gallium (^{68}Ga) is obtained from ^{68}Ge-Ga^{68} generator with certificate of analysis meeting the above specification.

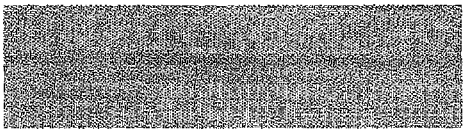
Tests

pH (2.4.24). Not more than 2.0.

Radioactivity assay. Radioactivity of an aliquot of test solution is assayed using precalibrated radioactivity dose calibrator or by comparison with a standardized fluorine F^{18}. It is expressed as MBq/ml at a particular time and date.

Radionuclidic purity. Determine by Gamma-ray-spectrometry.

Determine by gamma-ray spectrometry. Not less than 99.9 per cent of the observed gamma-ray spectrum is concordant with that of gamma photons with energy of 0.511 MeV, 1.077 MeV, 1.022 MeV and 1.883 MeV represent not more than 0.1 per cent of the total radioactivity.



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This above test is not mandatory if the gallium (^{68}Ga) is obtained from ^{68}Ge-Ga^{68} generator with certificate of analysis meeting the above specification.

The preparation may be released for use before completion of test.

Radiochemical purity. Determine by thin layer chromatography (2.4.17), coating the plate with silica gel.

Mobile phase. A mixture of 50 volumes of 7.7 per cent solution of *ammonium acetate* and 5 volumes of *methanol.*

Test solution. A solution containing 1.03 per cent v/v of substance under examination in *hydrochloric acid.*

Reference solution (a). Dilute 0.2 ml of the test solution adds 0.3 ml of 0.4 per cent w/v solution of *sodium hydroxide.* Use within 30 minute of preparation.

Reference solution (b). Dilute 1.0 ml of the test solution; add 1.0 ml of 1.0 per cent w/v solution of *pentetic acid* in a 0.4 per cent solution of *sodium hydroxide.* Use within 30 minute of preparation.

Apply to the plate 5.0 µl of reference solution (a) and (b). Apply to the plate 5 µl of test solution. Develop over a path of 10 cm using the above mobile phase in a suitable chamber presaturated with the mobile phase. Allow the plate to dry in air. Determine the radioactivity distribution in the chromatogram using a suitable method or a ratio chromatographic TLC scanner. The test is not valid unless the R_f of the principal peak in the chromatogram obtained with reference solution (a) is not more than 0.1 and the R_f of the principal peak in the chromatogram obtained with reference solution (b) is not less than 0.7. The (^{68}Ga) gallium(III) ion has an R_f of 0.0 to 0.2.

Not less than 95.0 per cent of the total radioactivity due to (^{68}Ga) gallium(III).

Chemical purity

Iron Content. Not more than 10 µg per GBq. Determine by atomic absorption spectrometry (2.4.2), measuring at 248.3 nm using a graphite furnace and iron hollow cathode lamp.

Modifier solution. A solution containing 1.4 per cent w/v solution of *magnesium nitrate.*

Test solution. Dilute the solution under examination with 1.0 per cent v/v solution of *nitric acid* to obtain a radioactivity concentration of 2.5 MBq per ml .

Reference solutions. Prepare the reference solutions using *iron standard solution (20 ppm Fe) R,* diluting with a 1 per cent v/v solution of *nitric acid* .

Internal and external protective gas: Argon

Injection temperature: 20°

Furnace programming:

Step	Final Temperature	Ramp time (s)	Hold time (s)	Internal protective gas flow rate (ml per min)
Drying	110	1	30	250
Drying	1301	5	30	250
Pyrolysis	1400	10	20	250
Atomization	2100	0	5	0
Cleaning	2450	1	3	250

Zinc Content. Not more than 10 µg/GBq. Determine by atomic absorption spectrometry (2.4.2), measuring at 248.3 nm using a air-acetylene flame and zinc hollow cathode lamp.

Test solution. Dilute the solution under examination with 1.0 per cent v/v solution of *nitric acid* to obtain a radioactivity concentration of 50 MBq per ml .

Reference solution. Prepare the reference solutions using *zinc standard solution (10 ppm Zn),* diluting with a 1.0 per cent v/v solution of *nitric acid* .

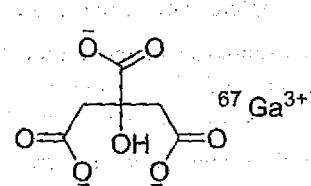
These above test are not mandatory if the gallium (^{68}Ga) is obtained from ^{68}Ge-Ga^{68} generator with certificate of analysis meeting the above specification.

Bacterial endotoxins (2.2.3). Test not more than 175 per V EU per ml, V being the maximum recommended dose in milliliters.

Sterility (2.2.11). Complies with the test for sterility. The test must be initiated within 24 hours however, the preparation may be released for use before completion of test.

Labelling. The label states that the solution is not intended for direct administration to humans; the maximum volume that can be used for the preparation of a single patient dose; the concentration of hydrochloric acid; the concentration of acetone, if present; that the solution is intended for use in the preparation of gallium-68-labelled radiopharmaceuticals; a procedure to reduce the level of germanium-68 below 0.001 per cent of the total radioactivity.

Gallium (^{67}Ga) Citrate Injection



Category. Diagnostic

Description. Gallium (^{67}Ga) Citrate Injection is a clear, colourless, sterile, aqueous solution and ready for use. The

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injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated content of gallium-67 on the label at the reference date stated on the label. Not less than 99 per cent of the total radioactivity is due to gallium-67. Not less than 95.0 per cent of the total gallium-67 radioactivity is present as gallium citrate. Not more than 0.2 per cent of the total radioactivity is due to gallium-66. Gallium (^{67}Ga) Citrate Injection should be used within 7 days from the date of preparation.

Identification

A. Determine by gamma-ray spectrometry as described in general chapter on Radiopharmaceutical preparations. The gamma-ray spectrum exhibits major peaks having energies of 0.093 MeV, 0.185 MeV and 0.300 MeV. Gallium-66 which is an impurity has a half-life of 9.4 hours with a main peak of 1.039 MeV.

B. To 0.2 ml of the injection under examination add 0.2 ml of a solution containing 0.1 per cent w/v solution of *ferric chloride* and 0.1 per cent v/v solution of *hydrochloric acid* and mix. Compare the colour with that of a 0.9 per cent w/v solution of *benzyl alcohol* and 0.7 per cent solution of *sodium chloride* treated in the same manner. A yellow colour develops in the test solution only.

Tests

pH (2.4.24). 5.0 to 8.0.

Radioactivity assay. Radioactivity of an aliquot of test solution is assayed using precalibrated radioactivity dose calibrator. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Record the gamma-ray spectrum using a suitable instrument and measure the half-life using a suitable method. Determine the relative amounts of gallium-67, gallium-66 and other radionuclidic impurities that may be present. Not less than 99.0 per cent of the total radioactivity is due to gallium-67 and not more than 0.2 per cent of the total radioactivity is due to gallium-66.

Radiochemical purity. Determine by ascending paper chromatography (2.4.15).

Paper. Chromatographic paper.

Mobile phase. Dissolve 1.36 g of *sodium acetate* and 0.58 ml of *glacial acetic acid* in 100 ml of *water*.

Apply 5 µl of the injection under examination to the paper strip. Allow the mobile phase to rise up to 8 to 10 cm from the point of spotting, allow the strip to dry and determine the radioactivity distribution by a gamma counter. It should be not less than 95.0 per cent of the total radioactivity is found as gallium citrate when measured at the solvent front. The R_f value is equal to or greater than 0.9.

Chemical purity

Zinc. Not more than 5 ppm

Test solution. To 0.1 ml of the injection under examination add 0.9 ml of *water*, 1 ml of a 25 per cent w/v solution of *sodium thiosulfate*, 5 ml of *acetate buffer solution pH 4.7* and 5.0 ml of a 0.001 per cent w/v solution of *dithizone* in *methyl ethyl ketone* allow to stand for 5 minutes, filter and immediately before use dilute the solution to 10 times its volume with *methyl ethyl ketone*. Shake vigorously for 2 minutes and allow the organic layer to separate.

Reference solution. 0.1 ml of *zinc standard solution (5 ppm Zn)* prepared in the same manner as the test solution.

Measure the absorbance of the organic layers at 530 nm, using the organic layer prepared from a blank solution as the compensation liquid. The absorbance of the organic layer obtained with the test solution is not greater than that of the organic layer obtained with the reference solution.

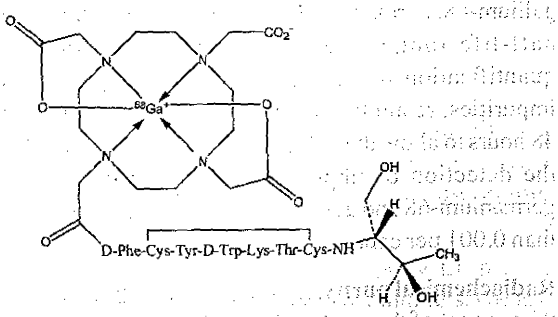
Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparations. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in ml. Alternatively the preparation should pass the pyrogen test (2.2.8).

Storage. Store at a temperature between 2° to 8°.

Labelling. The label states, in addition to the information specified under injections (1) the time and date of calibration; (2) the amount of ^{67}Ga as labelled gallium citrate expressed as total MBq (mCi or µCi) and concentration as MBq (mCi or µCi) per ml at the time of calibration; (3) the expiration date; and (4) the statement "Caution- Radioactive Material."

Gallium (^{68}Ga)Edotreotide Injection



$C_{65}H_{89}{}^{68}GaN_{14}O_{18}S_2$ Mol. Wt. 1487.0

Category. Diagnostic

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Description.A clear colourless solution.Gallium (^{68}Ga)Edotreotide Injection is a complex of gallium-68 with edotreotide (*N*-[[1,4,7,10- tetraazacyclododecan-l-yl]acetyl]-*D*-phenylalanyl-l-cysteinyl-*L*-tyrosyl-*D*-tryptophyl-*L*-lysyl-*L*-threonyl-*N*-[(1*R*,2*R*)-2-hydroxy-*L*-(hydroxymethyl)propyl]-*L*-cysteinamide cyclic (2 -7)-disulphide) (gallium-68 DOTATOC). Gallium (^{68}Ga) edotreotide injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared gallium-68 radioactivity at the date and time on the label. Edotreotide maximum 50 µg per maximum recommended dose in millilitres.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photons of have energy of 0.511 MeV and 1.077 MeV, depending on the measurement geometry, a sum peak of 1.022 Mev may be observed.

B. Determine the approximate half-life by no fewer than 3 measurements of the activity of a sample in the same geometrical conditions within a suitable period of time (for example, 15 minutes) is between 62 minutes to 74 minutes.

C. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of Gallium (^{68}Ga) injection. The principal peak in the radio chromatogram obtained with the test solution has a relative rention time of 1-3 with reference to the principal peak in the chromatogram obtained with reference solution (a).

Tests

pH(2.4.24). 4.0 to 8.0.

Radionuclidic purity. Determine by gamma ray spectrometry. Not less than 99.9 per cent of the total radioactivity due to gallium-68. Peaks in the gamma-ray spectrum corresponding to photons with an energy different from 0.511 MeV, 1.077 MeV,1.022 MeV and 1.883 MeV represent not more than 0.1 per cent of the total radioactivity. Determine the amount of gallium-68, germanium-68 and radionuclidic impurities with a half-life longer than 5 hours. For the detection and quantification of germanium-68 and gamma-ray-emitting impurities, retain the preparation to be examined for at least 48 hours to allow the gallium-68 to decay to a level that permits the detection of impurity. The total radioactivity due to germanium-68 and gamma-ray-emitting impurities is not more than 0.001 per cent.

Radiochemical purity. (^{68}Ga) gallium edotreotide: not less than 91 per cent of the total radioactivity due to gallium-68.

Impurity A [(^{68}Ga) gallium in colloidal form]. Not more than 3 per cent of the total radioactivity due to gallium-68.

Determine by thin-layer chromatography (2.4.17), coating the plates with *silica gel GF254*.

NOTE —Use the solution within 30 minutes.

Mobile phase. A mixture of 50 volumes of a buffer solution prepared by dissolving 77 g of *ammonium acetate* in 1000 ml of *water* and 50 volumes of *methanol*.

Test solution. The injection under examination.

Reference solution (a). Dilute *gallium (^{68}Ga) chloride solution* with *water* to obtain a final concentration of 10 g per liter of *hydrochloric acid*. To 1 ml of this solution add 1.5 ml of a 4 g per liter solution of *sodium hydroxide*.

Reference solution (b). Dilute *gallium (^{68}Ga) chloride solution* with *water* to obtain a final concentration of 10 g per liter of *hydrochloric acid*. To 1 ml of this solution add 1.0 ml of a solution containing 10 g per liter of *pentetic acid* and 4 g per liter solution of *sodium hydroxide*.

Retardation factor Impurity A is 0.0 to 0.1 and (^{68}Ga) gallium edotreotide is 0.8 to 1.0.

Apply 5 µl of each solution and dry with a current of air after each application. Development over 2/3 of the plate.The retardation factor of the principal signal in the chromatogram obtained with reference solution (a) is not more than 0.1; the retardation factor of the principal signal in the chromatogram obtained with reference solution (b) is more than 0.7.

Other radiochemical impurity. Determine by liquid chromatography (2.4.14) As described under edotreotide, galliumedotreotide and other related substances. If, necessary dilute the test solution with *water* to a radioactivity concentration suitable for the radioactivity detector.

Examine the chromatogram obtained using the radioactivity detector and locate the peak due to (^{68}Ga) gallium edotreotideby comparison with the chromatogram obtained with reference solution (a) and the spectrophotometer.

Relative retention with reference to [^{68}Ga] gallium edotreotide (retention time about 4.2 minutes and impurity B about 0.3.

Impurity B ([68 Ga] gallium (III) ion) is not more than 2 per cent of the total radioactivity due to gallium-68.

Calculate the percentage of radioactivity due to [^{68}Ga] gallium edotreotide using the following expression:

$$(100 - A) \times T$$

Where A is percentage of radioactivity due to impurity A determined in the test for impurity A under radiochemical purity and T is proportion of the area of the peak due to [^{68}Ga] gallium edotretide relative to the total areas of the peaks in the chromatogram obtained with the test solution.

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Edotreotide, gallium edotreotide and other related substances. Determine by liquid chromatography (2.4.14)

Test solution. The injection under examination.

Reference solution (a). Prepare a 50 µg per V solution of *edotreotide* in a 10.3 g per liter solution of *hydrochloric acid*, V being the maximum recommended dose in millilitres.

Reference solution (b). Prepare a 50 µg per V solution of *edotreotide acetate* in a 10.3 g per liter solution of *hydrochloric acid*, V is the maximum recommended dose in millilitres.

Reference solution (c). Mix 0.1 ml of reference solution (a) and 0.1 ml of reference solution (b).

Chromatographic system

- a stainless steel column 15 cm x 3.0 mm, packed with octadecylsilane base-deactivated bonded to porous silica gel (3 µm),
- column temperature: 20° to 30°,
- mobile phase: A. a mixture of 0.1 volume of *trifluoroacetic acid* and 99.9 volumes of *water*,
  B. a mixture of 0.1 volume of *trifluoroacetic acid* and 99.9 volumes of *acetonitrile*,
- a gradient programme using the conditions given below,
- flow rate: 0.6 ml per minute,
- spectrophotometer set at 220 nm,
- radioactivity detector connected in series,
- injection volume: 20 µl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	76	24
8	76	24
9	40	60
14	40	60

Inject reference solution (c). The test is not valid unless the resolution between the peaks due to edotreotide and octreotide is not less than 5.

Inject the reference solution (a). and (c). The test is not valid unless the edotreotide and metal complex of edotreotide (sum of the areas of the peaks with a relative retention with reference to edotreotide between 0.8 and 1.4) not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (50 µg per V). Unknown impurities: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (50 µg per V). Ignore any peak with a relative retention with reference to edotreotide of 0.5 or less.

Impurity D. Determine bythin-layer chromatography (2.4.17), coating the plates with *silica gel GF254*.

Mobile phase. A mixture of 25 volumes of *water* and 75 volumes of *acetonitrile*.

Test solution. The injection under examination.

Reference solution.Dissolve 10 mg of *impurity D IPRS* (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulphonic acid] in *water* and dilute to volume with the same solvent V being the maximum recommended dose in millilitres. Dilute to 1.0 ml of this solution to 50.0 ml with *water*.

Apply 1 µl of each solution and dry with a current of warm air after each application. Development over 2/3 of the plate. Detection expose to iodine vapour for 4 minutes. Retardation factor for impurity D is about 0.3 minute. Any spot due to impurity D is not more instance than the corresponding spot in the chromatogram obtained with the reference solution (200 µg per V).

Ethanol. Not more than 10 per cent v/v, and maximum 2.5 g per administration.

Determine by gas chromatography (2.4.13).

Internal standard solution. Dilute 1 ml of *propanol* to 1000 ml with *water*.

Test solution. Dilute 0.1 ml of the preparation under examination to 10.0 ml with the internal standard solution.

Reference solution. Dilute 1.0 ml of *anhydrous ethanol* to 100.0 ml with the internal standard solution. Dilute 1.0 ml of this solution to 10.0 ml with the internal standard solution.

Chromatographic system

- a fused silica column 30 m x 0.53 mm, packed with macrogol 20000 (film thickness 1.0 µm),
- temperature:
  column. 35°,
- inlet port 140° and detector. 220°,
- flow rate: 10 ml per minute, using helium as the carrier gas.

The retention time for ethanol is about 2 minutes to 4 minutes.

Inject 1 µl each of the reference solution. The test is not valid unless the resolution between peaks due to ethanol and propanol is not less than 5.0.

Inject the reference solution and the test solution.

Calculate the content of ethanol.

Sterility (2.2.11).Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

Bacterial Endotoxins (2.2.3). Less than 175 per V/EU per ml, V being the maximum recommended dose in milliliters.The test must be completed before release .

Labelling. The label states, the per cent content of ethanol in the preparation.

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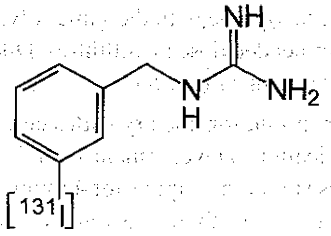
(^{131}I) Meta-Iodobenzyl Guanidine Injection for Diagnostic Use

Iobenguane Injection for Diagnostic Use



Category. Diagnostic.

Description. A clear, colourless or slightly yellow solution. On standing the solution may darken as a result of the effect of radiation. Radioactive concentration should be in the range of 0.25 to 1 mCi per ml.

(^{131}I) Meta-Iodobenzyl Guanidine Injection for diagnostic use is a sterile, apyrogenic solution of 1-(3-[^{131}I] iodobenzyl) guanidine or its salts. It may contain a suitable buffer. It may also contain a suitable labelling catalyst such as ionic copper and a suitable labelling stabiliser such as ascorbic acid. It may contain antimicrobial preservatives.

(^{131}I) Meta-Iodobenzyl Guanidine Injection for diagnostic use contains not less than 90.0 per cent and not more than 110.0 per cent of the stated iodine-131 radioactivity at the date and time stated on the label. Not less than 95 per cent of the radioactivity corresponds to iodine-131 in the form of Meta-Iodobenzyl Guanidine. Use the (^{131}I) Meta-Iodobenzyl Guanidine Injection for diagnostic use within 3 days from reference date.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon of iodine-131 has energy of 0.365 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of the (^{131}I) Meta-Iodobenzyl Guanidine.

Tests

pH (2.4.24). 5.0 to 8.0.

Radioactivity assay. Measure the radioactivity using a suitable counting apparatus by comparison with a reference solution of iodine-131 or by measurement in an instrument calibrated with the aid of such a solution.

Radionuclidic purity. Determine by Gamma-ray spectrometry. Not less than 99.9 per cent due to iodine-131. Examine the gamma ray spectrum of the preparation. The most prominent gamma photon has energy of 0.364 MeV.

Radiochemical purity. Determine by liquid chromatography (2.4.14).

Test solution. The injection under examination.

Reference solution. Dissolve an accurately weighed quantity of *mIBG sulphate IPRS* in *water* to obtain a solution having a known concentration of 100 to 500 µg per ml.

Chromatographic system:

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to silica gel (5µm),
- mobile phase: Add 3.04 g of *triethylamine* per liter of HPLC grade water, and adjust pH of 4.0 ± 0.4 with phosphoric acid (This may be called 'A'). The mobile phase consists of 80 volumes of 'A' and 20 volumes of *acetonitrile*. Degass before use,
- flow rate: 1.0 ml per minute,
- a suitable radioactivity detector,
- a spectrophotometer set at 229 nm,
- injection volume: 20 µl.

Inject the test solution and reference solution. In the chromatogram obtained with the test solution the radioactivity of the ^{131}I mIBG peak is not less than 95per cent of the total radioactivity measured, and its retention time is within 10 per cent of that of the Standard preparation, similarly chromatographed.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The test should be commenced within 48h of completion of preparation. However the injection may be released for use before completion of the test.

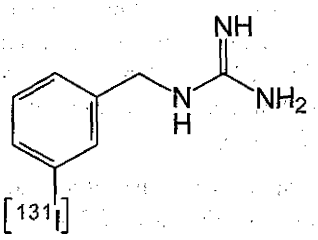
Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in milliliters. The test must be completed before release.

Storage. Store frozen at ≤ 0° with adequate shielding

Labelling. The label states in addition to the information specified under injections (1) the amount of ^{131}I as total megabecquerels (millicuries) and concentration as megabecquerels (millicuries) per ml on the date and time of preparation; (2) the expiration date and time; (3) the statement "Caution — Radioactive Material."

(^{131}I) Meta-Iodobenzyl Guanidine Injection for Therapeutic Use

Iobenguane Injection for Therapeutic Use



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Category. Therapeutic.

Description. A clear, colourless or slightly yellow solution. On standing, the solution may darken as a result of the effect of radiation. (^{131}I) Meta-Iodobenzyl Guanidine Injection for therapeutic use is a sterile solution and contains not less than 90.0 per cent and not more than 110.0 per cent of the stated iodine-131 radioactivity at the date and time stated on the label. Not less than 95 per cent of the radioactivity corresponds to iodine-131 in the form of Meta-Iodobenzyl Guanidine. The specific radioactivity is not less than 370 GBq (10 Ci) of iodine-131 per gram of iobenguane base.

(^{131}I) Meta-Iodobenzyl Guanidine Injection contains 1-(3-[^{131}I] iodobenzyl) guanidine or its salts. It may contain a suitable buffer. It may also contain a suitable labelling catalyst such as ionic copper and a suitable labelling stabilizer such as ascorbic acid. It may contain antimicrobial preservatives. Use the (^{131}I) Meta-Iodobenzyl Guanidine Injection for therapeutic use within 3 days from reference date.

Identification

A. Determine by gamma-ray spectrometry. The spectrum obtained corresponds to the spectrum obtained with the reference solution of Iodine 131. The most prominent gamma photon of iodine-131 has energy of 0.365 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of the (^{131}I) Meta-Iodobenzyl Guanidine.

Tests

pH (2.4.24). 5.0 to 8.0.

Radioactivity assay. Measure the radioactivity using a suitable counting apparatus or by measurement in an instrument calibrated with the aid of such a solution.

Radionuclidic purity. Determine by Gamma-ray spectrometry. Not less than 99.9 per cent due to iodine-131. The most prominent gamma photon of iodine-131 has energy of 0.364 MeV.

Radiochemical purity. Determine by liquid chromatography (2.4.14).

Test solution. The injection under examination.

Reference solution. Dissolve an accurately weighed quantity of mIBG sulfate in water to obtain a solution having a known concentration of 100 to 500 µg per ml.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to silica gel (5µm),
- mobile phase: Add 3.04 g of *triethylamine* per liter of HPLC grade water, and adjust pH of 4.0 ± 0.4 with phosphoric acid (This may be called 'A'). The mobile phase consists of 80 parts of 'A' and 20 parts of *acetonitrile*. Degass before use,
- flow rate: 1.0 ml per minute,
- a suitable radioactivity detector,
- a spectrophotometer set at 229 nm,
- injection volume: 20 µl.

Inject the test solution and reference solution. In the chromatogram obtained with the test solution the radioactivity of the ^{131}I mIBG peak is not less than 95 per cent of the total radioactivity measured, and its retention time is within 10 per cent of that of the Standard preparation, similarly chromatographed.

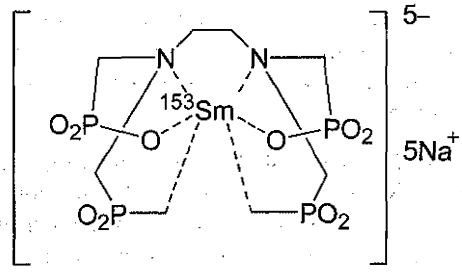
Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The test should be commenced within 48h of completion of preparation. However the injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum volume of injection. The test must be completed before release.

Storage. Store frozen at ≤ 0° with adequate shielding.

Labelling. The label states, in addition to the information specified under injections (1) the amount of ^{131}I as total megabecquerels (millicuries) and concentration as megabecquerels (millicuries) per ml at the time of preparation; (2) the expiration date and time; (3) the statement "Caution — Radioactive Material."

Samarium (^{153}Sm) Ethylene Diamine Tetramethylene Phosphonate (EDTMP) Injection



Category. Therapeutic.

Description. A clear, colourless to pale yellow, solution. Samarium (^{153}Sm) Ethylene Diamine Tetramethylene Phosphonate Injection is a sterile solution and contains not less than 90.0 per cent and not more than 110.0 per cent of the stated Samarium-153 radioactivity at the date and time stated

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on the label. Not less than 95 per cent of the radioactivity corresponds to samarium-153 in the form of Samarium-EDTMP. Use within three days from the reference date.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon of samarium-153 has an energy of 103KeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of ^{153}Sm-EDTMP.

Tests

pH (2.4.24). 7.0 to 8.0

Radioactivity assay. Measure the radioactivity using suitable counting equipment

Radionuclidic purity. Determine by gamma-ray spectrometry.

The total radioactivity due to radionuclidic impurities is not less than 99.8 per cent as samarium -153 and not more than 0.01 per cent of the total radioactivity is due to europium-154. The sum of all gamma-emitting impurities is not more than 0.2 per cent.

Calibrate the Gamma ray spectrometer using ^{152}Eu / ^{137}Cs as a standard source. Take a small aliquot of sample in a vial / tube and place on the detector connected to a multi channel analyzer. Acquire the counts for sufficient time interval. Identify the radionuclide ^{153}Sm by the photopeak corresponding to its principal gamma emission of 103 keV.

Radiochemical purity. Determine by liquid Chromatography (2.4.14).

Mobile phase. Transfer 8g of *sodium chloride*, 0.2g of *monobasic potassium phosphate*, 1.15g of *dibasic sodium phosphate* and 0.2g of *potassium chloride* to a 1 liter volumetric flask, dilute with distilled water to volume and mix.

Chromatographic system. A 10 x 40 mm glass chromatographic column is packed using gravity flow with a cation exchange resin (Such as Sephadex CM C-25) (prepared by mixing 5 g of resin with 25 ml of water in a suitable beaker) to a final resin volume of 0.5 ml.

Transfer the packed column to an ion-chamber or dose calibrator to determine the background count for Sm-153. Apply about 10 µl of the Injection on to the column and place it in the radioactivity counter, and record the total radioactivity. Elute the complexed radioactivity using about 20 ml of mobile phase and record the radioactivity retained on the column. Subtract the background radioactivity from all measured radioactivity values. Calculate the percentage of complexed radioactivity in the portion of the injection taken by the formula:

$$100\,(T-S/0.95)/T$$

In which T is the total amount of radioactivity; S is the quantity of Sm-153 retained on the column; and 0.95 is the correction factor (5 per cent of the uncomplexed Sm-153 passes through the column and into the Injection)

Not less than 95 per cent of the Sm-153 is complexed by EDTMP.

Bacterial endotoxins (2.2.3). Not more than 175 per V EU per ml, V being the maximum recommended dose in millilitres. The test must be completed before release.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

Storage. Store in a container with adequate shielding at 2 to 8°.

Labelling. The label states in addition to the information specified under injections (1) the name, time and date of Calibration; (2) the amount of ^{153}Sm as total megabecquerels (millicuries) and concentration as megabecquerels (millicuries) per ml at the time of calibration; (3) the expiration date; (4) the statement "Caution — Radioactive Material."

Samarium Phosphate (^{32}P) Colloid Injection

Category. Therapeutic

Description. Samarium phosphate (^{32}P) colloid injection is a white colloidal sterile solution of samarium phosphate (^{32}P) injection in isotonic solution. It should be used within 14 days from the date and time of preparation. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated ^{32}P-radioactivity at the date and time stated on the label.

Identification

A. Determine by β-ray spectrometry or half life method.

B. Measure radioactive concentration at time t_0 and after time t, (minimum one day). Calculate the expected radioactive concentration after time t using the equation $A_t = A_0\, e^{-\lambda t}$, where A_t = Activity measured after time t, A_0 = Activity measured earlier at time t_0, t = time elapsed between the 2 measurements, λ = decay constant and $e^{-\lambda t} = 0.953$ when t = 1day.

The measured radioactive concentration should be same as that expected.

Tests

pH (2.4.24). 5.0 to 8.0

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Radioactivity assay. Mix and dissolve 0.5 ml of colloidal samarium phosphate injection in 0.2 ml of *hydrochloric acid solution* and the total volume make up to 1 ml with distilled water. Radioactive concentration is then measure using a suitable precalibrated instrument.

Radionuclidic purity. No other extraneous β/γ emitting radionuclide shall be detectable as seen in a β-ray and γ-ray spectrometer .

Radiochemical purity. Determine by ascending paper chromatography (2.4.15). Not less than 95 per cent.

Mobile phase. Distilled water

Paper. Chromatographic paper (Whatman No.1) (2.5 cm x 15.0 cm)

Test solution. The injection under examination is diluted to approximately 50 µCi per ml.

Apply 5 to 10 µl of the injection to the two paper strips 3 cm from bottom end under examination. Allow the mobile phase to rise 10 cm from the point of spotting and dry the paper. Determine the distribution of radioactivity using a suitable radioactivity monitoring system. The R_f value of Samarium phosphate (^{32}P) colloid is 0 to 0.1 and of ionic phosphate (^{32}P) is at solvent front (R_f=1). The activity in the point of spotting should not be less the 95 per cent.

Bacterial Endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in ml. Alternatively the preparation should pass the pyrogen test (2.2.8).

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparations. The injection may be released for use before completion of the test.

Storage. Store in adequately shielded single dose or in multiple dose containers. Store at a temperature between 2° to 8°.

Labelling. The label states, in addition to the information specified under injections (1) the time and date of calibration; (2) the amount of ^{32}P as labelled samarium phosphate expressed as total megabecquerels (millicuries or microcuries) and concentration as megabecquerels (millicuries or microcuries) per ml at the time of calibration; (3) the expiration date and time and; (4) the statement "Caution- Radioactive Material."

Sodium Chromate (^{51}Cr) Injection

Category. Diagnostic

Description. Sodium Chromate (^{51}Cr) Injection is a sterile solution of radioactive chromium (^{51}Cr) processed in the form of sodium chromate in water for Injection. For those uses where an isotonic solution is required, sodium chloride may be added in appropriate amounts. Chromium 51 is produced by the neutron bombardment of enriched chromium 50. It contains not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of ^{51}Cr as sodium chromate expressed in megabecquerels (millicuries) per ml at the time indicated in the labelling. The sodium chromate content is not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount. The specific activity is not less than 370 megabecquerels (10 millicuries) per mg of sodium chromate at the end of the expiry period. Other chemical forms of radioactivity do not exceed 10.0 per cent of the total radioactivity.

Identification

Determine by gamma-ray spectrum is identical to that of a specimen of ^{51}Cr of known purity that exhibits a photopeak having an energy of 0.320 MeV.

Tests

pH (2.4.24). 7.5 to 8.5.

Radioactivity assay. Radioactivity of an aliquot of test solution is assayed using precalibrated radioactivity dose calibrator set for beta counting as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparations. It is expressed as MBq (µCi) per ml at a reference time and date.

Radiochemical purity. Place a volume of injection appropriately diluted so that it provides a count rate of about 20,000 counts per minute about 25 mm from one end of a 25 x 300 mm strip of chromatography paper (2.4.15) and immediately develop with a mixture of 5 volumes of *water*, 2 volumes of *dilute alcohol* (9.5 in 10) and 1 volume of *ammonium hydroxide*. Air dry the chromatogram and deterimine the radioactivity distribution by scanning the chromatogram with a suitable collimated radiation detector. The radioactivity of the chromate band is not less than 90 per cent of the total radioactivity. The R_f value for the chromate band falls within ± 10 per cent of the value found for a known sodium chromate specimen when determined under identical conditions.

Assay for sodium chromate. Determine by atomic absorption spectrophotometry (2.4.2).

Test solution. Use the injection.

Reference solution (a). Dissolve 3.735 g of *potassium chromate* in 1000 ml of water to obtain a solution having a known concentration of 1.0 mg per ml of chromium.

Reference solution (b). Pipette 0.025, 0.05, 0.075, 0.10, 0.125 and 0.150 ml of reference solution (a), accurately measured into separate 100-ml volumetric flasks. To each flask add 0.42 ml of *0.1 M sodium bicarbonate* and dilute with *water* to

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volume to obtain solutions having final concentration of 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50 µg of chromium per ml.

Blank solution. Transfer 0.42 ml of *0.1 M sodium bicarbonate* to a 100 ml volumetric flask and dilute with *water* to volume.

Concomitantly determine the absorbance of the test solution, reference solution (b) and the blank solution at the chromium emission line at 357.7 nm with a suitable equipped with a chromium hollow cathode lamp and an air acetylene (fuel rich) flame using *water* to set the instrument to zero. Plot the absorbance of the reference solution (b) and the blank solution versus concentration in µg per ml of chromium and perform a regression analysis. A suitable standard curve will have an intercept between -0.002 and + 0.002 and a regression coefficient of not less than 0.99. Using the standard curve so obtained determine the concentration C in µg per ml of chromium in the injection. Calculate the quantity of sodium chromate in µg per ml by the formula.

$$3.115 \times C$$

Where, 3.115 is the conversion factor.

Bacterial endotoxins (2.2.3). Less than 175 per VEU per ml, V being the maximum recommended dose in milliliters. Alternatively the preparation should pass the pyrogen test (2.2.8).

Storage. Store in single dose or in multiple-dose containers.

Labelling. The label states, in addition to the information specified under injections (1) the amount of sodium chromate expressed in µg per ml as ^{51}Cr expressed as total MBq (mCi or µCi) and concentration as MBq (mCi or µCi) per ml at the time of calibration; (2) the expiration date and time; (3) the statements "Caution-Radioactive Material". The radioactive half-life of ^{51}Cr is 27.8 days. " Do not use if cloudy or if it contains particulate matter".

Sodium Fluoride (^{18}F) Injection

Category. Diagnostic

Description. A clear, colourless solution. Sodium Fluoride (^{18}F) Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared fluorine-18 radioactivity at the date and time stated on the label. Fluorine in the form of fluoride is not more than 4.5 mg per maximum recommended dose, in milliliters. Use the Sodium Fluoride (^{18}F) Injection within 8 hours from the date and time of preparation.

Radionuclide Production. The radionuclide Fluorine-18 is most commonly produced by proton irradiation of *water* enriched in oxygen-18.

Radiochemical preparation. Sodium Fluoride (^{18}F) contains fluorine-18 in the form of sodium fluoride. Fluorine-18 in the form of fluoride is recovered from the irradiated [^{18}O] water, generally by adsorption on an anion exchange resin and desorption using 0.9 per cent sodium chloride solution from anion-exchange resins or electrochemical deposition and redissolution. It may contain carrier fluoride, sodium chloride and a suitable buffer.

Identification

A. Determine by gamma-ray spectrometry. The only gamma photons have energy of 0.511 MeV and, depending on the measurement geometry; a sum peak of 1.022 MeV may be observed.

B. Complies with test B for radionuclidic purity.

C. In the test for radiochemical purity by silica gel TLC, the principal peak in the radiochromatogram obtained with the test solution has an R_f of 0 is similar in retention time (remains at point of spotting) to the principal peak.

Tests

pH (2.4.24). 5.0 to 8.5.

Radioactivity Assay. Radioactivity of an aliquot of Test Solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Determine by gamma-ray spectrometry.

Either tests A and C or tests B and C may be applied. The test A or test B must be completed before release of the preparation. Test C is to be done on samples, after the decay of fluorine-18, periodically, once a month after the initial validation.

A. Determine by gamma-ray spectrometry using Sodium Iodide (Thallium) detector or Semiconductor detector. Not less than 90 per cent of the observed gamma-ray spectrum is concordant with that of gamma photons with energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed. The test must be completed before release of drug product.

B. Determine by gamma-ray counter. The half-life is between 105 min and 115 min determined with a gamma ray counter, by not less than three measurements of the radioactivity of a sample in the same geometrical conditions within a suitable period of decay time (for example, three readings at 10 minute intervals over 30 minutes) is between 105 and 115 minutes. The test must be completed before release of drug product.

C. The amount of Fluorine-18 and Radionuclidic impurities with a half-life longer than 2 hour. For the detection and quantification of impurities, retain the preparation under

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examination for minimum 24 hour to allow the Fluorine-18 to decay to a level that permits the detection of impurities. The total radioactivity due to Radionuclidic impurities is not more than 0.1 per cent. The injection may be released for use before completion of the test.

Radiochemical purity. Determine by Method A or Method B.

The test must be completed before release of the product. If necessary, dilute the test solution with *water* to obtain a radioactivity concentration suitable for the radioactivity detector. Not less than 98.5 per cent of the radioactivity is found in the peak corresponding to [^{18}F] fluoride.

Method A. Determine by liquid chromatography (2.4.14).

Test solution. The injection under examination.

Reference solution. Dissolve 10 mg of *sodium fluoride* in a volume equivalent to the maximum recommended dose in milliliters of *water*.

Chromatographic system
- a stainless steel column 25 cm × 4 mm, packed with anion-exchange resin (10 µm),
- column temperature: between 20° and 30°,
- mobile phase: *0.1 N sodium hydroxide*, protected from atmospheric carbon dioxide,
- flow rate: 1 ml per minute,
- spectrophotometer set at 220 nm,
- radioactivity detector connected in series,
- injection volume: 20 µl.

Inject the reference solution. The test is not valid unless the signal-to-noise ratio is not less than 10 for the principal peak and the retention time of fluoride is not less than 3 times the hold-up time. In the chromatogram obtained with the spectrophotometer the peak due to fluoride is not more than the area of the corresponding peak in the chromatogram obtained with the reference solution.

Method B. By Thin Layer Chromatography (2.4.17), using silica gel as stationary phase.

Mobile phase. A fresh mixture of 5 volumes of *water* and 95 volumes of *acetonitrile*.

Test solution. The injection under examination.

Apply to the plate 2 µl of test solution, appropriately diluted if necessary to give optimum count rate. Develop over a path of 8 cm using the above mobile phase in a suitable chamber pre-saturated with the mobile phase. Allow the plate to dry in air for 1-2 min. Determine the radioactivity distribution in the chromatogram using a suitable method or a radio-chromatographic TLC scanner. In this system [^{18}F] sodium fluoride has an R_f of 0. Not less than 98 per cent of the total radioactivity is in the spot corresponding to [^{18}F] fluoride.

The total fluoride content in the preparation may also be determined from the certificate of analysis provided with the [^{18}O] water used for making [^{18}F] fluoride, the water for injection (WFI) used and the sodium chloride used for making 0.9 per cent sodium chloride solution with WFI. If the total fluoride from all these sources is less than 4.5 mg per V, then it is not necessary to estimate the total fluoride content in each preparation of [^{18}F] NaF.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Not more than 175 per V EU per ml, V being the maximum recommended dose in milliliters. A parametric release of the injection for use may be made before completion of the test. However, there is a kinetic LAL test, which can be completed within 20 minutes before the release of the product.

Storage. Store in sterile and endotoxin free containers either as a single-dose or multiple-doses. The containers should be adequately shielded for radioactivity.

Labelling. The label states, in addition to the information specified under injections (1) the amount of ^{18}F as fluoride anion expressed in megabecquerels (microcuries or millicuries) per ml at the time of; (2) the date of calibration; (3) calibration the maximum recommended dose in milliliters; (4) the expiration date and time; (5) the statement " Caution — Radioactive Material" and "do not use if cloudy or if it contains particulate matter"; (6) in making dosage calculations, correction is to be made for radioactive decay; (7) radioactive half life of ^{18}F is 109.7 minutes.

Sodium Iodide (^{123}I) Injection (From ^{124}Xe)

Category. Diagnostic.

Description. Sodium Iodide (^{123}I) Injection is a clear colourless solution, Sodium (^{123}I) Iodide Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated Sodium (^{123}I) Iodide radioactivity at the date and time stated on the label. Sodium (^{123}I) Iodide Injection should be used within 48 hours from date and time of preparation. Iodine-123 is obtained by proton irradiation of xenon enriched in xenon-124 (minimum 98 per cent) followed by the decay of xenon.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon has energy of 0.159 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of Sodium Iodide (^{123}I) Injection.

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Tests

pH (2.4.24) 7.5 to 9.0.

Radioactivity assay. Radioactivity of an aliquot of test solution is assayed using precalibrated radioactivity dose calibrator set for gamma counting as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparations. It is expressed as MBq (μCi) per ml at a reference time and date.

Radionuclidic Purity. Determine by gamma ray spectrometry. Not less than 99.5 per cent of the total radioactivity due to Iodine-123, at the time of preparation and not less than 87 per cent at the time of expiration.

Determine the relative amounts of I-123. Retain the preparation to be examined for a sufficient time to allow I-123 to decay to a level which permits the detection of other radionuclidic impurities. No radionuclides with a half-life longer than that of I-125 are detected. The preparation may be released for use before completion of the test.

Radiochemical purity. Determine by liquid chromatography (2.4.14)

Test solution. The injection under examination diluted with 2.0 gm solution of *sodium hydroxide* in 1000 ml of *water*, radioactive concentration suitable for detector. Add an equal volume of a solution containing 1 gm of *potassium iodide*, 2 gm of *potassium iodate* and 10 gm of *sodium hydrogen carbonate* in 1000 ml of *water* and mix.

Reference solution (a) Dissolve 2.62 mg of *potassium iodide* in 100 ml of *water*. Dilute 1.0 ml to 10.0 ml with *water*.

Reference solution (b) Dissolve 2.45 mg of *potassium iodate* in 100 ml of *water* Dilute 1.0 ml to 10.0 ml with *water*. Mix equal volume of *reference solution* (a) and *reference solution* (b).

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica or ceramic microparticles (5 μm),
- column temperature: 20° to 30°,
- mobile phase: a mixture of a buffer solution prepared by dissolving 5.85 g of *sodium chloride* and 0.65 ml of *octylamine* in 1000 ml of *water* and adjusted to pH 7.0 with dilute *phosphoric acid*, add 50 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 μl,
- run time: about 12 minutes.

The relative retention time with reference to iodide (retention time = about 5 min) for a iodate is about 0.2 to 0.3.

Inject reference solution solution (b) The test is not valid unless the resolution between the peaks due to the iodide and the iodate is not less than 2.0.

Inject the test solution and reference solution (a). In the chromatogram obtained with the test solution the radioactivity of the ^{123}I peak is not less than 95 per cent of the total radioactivity measured.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

Bacterial Endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in milliliters. Alternatively the preparation should pass the pyrogen test (2.2.8).

Storage. At 25° room temperature with adequate shielding.

Labelling. The label states, in addition to the information specified under injections (1) the amount of ^{123}I as labeled sodium iodide expressed as total MBq (mCi or μCi) and concentration as MBq (mCi or μCi) per ml at the time of calibration; (2) the expiration date and time; (3) the statements "Caution–Radioactive Material".

Sodium Iodide (^{123}I) Solution For Radiolabelling

Description. Sodium Iodide (123I) Solution For Radiolabelling is a clear colourless solution, Sodium Iodide (123I) Solution For Radiolabelling contains not less than 90.0 per cent and not more than 110.0 per cent of the stated iodine-123 radioactivity at the date and time stated on the label. Sodium Iodide (123I) Solution For Radiolabelling should be used within 24 hours from date and time of preparation. Iodine-123 is obtained by proton irradiation of xenon enriched in xenon-124 followed by the decay of xenon-123 which is formed directly and by the decay of caesium-123. No carrier iodide and reducing agent are added.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon of iodine- 123 has an energy of 0.159 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of Sodium Iodide (^{123}I) Solution For Radiolabelling.

Tests

pH (2.4.24). greater than 9.0.

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Radioactivity assay. Radioactivity of an aliquot of test solution is assayed using precalibrated radioactivity dose calibrator set for beta counting as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparations. It is expressed as MBq (μCi) per ml at a reference time and date.

Radionuclidic Purity. Determine by gamma ray spectrometry. Not less than 99.5 per cent of the total radioactivity due to Iodine-123, at the time of preparation and 94 per cent after the time of expiration.

Determine the relative amounts of I-123. Retain the preparation to be examined for a sufficient time to allow I-123 to decay to a level which permits the detection of other radionuclidic impurities. No radionuclides with a half-life longer than that of I-125 are detected. The preparation may be released for use before completion of the test.

Radiochemical purity. Determine by liquid chromatography (2.4.14)

Test solution. The injection under examination diluted with 2 gm solution of *sodium hydroxide* in 1000 ml of *water*, radioactive concentration suitable for detector. Add an equal volume of a solution containing 1.0 gm of *potassium iodide*, 2 gm of *potassium iodate* and 10 gm of *sodium hydrogen carbonate* in 1000 ml of *water* and mix.

Reference solution (a). Dissolve 10 mg of *potassium iodide* in 10 ml of *water*.

Reference solution (b). Dissolve 20 mg of *potassium iodate* in 10 ml of *water*. Mix equal volume of *reference solution (a)* and *reference solution (b)*.

Chromatographic system

- a stainless steel column 25 cm x 4.0 mm, packed with octadecylsilane bonded to porous silica or ceramic microparticles (5 μm),
- column temperature: 20° to 30°,
- mobile phase: a mixture of a buffer solution prepared by dissolving 5.85 g of *sodium chloride* and 0.65 ml of *octylamine* in 1000 ml of *water* and adjusted to pH 7.0 with dilute *phosphoric acid*, add 50 volumes of *acetonitrile*,
- flow rate: 1.5 ml per minute,
- spectrophotometer set at 220 nm,
- injection volume: 20 μl,
- run time: about 12 minutes.

The relative retention time with reference to iodide (retention time = about 5 min) for a iodate is about 0.2 to 0.3.

Inject reference solution solution (b) The test is not valid unless the resolution between the peaks due to the iodide and the iodate is not less than 2.0.

Inject the test solution and reference solution (a). In the chromatogram obtained with the test solution the radioactivity of the ^{123}I peak is not less than 95 per cent of the total radioactivity measured.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

Bacterial Endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in milliliters. Alternatively the preparation should pass the pyrogen test (2.2.8).

Storage. At 25° room temperature with adequate shielding.

Labelling. The label states, in addition to the information specified under injections (1) the amount of ^{123}I as labeled sodium iodide expressed as total MBq (mCi or μCi) and concentration as MBq (mCi or μCi) per ml at the time of calibration; (2) the expiration date and time; (3) the statements *"Caution—Radioactive Material" and " The solution is not for direct administration to humans"*.

Sodium Iodide (^{131}I) Capsules for Diagnostic Use

Category. Diagnostic.

Description. Sodium Iodide (^{131}I) Capsules for Diagnostic Use containing iodine-131 in the form of sodium iodide on a solid support prepared by using not more than 150 μl of sodium iodide (^{131}I) solution IP per capsule. They may contain sodium thiosulphate or some other suitable reducing agents and a suitable buffering substance. A package contains 1 or more capsules.

Sodium Iodide (^{131}I) Capsules for Diagnostic use contains Iodine-131not more than 37 MBq per capsule. The average radioactivity determined in the test for uniformity of content is 90.0 per cent to 110.0 per cent of the stated iodine-131 radioactivity at the date and time stated on the label. Use the Sodium Iodide (^{131}I) Capsules within 4 weeks from the date and time of preparation.

Identification

Determine by gamma-ray spectrometry. The most prominent gamma photon has energy of 0.364 MeV.

Tests

Radioactivity assay. Radioactivity of capsules are assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity. It is expressed as MBq (mCi) per capsule at a particular time and date.

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Disintegration. Not more than15 minutes.

In a water bath at 37°, warm in a small beaker about 20 ml of a 0.2 per cent w/v solution of *potassium iodide*. Add a capsule under examination. Stir magnetically at 20 revolutions per minute.

Uniformity of content. Determine the radioactivity of not less than 10 capsules. Calculate the average radioactivity per capsule. The radioactivity of no capsule differs by more than 10 per cent from the average, the relative standard deviation is not more than 3.5 per cent.

Radionuclidic purity. Determine by gamma-ray spectrometry.

Iodine-131 is not less than 99.9 per cent of the total radioactivity.

Dissolve the capsule in approximately 5 ml of warm water and take a drop of this solution in glass vial and place the vial on the detector for gamma spectrometry. Alternatively the spectra can be taken directly using the capsule.

Examine the gamma ray spectrum of the preparation. The most prominent gamma photon has energy of 0.364 MeV.

Radiochemical purity. Determine by Method A or method B.

Prepare a solution by dissolving one or more capsules in sufficient warm water to yield a concentration of not less than 25 µCi per ml.

Method A. Paper chromatography (2.4.15)

Support. Chromatography Paper Whatman No.1 (3 x 23cm).

Mobile phase. 70 per cent *methanol* in distilled water.

Spot the carrier solutions of 1.5 per cent potassium iodide and 1.5 per cent potassium iodate and the sample solution on two chromatography strips, at the point of spotting marked 3 cm from one end of the strips. Develop the chromatogram by ascending chromatography till the solvent front reaches 15 cm from the point of spotting. Allow the strip to dry.

Spray the strips with the reagent solutions and observe the colour as follows

(i) *0.1 per cent palladous chloride solution* – Brown coloration indicates iodide zone.

(ii) *1.5 per cent potassium iodide + acetic acid + starch solution, iodide free* – Blue coloration indicates iodate zone.

R_f of Iodide = 0.7 and R_f of Iodate = 0.4

Scan the strip using the radiochromatogram scanner with the collimator suitable adjusted to measure ^{131}I or cut into 1cm sections and count in a NaI (Tl) well type scintillation counter. Calculate Radiochemical Purity as the per cent of radio activity present in the form of Na^{131}I as follows

(per cent) Radiochemical Purity = Radioactivity in the Iodide zone x 100 / Total Activity

The radioactivity in the iodide zone should not be less than 95per cent of the total activity.

Method B. Paper electrophoresis (2.4.12).

Support. Whatman No.1 / No.3 Chromatography Paper (3 x 34 cm)

Mobile phase. *0.05 M disodium hydrogen ortho phosphate*, pH 7.5.

Wash the electrophoresis bath with distilled water; add 150 ml of *0.05M disodium hydrogen ortho phosphate* solution in each compartment. Take two strips of paper, mark the point of spotting at 6 cm from one end. Moisten the strips with *0.05 M disodium hydrogen ortho phosphate* solution; remove the excess solution by blotting on an absorbent sheet. Mount them on the bridge with the point of spotting at the cathode end. Switch on the electrophoresis unit and run for 5 min at 240 volts for saturation. Put off the electrophoresis unit and spot the carrier solutions of potassium iodide and potassium iodate and the sample (dissolve one capsule in 5 ml of warm water). Switch on the unit and allow the strip to run for 35 min. at 240 V. Switch off, remove the strips, allow to dry. Identify the iodide and iodate zones as described above.

Scan the strip using radiochromatogram scanner with the collimator suitable adjusted to measure ^{131}I or cut the strips into 1cm sections and count in a NaI(Tl) well type scintillation counter. Calculate the Radiochemical Purity as per cent of radioactivity present in the form of iodide as described under paper Chromatography.

The activity in the iodide zone should not be less than 95 per cent of the total activity.

Storage. Store in a cool and dark place with adequate shielding.

Labelling. The label states, (1) the time and date of calibration; (2) the amount of ^{131}I as total megabecquerels (millicuries) and concentration as megabecquerels (millicuries) per capsule at the time of calibration; (3) the expiration date and time; (4) the statement "Caution — Radioactive material".

Sodium Iodide (^{131}I) Capsules for Therapeutic Use

Category. Therapeutic.

Description. Sodium Iodide (^{131}I) Capsules for Therapeutic Use containing iodine-131 in the form of sodium iodide on a solid support prepared by using not more than 150 µl of sodium iodide (^{131}I) solution IP per capsule. They may contain sodium thiosulphate or some other suitable reducing agents and a

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suitable buffering substance. A package contains 1 or more capsules. Sodium Iodide (^{131}I) Capsules for Therapeutic Use contains not more than 5550 MBq (150 mCi) Iodine131 per capsule.

The average radioactivity determined in the test for uniformity of content is 90.0 per cent to 110.0 per cent of the stated iodine-131 radioactivity at the date and time stated on the label. Use the Sodium Iodide (^{131}I) Capsules within 4 weeks from the date and time of preparation.

Identification

Determine by Gamma-ray spectrometry. The most prominent gamma photon of iodine-131 has energy of 0.364 MeV.

Tests

Radioactivity assay. Radioactivity of the capsules is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity. It is expressed as MBq (mCi) per capsule at a particular time and date.

Disintegration. Not more than 15 minutes

In a water-bath at 37°, warm in a small beaker about 20 ml of a 0.2 per cent w/v solution of *potassium iodide*. Add a capsule under examination. Stir magnetically at a rate of 20 revolutions per minute.

Radionuclidic purity. Determine by gamma-ray spectrometry. Not less than 99.9 per cent due to Iodine-131.

Dissolve the capsule in approximately 5 ml of warm water and take a drop of this solution in glass vial and place the vial on the detector for gamma spectrometry. The most prominent gamma photon has energy of 0.364 MeV.

Radiochemical purity. Determine by method A or alternatively by method B.

Prepare a solution by dissolving one or more capsules in sufficient warm water to yield a concentration of not less than 25 μCi per ml.

Method A. By Paper chromatography (2.4.15)

Support. Chromatography Paper Whatman No1/ No3 (3x 23cm)

Mobile phase. 70 per cent *methanol* in distilled water

Spot the carrier solutions of 1.5 per cent potassium iodide and 1.5 per cent potassium iodate and the sample solution on two chromatography strips, at the point of spotting marked 3 cm from one end of the strips. Develop the chromatogram by ascending chromatography till the solvent front reaches 15 cm from the point of spotting. Allow the strip to dry.

Identify the iodide and iodate zones as follows:

Spray the strips with the reagent solutions and observe the colour as follows

(i) *0.1 per cent palladous chloride solution* – Brown coloration indicates iodide zone.

(ii) *1.5 per cent potassium iodide + Acetic acid + starch solution, iodide free* – Blue coloration indicates iodate zone.

R_f of Iodide = 0.7 and R_f of Iodate = 0.4

Scan the strip using the radiochromatogram scanner with the collimator suitable adjusted to measure ^{131}I or cut into 1cm sections and count in a NaI (Tl) well type scintillation counter.

Calculate Radiochemical Purity as the per cent of radio activity present in the form of Na^{131}I as follows

(per cent) Radiochemical Purity = Radioactivity in the Iodide zone x 100 / Total Activity

The radioactivity in the iodide zone should not be less than 95 per cent of the total activity.

Method B. By Paper electrophoresis (2.4.12).

Support. Whatman No.3/ No 1 Chromatography Paper (3 x 34 cm).

Mobile phase. 0.05M disodium hydrogen ortho phosphate, pH 7.5.

Wash the electrophoresis bath with distilled water; add 150 ml of 0.05M *disodium hydrogen ortho phosphate solution* in each compartment. Take two strips of paper, mark the point of spotting at 6 cm from one end. Moisten the strips with *0.05M disodium hydrogen ortho phosphate solution*; remove the excess solution by blotting on an absorbent sheet. Mount them on the bridge with the point of spotting at the cathode end. Switch on the electrophoresis unit and run for 5 min at 240 volts for saturation. Put off the electrophoresis unit and spot the carrier solutions of potassium iodide and potassium iodate and the sample (dissolve one capsule in 5 ml of warm water). Switch on the unit and allow the strip to run for 35 min. at 240 V. Switch off, remove the strips, allow to dry. Identify the iodide and iodate zones as described above.

Scan the strip using radiochromatogram scanner with the collimator suitable adjusted to measure ^{131}I or cut the strips into 1 cm sections and count in a NaI (Tl) well type scintillation counter. Calculate the Radiochemical Purity as per cent of radioactivity present in the form of iodide as described under paper Chromatography.

The activity in the iodide zone should not be less than 95 per cent of the total activity.

Storage. To be store in a cool and dark place with adequate shielding.

Labelling. The label states (1) the time and date of calibration; (2) the amount of ^{131}I as total megabecquerels (millicuries) and concentration as megabecquerels (millicuries) per capsule at the time of calibration; (3) the expiration date and time; (4) the statement " Caution — Radioactive material".

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Sodium Iodide (^{131}I) Solution

Category. Therapeutic and Diagnostic

Description. A clear colourless solution. The radioactivity in the solution is not less than 90.0 per cent to not more than 110.0 per cent of the stated iodine-131 radioactivity at the date and time stated on the label. It is a solution of [^{131}I] Sodium iodide in dilute sodium thiosulphate solution. It is suitable for oral use and not for injection. Radioactive concentration should not exceed 100 mCi per ml. Use the Sodium iodide [^{131}I] orally within 4 weeks from the date and time of preparation.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon has energy of 0.365 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of the (^{131}I) Sodium iodide.

Tests

pH (2.4.24). 7.0 to 10.0.

Radioactivity assay. Radioactivity of an aliquot of Test Solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Determine by gamma ray spectrometry. Not less than 99.9 per cent due to Iodine-131.

Examine the gamma ray spectrum of the preparation. The most prominent gamma photon has energy of 0.364 MeV.

Radiochemical purity. Determine by Paper chromatography (2.4.15).

Support. Whatman Chromatography Paper No.1 (3x 23cm).

Mobile phase. 70 per cent *methanol* in WFI.

Spot the carrier solutions 1.5 per cent potassium iodide and 1.5per cent potassium iodate and the sample solution on two chromatography strips, at the point of spotting marked 3cms from one end of the strips. Develop the chromatogram by ascending chromatography till the solvent front reaches 15 cm from the point of spotting. This takes about 90 min. Allow the strip to dry

Identification of Iodide and Iodate Zones by Spray the strips with

(i) *0.1per cent palladous chloride solution* – Brown coloration indicates iodide zone.

(ii) *potassium iodide + acetic acid + starch solution, iodide free* – Blue coloration indicates iodate zone.

R_f of Iodide = 0.7 and R_f of Iodate = 0.4

Scan the strip using the radiochromatogram scanner or cut into 1cm sections and count in a well type scintillation counter.

Calculate Radiochemical Purity as the per cent of activity present in the form of Na^{131}I as follows:

per cent Radiochemical Purity = Activity in the Iodide zone x 100 / Total Activity

The activity in the iodide zone should not be less than 95 per cent of the total radioactivity.

Sodium thiosulphate content

Thiosulphate content is determined by titrating the sample against the 0.005 N standard iodine solution.

Titration of blank solution. Dissolve 250 mg of sodium bicarbonate powder in 10 ml distilled water in a conical flask. Add 0.5 ml of 1per cent starch solution as indicator and titrate against 0.005 N standard iodine solution till the end point (color changes from colourless to blue).

Volume of standard iodine solution consumed for blank = 'B' ml

Titration of sample solution. Dissolve 250 mg of sodium bicarbonate powder in 10 ml distilled water in a conical flask. Add 0.5 ml of 1per cent starch solution as indicator add 250 µl sample and titrate against 0.005 N standard iodine solution till the end point (colour changes from colourless to blue).

Volume of standard iodine solution consumed by 250 µl sample = 'A' ml.

The actual volume of standard iodine solution consumed for 1 ml sample = (A-B) x 4 ml = C ml.

Sodium thiosulphate content is calculated as follows:

C x 0.005 x 158 = D mg *sodium thiosulphate* per ml

Sodium thiosulphate content in mg per Ci of ^{131}I calculated by the formula:

$$\frac{D \times 1000 \text{mg/Ci}}{\text{Activity (mCi/ml)}}$$

The limits should be 25-100mg per Ci of I-131on date of preparation.

Storage. Store at room temperature with adequate shielding.

Labelling. The label states (1) the time and date of calibration; (2) the amount of ^{131}I as iodide expressed in megabecquerels (microcuries or millicuries) at the time of calibration; (3) the expiration date; (4) the statement "Caution — Radioactive material".

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Sodium Pertechnetate (^{99m}Tc) Injection (Fission)

Category. Diagnostic.

Description. A clear, colourless solution. Sodium Pertechnetate (^{99m}Tc) Injection (Fission) is a sterile solution containing technetium-99m in the form of pertechnetate ion and made isotonic by the addition of sodium chloride. Sodium Pertechnetate (^{99m}Tc) Injection (Fission) contains not less than 90.0 per cent and not more than 110.0 per cent of the stated technetium-99m radioactivity at the date and time stated on the label.

Radiopharmaceutical Preparation. Sodium Pertechnetate (^{99m}Tc) is extracted from Technetium-99m column chromatography generator containing fission-produced Molybdenum-99 adsorbed on acidic alumina column. Molybdenum-99 is extracted from uranium fission products. Sodium chloride solution, 0.9 per cent w/v, is used to elute Sodium Pertechnetate (^{99m}Tc) solution which is sterilized by ultrafiltration through 0.22 μ filter.

Identification

A. Determine by gamma-ray spectrometry. The gamma-ray spectrum obtained from an aliquot test solution corresponds to the spectrum obtained with reference solution of Technetium-99m either by direct comparison or by using an instrument calibrated with the aid of such a solution. The most prominent gamma photon of technetium-99m has energy of 0.140 MeV.

B. Half life of Technetium-99m in test solution as determined with a calibrated gamma ray counter or a radioactivity dose calibrator, by not less than three measurements of the radioactivity of a sample in the same geometrical conditions within a suitable period of decay time (for example, three readings at 30 minute intervals over 90 minutes) is between 5.7 hours to 6.3 hours.

C. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of the distribution of radioactivity in the preparation.

Tests

pH (2.4.24). 4.0 to 8.0 using pH paper strips.

Radioactivity Assay. Radioactivity of an aliquot of Test Solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Not less than 99.9 per cent due to Technetium-99m.

The fission ^{99}Mo to be used for extraction of Technetium-99m (fission) should meet specification with respect to the following fission products:

However ^{99m} Tc or Fission ^{99}Mo need not be reanalyzed for radionuclide impurity for the fission products, if fission ^{99}Mo to be used for the extraction of ^{99m}Tc is procured from reputed manufacturers or suppliers who certifies the specification on radionuclide purity of fission ^{99}Mo for the following radionuclide.

Radionuclide	Limit (not more than)
iodine-131	5×10^{-3} per cent
ruthenium-103	5×10^{-3} per cent
strontium-89	6×10^{-5} per cent
strontium-90	6×10^{-6} per cent
alpha-emitting impurities	1×10^{-7} per cent
other gamma-emitting impurities	0.01 per cent

Molybdenum-99, Iodine-131, Ruthenium-103, Strontium-89, Yttrium-90 and other gamma-emitting impurities should not be more than 0.1 per cent of total radioactivity.

Molybdenum-99. Molybdenum-99 is the principal radionuclidic impurity which is required to be determined in the test solution. The prescribed Molybdenum-99 impurity in test solution should not exceed 37 KBq (1 μCi) of Molybdenum-99m per 37 MBq (1 mCi) of Technetium-99m. The radionuclidic purity of Technetium-99m in test solution should not be less than 99.9 per cent. Determination of Molybdenum-99 impurity in test solution is referred to "Molybdenum-99 Breakthrough Test". This test can be done either by Method A or Method B.

Method A. By using 4-7 mm thick lead canister (radiation shield) and calibrated dose calibrator for assaying Molybdenum-99 activity in test solution.

An empty 4 -7 mm thick lead radiation shield is placed in dose calibrator and background activity (a kBq) is measured at Molybdenum-99 setting of the dose calibrator. Pertechnetate (^{99m}Tc) test solution vial is placed inside the lead canister and Molybdenum-99 activity (b kBq) is measured in dose calibrator at the calibration factor for Molybdenum-99. The background activity (a kBq) for Molybdenum-99 obtained is subtracted from Molybdenum-99 activity reading (b kBq). The correction factor for Molybdenum-99 gamma photon attenuation by lead shield is taken as recommended for the dose calibrator to determine Molybdenum-99 radioactivity. The Technetium-99m test solution vial is removed from the Molybdenum-99 assay shield container and assayed for Technetium-99m radioactivity (c MBq) in dose calibrator at setting for Technetium-99m. Molybdenum-99 activity ((b-a) kBq) divided by Technetium-99m activity (c MBq) gives Molybdenum-99 Breakthrough in kBq/MBq Technetium- 99m.

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Method B. Molybdenum-99 breakthrough in Technetium-99m test solution can also be determined by Gamma ray spectrometry by the following procedure.

The activity (s kBq), of 0.3 ml of the Sodium Pertechnetate (^{99m}Tc) test solution is measured in a pre-calibrated dose calibrator. Similarly, the activity (r kBq) of 0.3 ml of the Molybdenum-99 sodium molybdate (reference standard) solution is measured. This Molybdenum-99 reference standard, taken in a lead pot of 4 -7 mm lead thickness and its count rate determined in a scintillation counter, set to 700-800 keV range, at a reproducible distance from the detector (q Bq). In the same manner the count rates of the test Sodium Pertechnetate ^{99m}Tc (s kBq) test solution is determined in the same range and at the same distance (p Bq). The count rates are corrected for background and the percentage of Molybdenum-99 content in Sodium Pertechnetate (^{99m}Tc), calculated by the expression, (100rp)/(qs), where p = net count rate of Molybdenum-99 radionuclidic impurity in 0.3 ml the Sodium Pertechnetate (^{99m}Tc) test solution, q= net count rate kBq) of the Molybdenum-99 sodium molybdate solution (0.3ml).

Retain a sample of the injection for a sufficient time to allow the technetium-99m radioactivity to decay to a sufficiently low level to permit the detection of radionuclidic impurities. All measurements of radioactivity are expressed with reference to the date and time of administration.

Iodine-131. By gamma ray spectrometry. The most prominent photon has energy of 0.364 MeV. Iodine-131 has a half-life of 8.08 days. Not more than 5×10^{-3} per cent of the total radioactivity is due to iodine-131.

Ruthenium-103. By gamma ray spectrometry. The most prominent photon has energy of 0.497 MeV. Ruthenium-103 has a half-life of 39.3 days. Not more than 5×10^{-3} per cent of the total radioactivity is due to ruthenium-103.

Strontium-89. By beta ray counter. Determine the presence of strontium-89 in the injection by a counting system appropriate for the detection of particulate radiations. Strontium-89 decays with a beta emission of 1.463 MeV maximum energy and has a half-life of 52.7 days. Not more than 6×10^{-5} per cent of the total radioactivity is due to strontium-89.

Strontium-90. By beta ray counter. Determine the presence of strontium-90 in the injection by a counting system appropriate for the detection of particulate radiations. Strontium-90 decay with beta emissions of 0.546 MeV maximum energy and half-lives of 27.7 years and 64.0 hours. Not more than 6×10^{-6} per cent of the total radioactivity is due to strontium-90.

Other gamma-emitting impurities. The total gamma radioactivity due to these impurities is not more than 0.01 per cent of the total radioactivity.

Alpha-emitting impurities. Determine the alpha radioactivity of the decayed material to detect any alpha-emitting radionuclidic impurities, which should, where possible, be identified and quantified. The total alpha radioactivity due to these impurities is not more than 1×10^{-7} per cent of the total radioactivity.

Radiochemical purity. Determine by Method A or Method B. Not less than 95 per cent due to Technetium-99m as ^{99m}Tc Pertechnetate.

Method A. By ascending paper chromatography (2.4.15).

Mobile phase. A mixture of 15 volumes of *water* and 85 volumes of *methanol.*

Support. Whatman Chromatography paper No 1/No 3.

Test solution. Dilute the injection under examination with *water* or *saline* (0.9 per cent *sodium chloride* w/v solution) to a suitable radioactive concentration.

Apply 2-5 µl of the test solution at 1cm above the bottom of the strip of a Whatman No1/No3 strip. Develop for 2 hour and allow to dry in air. Determine the distribution of radioactivity using a suitable detector. Not less than 95 per cent of the total radioactivity is found in the spot corresponding to pertechnetate ion, which has an R_f value of about 0.5 to 0.7.

Method B. Determine by rapid paper chromatography (2.4.15).

Mobile phase. acetone or *methyl ethyl ketone.*

Test solution. Dilute the injection under examination with water or 0.9 per cent *sodium chloride* w/v solution to a suitable radioactive concentration.

Apply 2 to 5µl of the test solution at 1 cm above the bottom of a Whatman No. 3/No 1 paper strip. Run the chromatogram in *acetone* or *methyl ethyl ketone* up to a distance of 6 cm. The R_f value for ^{99m}Tc Pertechnetate is 1 and R_f value for reduced hydrolysed ^{99m}Tc is 0. The chromatographic strip is dried in air and cut into two equal sections at 4.0 cm. The upper half section (section A) and bottom half section (section B) are counted separately in counting tubes in NaI(Tl) scintillation counter and corrected for background counts. Radiochemical purity is calculated as under

$$\text{per cent RCP} = \frac{\text{Counts in section A}}{\text{Counts in section A} + \text{Counts in section B}} \times 100$$

Aluminium. By spot test. Apply 10 µl of the test solution by micropipette on spot plate. Drop 30 µl *acetate buffer solution pH 4.6.* Add 10 µl of a 0.1 per cent w/v solution of *chromazurol S.* After 3 minutes, red or pink colour of the solution is not more intense than that of a standard prepared at the same time and in the same manner using 10 µl of *aluminium standard solution* (10 ppm).

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Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Not more than 175 per V EUper ml, V being the maximum recommended dose in milliliters.

Labelling The label states, in addition to the information specified under injection (1) the amount of ^{99m}Tc as total MBq (mCi) and concentration as MBq (mCi) per ml at the time of production; (2) the expiration (use before) date and time; (3) the statement "Caution—Radioactive Material" and "do not use if cloudy or if it contains particulate matter"; (4) in making dosage calculations, correction is to be made for radioactive decay; (5) radioactive half life of ^{99m}Tc is 6.02 hours.

Sodium Pertechnetate (^{99m}Tc) Injection (Non-fission)

Category. Diagnostic.

Description. A clear, colourless solution. Sodium Pertechnetate (^{99m}Tc) Injection is a sterile solution containing technetium-99m in the form of pertechnetate ion and made isotonic by the addition of sodium chloride. Sodium Pertechnetate (^{99m}Tc) Injection (Non-fission) contains not less than 90.0 per cent and not more than 110.0 per cent of the stated technetium-99m radioactivity at the date and time stated on the label. Not less than 95 per cent of the radioactivity corresponds to technetium-99m in the form of pertechnetate ion.

Radiopharmaceutical Preparation. Sodium Pertechnetate (^{99m}Tc) is extracted from aqueous alkaline solution (*5 N sodium hydroxide)* of Sodium molybdate (Mo^{99}) in distilled *methyl ethyl ketone* using (^{99m}Tc) Solvent Extraction Generator. The *methyl ethyl ketone solution* containing Pertechnetate (^{99m}Tc) is purified by passing through short column of alumina and solvent is evaporated by heating. The residual Sodium Pertechnetate (^{99m}Tc) is dissolved in 0.9 per cent w/v *sodium chloride* aqueous solution and rendered sterile by terminal ultra filtration using 0.22 μ filter or autoclave. Alternatively Sodium Pertechnetate (^{99m}Tc) is eluted from Technetium-99m gel generator containing Molybdenum-99 immobilized as a zirconium molybdate-^{99}Mo column matrix. 0.9 per cent sodium chloride solution is used as eluant for Sodium Pertechnetate (^{99m}Tc) solution and is sterilized by ultrafiltration through 0.22 μ filter or heating in an autoclave.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon of technetium-99m has energy of 0.140 MeV.

B. Half life of Technetium-99m in test solution should be between 5.7 to 6.3 hours.

C. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of the distribution of radioactivity in the preparation.

Tests

pH (2.4.24). 4.0 to 8.0 using pH paper strips.

Radioactivity Assay. Radioactivity of an aliquot of Test Solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity. It is expressed as MBq(mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Not less than 99.9 per cent due to Technetium-99m.

The radioactivity due to radionuclides other than Technetium-99m, apart from that due to Technetium-99 resulting from the decay of Technetium-99m is not more than that shown below, expressed as a percentage of the total radioactivity and calculated with reference to the date and time of administration.

Molybdenum-99 breakthrough not more than 0.1 per cent.

Molybdenum-99. Molybdenum-99 is principal radionuclidic impurity which is required to be determined in test solution. The prescribed Molybdenum-99 impurity in test solution should not exceed 37 KBq (1μCi) of Molybdneum-99 m per 37 MBq (1 mCi) of Technetium-99m. Determination of Molybdenum 99 impurity in test solution is referred to "Molybdenum Breakthrough Test". This test can be done either by Method A or Method B.

Method A. By using 4-7 mm thick lead radiation shield and calibrated dose calibrator for assaying Molybdenum 99 activity in test solution.

An empty 4-7 mm thick lead radiation shield is placed in dose calibrator and background activity (a kBq) is measured at Molybdenum-99 setting of the dose calibrator. Pertechnetate (^{99m}Tc) test solution vial is placed inside the lead canister and Molybdenum-99 activity (b kBq) is measured in dose calibrator at the calibration factor for Molybdenum-99. The background activity (a kBq) for Molybdenum-99 obtained is subtracted from Molybdenum-99 activity reading (b kBq). The correction factor for Molybdenum-99 gamma photon attenuation by lead shield is taken as recommended for the dose calibrator to determine Molybdenum-99 radioactivity. The Technetium-99m test solution vial is removed from the Molybdenum-99 assay shield container and assayed for Technetium-99m radioactivity (c MBq) in dose calibrator at setting for Technetium-99m. Molybdenum-99 activity ((b-a) kBq) divided by Technetium-99m activity (c MBq) gives Molybdenum-99 Breakthrough in kBq/MBq Technetium- 99m.

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Method B. Molybdenum-99 breakthrough in Technetium-99m test solution can also be determined by Gamma ray spectrometry by the following procedure.

The activity (s kBq), of 0.3 ml of the Sodium Pertechnetate (^{99m}Tc) test solution is measured in a pre-calibrated dose calibrator. Similarly, the activity (r kBq) of 0.3 ml of the Molybdenum-99 sodium molybdate (reference standard) solution is measured. This Molybdenum-99 reference standard, taken in a lead pot of 4-7 mm lead thickness and its count rate determined in a scintillation counter, set to 700-800 keV range, at a reproducible distance from the detector (q Bq). In the same manner the count rates of the test Sodium Pertechnetate ^{99m}Tc (s kBq) test solution is determined in the same range and at the same distance (p Bq). The count rates are corrected for background and the percentage of Molybdenum-99 content in Sodium Pertechnetate (^{99m}Tc), calculated by the expression, (100rp)/(qs), where p = net count rate of Molybdenum-99 radionuclidic impurity in 0.3 ml the Sodium Pertechnetate (^{99m}Tc) test solution, q= net count rate kBq) of the Molybdenum-99 sodium molybdate solution (0.3 ml).

Retain a sample of the injection for a sufficient time to allow the technetium-99m radioactivity to decay to a sufficiently low level to permit the detection of radionuclidic impurities. All measurements of radioactivity are expressed with reference to the date and time of administration.

Other gamma-emitting impurities does not exceed 0.01 percent of the total radioactivity.

Determine the gamma-ray spectrum of the decayed material for the presence of other radionuclidic impurities, which should, where possible, be identified and quantified. The total radioactivity due to other radionuclidic impurities does not exceed 0.01 per cent of the total radioactivity.

Radiochemical purity. Determine by Method A or Method B. Not less than 95 per cent due to Technetium-99m as ^{99m}Tc Pertechnetate.

Method A. Determine by ascending paper chromatography (2.4.15).

Mobile phase. 85 per cent *methanol* in *water.*

Support. Whatman no 1 /No 3 chromatography paper.

Test solution. Dilute the injection under examination with *water* or *saline* (0.9 per cent *sodium chloride* w/v solution) to a suitable radioactive concentration.

Apply 5 µl of the test solution. Develop for 90 min or up to 15 cm and allow to dry in air. Scan the strips using a radiochromatogram scanner with collimator suitably adjusted to measure ^{99m}Tc or cut into one cm sections and count in a well type NaI(Tl) scintillation counter. Not less than 95 per cent of the total radioactivity is found in the spot corresponding to pertechnetate ion, which has an R_f value of about 0.5 to 0.7.

Method B. Determine by rapid paper chromatography. (2.4.15).

Mobile phase. acetone or *methyl ethyl ketone.*

Test solution. Dilute the injection under examination with *water* or *saline* (*0.9 per cent sodium chloride* w/v solution) to a suitable radioactive concentration.

Apply 2 to 5 µl of the test solution at 1 cm above the bottom of the strip on Whatman No. 3/No 1 paper strip. Run the chromatogram in *acetone* or *methyl ethyl ketone* up to a distance of 6 cm. The R_f value for ^{99m}Tc Pertechnetate is 1 and R_f value for reduced hydrolysed ^{99m}Tc is 0. The chromatographic strip is dried in air and cut into two equal sections at 4.0 cm. The upper half section (section A) and bottom half section (section B) are counted separately in counting tubes in NaI(Tl) scintillation counter and corrected for background counts. Radiochemical purity is calculated as under

$$\text{per cent RCP} = \frac{\text{Counts in section A}}{\text{Counts in section A} + \text{Counts in section B}} \times 100$$

Aluminium. By spot test. Apply 10 µl of the test solution by micropipette on spot plate. Drop 30 µl acetate buffer pH 4.6. Add 10 µl of a 0.1 per cent w/v solution of *chromazurol S.* After 3 minutes, red or pink colour of the solution is not more intense than that of a standard prepared at the same time and in the same manner using 10 µl of *aluminium standard solution (10 ppm).*

Molybdenum. By spot test. Take 50 µl of test solution by micropipette on the spot plate. Add 50 µl of 10 per cent of *potassium thiocyanate solution* followed by 50 µl of 10 per cent *tin(II) chloride solution.* The orange red colour developed should be less intense than that of a standard prepared at the same time and in the same manner using 50 µl of *molybdenum standard solution (10 ppm).*

Methyl ethyl ketone By spot test: (Only for pertechnetate (^{99m}Tc) obtained from solvent extraction method): In a test tube take 200 µl of 1 M *sodium hydroxide solution* followed by 200 µl of *0.1M iodine solution.* Add 50 µl of test solution followed by 150 µl of *double distilled water.* The turbidity in the solution should be less than that of the turbidity produced by standard *methyl ethyl ketone solution* (0.1 per cent v/v) prepared at the same time in the similar manner.

Zirconium. (Only for Technetium obtained from *Zr* molybdate gel generator).

By spot test. Take 10 µl of test solution by micropipette on the spot plate. Add 80 µl of *2 N hydrochloric acid solution* followed by 10 µl of 0.05 per cent *Alizarin S solution.* The Reddish Yellow colour developed should be less intense than

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that of a standard prepared at the same time and in the same manner using 10 µl of *Zirconium standard solution (10ppm)*.

Bacterial endotoxins (2.2.3). Not more than 175 per V EU per ml, V being the maximum recommended dose in milliliters. The test must be completed before release of the preparation.

Sterility (2.2.11) Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

Labelling. The label states, in addition to the information specified under injection (1) the amount of ^{99m}Tc as total MBq (mCi) and concentration as MBq (mCi) per ml at the time of production; (2) the expiration (use before) date and time; (3) the statement "Caution — Radioactive Material" and "do not use if cloudy or if it contains particulate matter"; (4) in making dosage calculations, correction is to be made for radioactive decay; (5) radioactive half life of ^{99m}Tc is 6.02 hour.

Sodium Phosphate (^{32}P) Injection

Category. Therapeutic.

Description. A clear, colourless solution. Sodium Phosphate (^{32}P) Injection is a sterile solution and contains not less than 90.0 per cent and not more than 110.0 per cent of the stated phosphorus-32 radioactivity at the date and time stated on the label. Not less than 95.0 per cent of the radioactivity corresponds to phosphorus-32 in the form of orthophosphate ion. Sodium Phosphate (^{32}P) Injection is a solution of disodium and monosodium (^{32}P) orthophosphates made isotonic by the addition of sodium chloride.

Identification

Measure radioactive concentration at time t_0 and after time t, (minimum one day). Calculate the expected radioactive concentration after time t using the equation $A_t = A_o e^{-\lambda t}$, where A_t = Activity measured after time t, A_o = Activity measured earlier at time t_o, t = time elapsed between the 2 measurements, λ = decay constant and $e^{-\lambda t}$ =0.953 when t=1 day.

The measured radioactive concentration should be same as that expected.

Tests

pH (2.4.24). 6.0 to 8.0.

Radioactivity assay. Measure the radioactivity using suitable precalibrated counting equipment

Radionuclidic purity. No gamma emitting radionuclides should be present when checked by gamma spectrometry. Half life determination to be carried out as described under Identification.

Radiochemical purity. Determine by ascending paper chromatography (2.4.15).

Support. chromatography paper Whatman No 1/No.3; 2 strips (3 x 33 cm)

Mobile phase. A mixture of 75 volumes of *isopropanol*, 10 volumes of 50 per cent w/v *trichloroacetic acid* (10 ml), 14.4 volumes of *distilled water* and 0.6 volume of 10 per cent *ammonia solution*.

Spot about 2-5 µl of test solution with a capillary or micropipette at the point of spotting marked at 3 cm from one end of the strip (3 x 33 cm) in duplicate. Develop the chromatogram by ascending chromatography till the solvent front reaches 27 cm from the point of spotting. This takes about 18 hours (Overnight). Allow the strips to dry. Scan the strip using a radiochromatogram scanner or cut into one cm sections and count in a well type scintillation counter. The R_f values of metaphosphate, pyrophosphate and orthophosphate are 0, 0.4, and 0.6-0.8 respectively.

Calculate the per cent of ^{32}P present as ortho phosphate as follows:

(per cent) Radiochemical Purity = Activity in the ^{32}P ortho phosphate zone x 100 / Total Activity

The activity in the ^{32}P orthophosphate zone should not be less than 95 per cent of the total activity.

Phosphorous Content

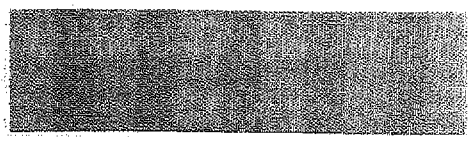
Take 3 vials of 10 ml capacity and label them blank, standard, active sample and dispense reagents as follows

Reagents	Blank	Standard	Active Sample
Standard Phosphorous solution 100 µg/ml	NIL	1 ml	NIL
Sodium Phosphate (^{32}P) Injection	NIL	NIL	0.1 ml
Distilled Water	3.5 ml	2.5 ml	3.4 ml
Mixed Reagent*	1.5 ml	1.5 ml	1.5 ml

*Mixed reagent: 0.4 N hydrochloric acid, 3.53per cent ammonium molybdate solution, 0.236 per cent ammonium meta vanadate solution

The spectrophotometer is set for measurement at 460 nm. Ensure, while filling the cell that it is clean and devoid of drops. In the fixed wavelength measurement setup, take the blank reading. Take the absorbance for the standard and active sample as prepared above.

Phosphorus content = (Aa) / (As) mg per ml



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where, Aa = absorbance of active sample,
As = absorbance of standard phosphorus.

The phosphorus content should not exceed 1 mg per ml.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Not more than 175 per V EU per ml, V being the maximum recommended dose in milliliters. The test must be completed before release.

Storage. Store at room temperature not exceeding 30°.

Labelling. The label states, in addition to the information specified under injections (1) the time and date of calibration; (2) the amount of ^{32}P as total megabecquerels (millicuries) and concentration as megabecquerels (millicuries) per ml at the time of calibration; (3) the expiration date; (4) the statement "Caution—Radioactive Material."

Strontium (^{89}Sr) Chloride Injection

Category. Therapeutic

Description. Strontium (^{89}Sr) Chloride Injection is a clear, colourless, sterile solution of strontium-89 as strontium dichloride and ready for use. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated content of strontium-89 on the label at the reference date stated on the label. Not less than 99.0 per cent of the total radioactivity is due to strontium-89. The specific radioactivity is not less than 1.8 MBq (48.6 μCi) per mg of strontium at the reference date stated on the label. Not more than 0.6 per cent of the total radioactivity is due to radionuclides other than strontium-89. The injection contains not less than 6.0 mg per ml and not more than 12.5 mg per ml of strontium. Strontium-89 is produced by neutron irradiation of strontium enriched in strontium-88. Strontium (^{89}Sr) Chloride Injection should be used within 7 days from the date of preparation.

Identification

A. Determine by beta-ray spectrum using liquid scintillation counter as described in general chapter on Radiopharmaceutical preparations, with a sample of strontium-89, suitably diluted if needed. The spectrum exhibits a major peak of 1.495 MeV. A photon of 0.909 MeV is due to the short lived daughter product, yttrium-89m formed in 0.01 per cent of the disintegrations, in equilibrium with strontium-89 can be detected by using a gamma spectrophotometer.

B. 0.1 ml of the injection under examination is added with a freshly prepared 0.1 per cent w/v solution of *sodium rhodizonate dibasic R*, a reddish-brown precipitate is formed.

Tests

pH (2.4.24). 4.0 to 7.5.

Radioactivity assay. Radioactivity of an aliquot of the test solution is assayed using precalibrated radioactivity dose calibrator set for beta counting as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparations. It is expressed as MBq (mCi) in volume V ml at a reference time and date.

Radionuclidic purity

Gamma emitting impurities. Determine by gamma-ray and X-ray spectroscopy, using a suitable instrument and measure the half life using a suitable method. Determine the relative amount of gamma emitting impurities. Not more than 0.4 per cent of the total radioactivity is due to gamma emitting radionuclides other than yttrium-89m.

Beta emitting impurities. Determine by using a suitable cationic exchange resin and a suitable chemical separation method, determine the relative amounts of sulphur-35 and phosphorus-32. Not more than 0.2 per cent of the total radioactivity is due to beta emitting impurities.

Chemical purity

Aluminium. Not more than 2 μg per ml.

Determine by atomic emission spectrometry (2.4.3).

Test solution. 0.2 ml of the injection under examination, dilute to a suitable volume with *dilute nitric acid*.

Reference solutions. Prepare the reference solutions using *aluminium standard solution (10 ppm Al)* diluted as necessary with *dilute nitric acid*.

Iron. Not more than 5 μg per ml.

Determine by atomic emission spectrometry (2.4.3).

Test solution. 0.2 ml of the injection under examination, dilute to a suitable volume with *dilute nitric acid*.

Reference solutions. Prepare the reference solutions using *iron standard solution (20 ppm Fe)* diluted as necessary with *dilute nitric acid*.

Lead. Not more than 5 μg per ml.

Determine by atomic emission spectrometry (2.4.3).

Test solution. 0.2 ml of the injection under examination, dilute to a suitable volume with *dilute nitric acid*.

Reference solution. Prepare the reference solutions using *lead standard solution (10 ppm Pb)* diluted as necessary with *dilute nitric acid*.

Strontium. Not less than 6.0 mg per ml and not more than 12.5 mg per ml.

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Determine by atomic emission spectrometry (2.4.3).

Test solution. 0.2 ml of the injection under examination, dilute to a suitable volume with *dilute nitric acid.*

Reference solutions. Prepare the reference solutions using *strontium standard solution (1.0 per cent Sr)* diluted as necessary with *dilute nitric acid.*

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparations. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in ml. Alternatively the preparation should pass the pyrogen test (2.2.8).

Storage. Store at a temperature between 2° to 8°.

Labelling. The label states, in addition to the information specified under injections (1) the time and date of calibration; (2) the amount of strontium (^{89}Sr) chloride expressed as mg of strontium per ml; (3) the amount of ^{89}Sr as labelled strontium chloride expressed as total MBq (mCi) and concentration as MBq per ml (or mCi per ml) on the date of calibration; (4) the expiration date; (5) and the statement "Caution- Radioactive Material."

Technetium (^{99m}Tc) Colloidal Rhenium Sulphide Injection

Category. Diagnostic

Description. Technetium (^{99m}Tc) Colloidal Rhenium Sulphide Injection is a light brown sterile colloidal dispersion of rhenium sulphide, the micelles of which are labelled with technetium-99m. It contains not less than 90.0 per cent and not more than 110.0 per cent of the stated technetium-99m radioactivity at the date and time stated on the label. Technetium (^{99m}Tc) Colloidal Rhenium Sulphide Injection should be used within 8 hours from date and time of preparation.

Radiopharmaceutical Preparation. Technetium (^{99m}Tc) Colloidal Rhenium Sulphide Injection is prepared aseptically following instructions provided with the kit. The kit contains *sodium pertechnetate (^{99m}Tc) injection (fission or non - fission)* and other suitable ingredients and stabilizers. The pH of the injection may be adjusted by the addition of a suitable buffer such as a citrate buffer solution.

Identification

A. Determine by gamma ray spectrometry as described in general chapter on Radiopharmaceutical preparations. The most prominent gamma photon of technetium-99m has energy of 0.141 MeV.

B. In the test for radiochemical purity, the chromatogram obtained contributes to the identification of Technetium (^{99m}Tc) Colloidal Rhenium Sulphide.

C. To 1 ml of the injection add 5 ml of *hydrochloric acid*, 5 ml of a 5.0 per cent w/v solution of *thiourea* and 1 ml of a 20.0 per cent w/v solution of *stannous chloride* in *hydrochloric acid.* A yellow colour is produced.

Tests

pH (2.4.24): 4.0 to 7.0.

Radioactivity assay. Radioactivity of an aliquot of test solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparations. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radiochemical purity. Determine by ascending paper chromatography (2.4.15).

Mobile phase. 0.9 per cent w/v solution of *sodium chloride.*

Apply to the paper strip 10 µl of the injection under examination. Allow the mobile phase to rise 10 cm to 15 cm and dry the paper. Determine the distribution of radioactivity using a suitable detector. The retardation factor of Technetium-99m in colloidal form is 0.0 to 0.1 and pertechnetate ion migrates with an R_f of about 0.6. There may be other impurities with an R_f of 0.8 to 0.9. The radioactivity corresponding to technetium-99m in colloidal form represents not less than 92.0 per cent of the total radioactivity of the chromatogram.

Rhenium. Determine by UV spectrophotometer (2.4.7). Not more than 0.22 mg per milliliter.

Test solution. 1 ml of the injection under examination.

Reference solution. Using a solution containing 100 µg of *potassium perrhenate* (equivalent to 60 ppm of Re) and 240 µg of *sodium thiosulphate* per millilitre, prepare a range of solutions and dilute to the same final volume with *water.*

To the test solution and to 1 ml of each of the reference solutions add 5 ml of *hydrochloric acid*, 5 ml of a 5.0 per cent w/v solution of *thiourea* and 1 ml of a 20.0 per cent w/v solution of *stannous chloride* in *hydrochloric acid* and dilute to 25.0 ml with *water.* Allow to stand for 40 minutes and measure the absorbance of each solution at 400 nm, using a reagent blank as the compensation liquid. Using the absorbances obtained with the reference solutions, draw a calibration curve and calculate the concentration of rhenium in the injection under examination.

Physiological distribution. To be done in laboratory animals as described in section 1.6 in general chapter on Radiopharmaceutical preparations. Inject a volume, not more than 0.2 ml into a caudal vein of each of three mice, each

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weighing 20 g to 25 g. Euthanise the animals 20 minutes after the injection, remove the liver, spleen and lungs and measure the radioactivity in the organs using a gamma counter. Measure the radioactivity in the rest of the body after having removed the tail. Determine the percentage of radioactivity in the liver, the spleen and the lungs from the following expression:

$$\frac{A}{B} \times 100$$

where, A = radioactivity of the organ concerned,
B = total radioactivity in the liver, the spleen, the lungs and the rest of the body.

In each of the 3 animals not less than 80.0 per cent of the radioactivity is found in the liver and spleen and not more than 5.0 per cent in the lungs. If the distribution of radioactivity in 1 of the 3 animals does not correspond to the prescribed proportions, repeat the test on a further three animals. The preparation complies with the test if the prescribed distribution of radioactivity is found in 5 of the 6 animals used.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparations. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Less than 175 per *V* EU per ml, *V* being the maximum recommended dose in milliliters. Alternatively the preparation should pass the pyrogen test (2.2.8).

Storage. Store at a temperature between 2° to 8°.

Labelling. The label states, in addition to the information specified under injections (1) the amount of ^{99m}Tc as labelled rhenium sulphide expressed as total MBq (mCi or μCi) and concentration as MBq (mCi or μCi) per ml at the time of calibration; (2) the expiration date and time; (3) the statements "Caution—Radioactive Material".

Technetium (^{99m}Tc) DMSA Injection

Technetium (^{99m}Tc) Succimer Injection

Category. Diagnostic.

Description. A clear, colourless solution. Technetium (^{99m}Tc) DMSA Injection is a sterile solution and contains not less than 90.0 per cent and not more than 110.0 per cent of the stated technetium-99m radioactivity at the date and time stated on the label. Not less than 95 percent of the total technetium-99m is present as ^{99m}Tc-DMSA. Use the Technetium (^{99m}Tc) DMSA Injection within 4 hours from the date and time of preparation.

Radiopharmaceutical preparation. Technetium (^{99m}Tc) Succimer Injection is prepared aseptically following manufacturer's instructions provided with the kit. The kit contains a sterile solution of *meso*-2, 3-dimercaptosuccinic acid. It also contains a reducing substance, such as a tin salt and may contain stabilizers, antioxidants such as ascorbic acid, and inert additives. Sodium pertechnetate ^{99m}Tc (fission or non-fission), complying with specification given for it, is used with the kit to make the Technetium (^{99m}Tc) DMSA Injection.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon of Technetium (^{99m}Tc) has energy of 0.140 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of the Technetium (^{99m}Tc) DMSA.

Tests

pH (2.4.24). 2.3 to 3.5.

Radioactivity assay. Radioactivity of an aliquot of Test Solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparation. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Not less than 99.9 per cent due to Technetium-99m.

Complies with the radionuclidic purity tests as described under monograph on Sodium Pertechnetate (^{99m}Tc) Injection (Fission) or Sodium Pertechnetate (^{99m}Tc) Injection (Non Fission) used for preparation of Technetium (^{99m}Tc) DMSA Injection.

Radiochemical purity. Determine by Method A or by Method B.

Method A. Determine by paper chromatography (2.4.15).

Support. Whatman chromatography paper No 3/ No 1: 2 strips (1 x 8 cm).

Mobile phase. acetone.

Spot about two to five microlitres of ^{99m}Tc(III)-DMSA injection (corresponding activity) with a capillary or a micropipette at the point of spotting marked at 1 cm from one end of the strip (1 x 8 cm) in duplicate. Develop the chromatogram by ascending chromatography in the solvents till the solvent front reaches 6 cm from the point of spotting.

Cut the strip into 2 sections lower half (0–3 cm) and upper half (3-6 cm) and count in a well type scintillation counter. Per cent of impurity free pertechnetate (R_f – upper half) is determined in the solvent, as follows.

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Per cent free pertechnetate = Activity in the ^{99m}Tc pertechnetate zone x 100 /Total Activity.

The percentage of activity in the ^{99m}Tc-pertechnetate zone should not be more than 2 per cent of the total activity.

The radiochemical purity of ^{99m}Tc (III) DMSA (R_f=0) should not be less than 95per cent.

Method B. Determine by thin-layer chromatography (2.4.17), coating the plate with *silica gel.*

Mobile phase. methyl ethyl ketone.

Heat the plate at 110° C for 10 minutes. Use a plate such that during development the mobile phase migrates over a distance of 10 cm to 15 cm in about 10 minutes.

Apply to the plate 5 µl to 10 µl of the injection to be examined. Allow the mobile phase to rise 10 cm to 15 cm and dry the plate. Determine the distribution of radioactivity using a suitable detector. Technetium succimer complex remains at the starting point. Pertechnetate ion migrates near to the solvent front. Not less than 95.0 per cent of the total radioactivity is found in the spot corresponding to technetium succimer complex. The radioactivity corresponding to pertechnetate ion represents not more than 2.0 per cent of the total radioactivity.

Physiological distribution. To be done in laboratory animals as described in section 1.6 in general chapter on radiopharmaceuticals. If not fewer than two of the three rats used, the radioactivity in the kidneys is not less than 40 per cent, that in the liver is not more than 10.0 per cent, that in the stomach is not more than 2.0 per cent and that in the lungs is not more than 5.0 per cent of the injected dose at 1 hour post injection.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

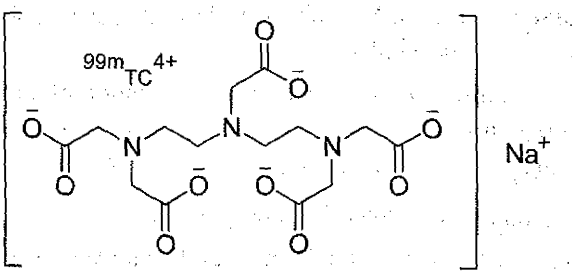
Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in ml, alternatively the preparation should pass the pyrogen test (2.2.8) in general chapter.

Storage. Store at room temperature with adequate shielding.

Labelling. The label states, in addition to the information specified under injection (1) the amount of ^{99m}Tc as labelled succimer expressed as total megabecquerels (or millicuries) and the radioactive concentration as megabecquerels per ml (or as millicuries per ml) on the date and time of reconstitution; (2) the expiration date and time; (3) the statement, "Caution — Radioactive Material".

Technetium (^{99m}Tc) DTPA Injection

Technetium (^{99m}Tc) Pentetate Injection



Category. Diagnostic.

Description. A clear, colourless solution. Technetium (^{99m}Tc) Pentetate Injection is a sterile solution and contains not less than 90.0 per cent and not more than 110.0 per cent of the stated technetium-99m radioactivity at the date and time stated on the label. Not less than 95.0 per cent of the total technetium-99m is present as Technetium (^{99m}Tc) Pentetate. Use the Technetium (^{99m}Tc) Pentetate Injection within 4 hours from the date and time of preparation.

Radiopharmaceutical preparation. Technetium (^{99m}Tc) Pentetate Injection is prepared aseptically following manufacturer's instructions provided with the kit. The kit contains diethylenetriaminepentaacetic acid, stannous chloride and may contain antimicrobial agents and buffers. Sodium pertechnetate ^{99m}Tc (fission or non-fission), complying with specification given for it, is used with the kit to make the (^{99m} Tc) Pentetate Injection IP.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon of technetium-99m has energy of 0.140 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of the Technetium (^{99m}Tc) Pentetate.

Tests

pH (2.4.24). 5.0 to 8.0.

Radioactivity assay. Radioactivity of an aliquot of Test Solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparation. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Not less than 99.9 per cent due to Technetium-99m.

Complies with the radionuclidic purity tests as described under monograph on Sodium Pertechnetate (^{99m}Tc) Injection (Fission) or Sodium Pertechnetate (^{99m}Tc) Injection (Non

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Fission) used for preparation of Technetium (^{99m}Tc) Pentetate Injection.

Radiochemical purity. Use method A or method B.

Method A. Determine by thin-layer chromatography (2.4.17), *silica gel G* coated plates.

Mobile phase (a). A 0.9 per cent w/v solution of *sodium chloride* in *water*.

Mobile phase (b). *methyl ethyl ketone*.

Test solution. Injection under examination.

Heat the plate at 110° for 10 minutes. Use a plate such that during development the mobile phase migrates over a distance of 10 cm to 15 cm in about 10 minutes.

Use two plates, one for mobile phase (a) and one for mobile phase (b). Spot about two to five microlitres of ^{99m}Tc- DTPA Injection with a capillary or a micropipette at the point of spotting marked at 3 cm from one end of the TLC plate in duplicate. Develop the chromatogram by ascending chromatography in both the mobile phases (a) and (b) till the solvent front reaches 10 - 15 cm from the point of spotting. Allow the plates to dry.

Scan the plates using a radiochromatogram scanner with its collimator suitably adjusted to measure the distribution of ^{99m}Tc. In TLC, with mobile phase (a) , impurities in colloidal form remain at the starting point while Technetium DTPA complex and pertechnetate ions migrate near to the solvent front. With mobile phase (b), the radioactivity at the point of spotting gives the technetium DTPA complex and impurities in colloidal form while pertechnetate ions migrate to the solvent front.

The sum of the percentages of radioactivity corresponding to impurities in the chromatograms obtained in test (a) and (b) does not exceed 5.0 per cent.

Method B. Determine by Paper chromatography (2.4.15) with Whatman No 1/No.3 paper.

Mobile phase (a). 0.9 per cent w/v solution of *sodium chloride* in water.

Mobile phase (b). *methyl ethyl ketone*.

Spot about two to five microlitres of ^{99m}Tc- DTPA Injection with a capillary or a micropipette at the point of spotting marked at 3 cm from one end of the strip (3 X 23 cm) in duplicate. Develop the chromatogram by ascending chromatography in both the mobile phases (a) and (b) till the solvent front reaches 15 cm from the point of spotting. Allow the strips to dry.

Scan the strips using a radiochromatogram scanner with its collimator suitably adjusted to measure ^{99m}Tc or alternately, cut the strip into one cm sections and count in a well type NaI(Tl) scintillation counter. In paper chromatography with mobile phase (a) , impurities in colloidal form remain at the starting point while Technetium DTPA complex and pertechnetate ions migrate near to the solvent front. With mobile phase (b), the radioactivity at the point of spotting gives the hydrolysed technetium and the technetium DTPA complex whereas, the pertechnetate impurity moves to the solvent front.

The sum of the percentages of radioactivity corresponding to impurities in the chromatograms obtained in test (a) and (b) does not exceed 5.0 per cent.

Physiological distribution. To be done in laboratory animals as described in section 1.6 in General Chapter on Radiopharmaceuticals. The whole body radioactivity retention should not be more than 5per cent of the injected dose in 24 hours post injection in 2 out of 3 mice.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in ml. Alternatively the preparation should pass the pyrogen test (2.2.8) in general chapter.

Storage. Store at room temperature with adequate shielding.

Labelling. The label states, in addition to the information specified under injection (1) the amount of ^{99m}Tc as labelled Pentetate expressed as total megabecquerels (or millicuries) and the radioactive concentration as megabecquerels per ml (or as millicuries per ml) on the date and time of reconstitution; (2) the expiration date and time; (3) the statement, "Caution — Radioactive Material".

Technetium (^{99m}Tc) EC Injection

Category. Diagnostic.

Description. A clear, colourless solution. Technetium (^{99m}Tc) EC Injection is a sterile solution and contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of technetium-99m radioactivity at the date and time stated on the label. Not less than 90 percent of the total technetium -99m is present as ^{99m}Tc-EC. Use the Technetium (^{99m}Tc) EC Injection within 4 hours from the date and time of preparation.

Radiopharmaceutical preparation. Technetium (^{99m}Tc) EC Injection is prepared aseptically following manufacturer's instructions provided with the kit. The kit contains diethyl ethylene dicysteine, stannous chloride and may contain antimicrobial agents and buffers. Sodium pertechnetate ^{99m}Tc

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(fission or non-fission), complying with specification given for it, is used with the kit to make the Technetium (^{99m}Tc) EC Injection.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon of Technetium (^{99m}Tc) has energy of 0.140 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of the Technetium (^{99m}Tc) EC.

Tests

pH (2.4.24). 6.0 to 8.0.

Radioactivity assay. Radioactivity of an aliquot of Test Solution is assayed using pre-calibrated radioactivity dose calibrator as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparation. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Not less than 99.9 per cent due to Technetium-99m.

Comply with the radionuclidic purity tests as described under monograph on Sodium Pertechnetate (^{99m}Tc) Injection (Fission) or Sodium Pertechnetate (^{99m}Tc) Injection (Non Fission) used for preparation of Technetium (^{99m}Tc) EC Injection.

Radiochemical purity. Determine by the paper chromatography (2.4.15)

Support. Whatman chromatography paper No 1/ No 3; 4 strips (1 x 8 cm)

Mobile phase (a). *acetone*

Mobile phase (b). 50 per cent *acetonitrile* in distilled water

Spot about 2-5 microlitres of ^{99m}Tc-EC injection with a capillary or a micropipette at the point of spotting marked at 1 cm from one end of the strip in duplicate.

Develop the chromatogram by ascending chromatography in both the solvents till the solvent front reaches 6 cm from the point of spotting. This takes about 8 - 10 min. Allow the strips to dry.

Cut the strips into 2 sections; lower half (0 –3 cm) and upper half (3-6 cm) and count in a well type scintillation counter. Percentage of impurities viz. free pertechnetate and reduced hydrolyzed technetium are determined in the solvents, acetone (R_f – upper half) and 50 per cent acetonitrile (R_f – lower half) respectively as follows.

$$\text{(per cent) free pertechnetate} = \frac{(\text{Activity in the } [^{99m}\text{Tc}] \text{ pertechnetate zone} \times 100)}{\text{Total Activity}}$$

$$\text{(per cent) reduced hydrolysed technetium (RHT)} = \frac{(\text{Activity in RHT zone} \times 100)}{\text{Total Activity}}$$

Percentage of each impurity should not be more than 5 per cent of the total activity.

The radiochemical purity of ^{99m}Tc EC = 100 – (per cent free pertechnetate + per cent reduced hydrolyzed technetium).

The Radiochemical Purity should not be less than 90per cent of the total activity.

Physiological distribution. To be done in laboratory animals as described in section 1.6 in general chapter on radiopharmaceuticals. Not less than 80per cent of the injected radioactivity is present in the urine and bladder together and not more than 5per cent of the injected radioactivity is present in the kidneys in at least 2 out of 3 swiss mice at 30 minutes post injection.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

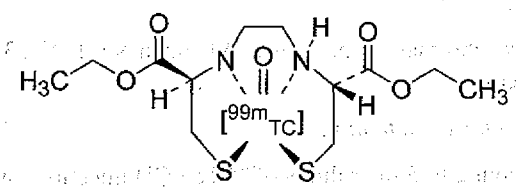
Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in ml. Alternatively the preparation should pass the pyrogen test (2.2.8) in general chapter.

Storage. Store at room temperature with adequate shielding.

Labelling. The label states, in addition to the information specified under injection (1) the amount of ^{99m}Tc as labelled EC expressed as total megabecquerels (or millicuries) and the radioactive concentration as megabecquerels per ml (or as millicuries per ml) on the date and time of reconstitution; (2) the expiration date and time; (3) the statement, "Caution — Radioactive Material".

Technetium (^{99m}Tc) ECD Injection

^{99m}Tc-Bicisate injection



Category. Diagnostic.

Description. A clear, colourless solution. Technetium (^{99m}Tc) ECD Injection is a sterile solution and contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of technetium-99m radioactivity at the date and time stated on the label. Not less than 90 percent of the total technetium-99m is present as ^{99m}Tc-ECD. Use the Technetium

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(^{99m}Tc) ECD Injection within 4 hours from the date and time of preparation.

Radiopharmaceutical preparation. Technetium (^{99m}Tc) Bicisate Injection is prepared aseptically following manufacturer's instructions provided with the kit. The kit contains *N,N'*-(1,2-ethylenediyl)bis[(2*R*)-2-amino-3-sulphanylpropanoic acid] diethyl ester and reducing agents such as a stannous salt and it may contain stabilizers and inert additives such as mannitol and disodium edetate. Sodium pertechnetate ^{99m}Tc (fission or non-fission), complying with specification given for it, is used with the kit to make the Technetium (^{99m}Tc) ECD Injection.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon of Technetium (^{99m}Tc) has energy of 0.140 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of the Technetium (^{99m}Tc) ECD.

Tests

pH (2.4.24). 6.0 to 8.0.

Radioactivity assay. Radioactivity of an aliquot of Test Solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparation. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Not less than 99.9 per cent due to Technetium-99m.

Complies with the radionuclidic purity tests as described under monograph on Sodium Pertechnetate (^{99m}Tc) Injection (Fission) or Sodium Pertechnetate (^{99m}Tc) Injection (Non Fission) used for preparation of Technetium (^{99m}Tc) ECD Injection.

Radiochemical purity. Determine by paper chromatography (2.4.15).

Support. chromatography paper Whatman No. 1/ No. 3 strip (3 x 23 cm).

Mobile phase. methanol.

Spot about 2 to 5 microlitres of ^{99m}Tc ECD injection with a capillary or a micropipette at the point of spotting marked at 3 cm from one end of the strip in duplicate. Develop the chromatogram by ascending chromatography till the solvent front reaches 15 cm from the point of spotting. This takes about 60 min. Allow the strip to dry. Scan the strip using a radiochromatogram scanner with collimator adjusted for ^{99m}Tc (0.140MeV) or cut into one cm sections and count in a well type NaI(Tl) scintillation counter. The R_f values of ^{99m}Tc-ECD, ^{99m}Tc-pertechnetate and ^{99m}Tc -reduced, hydrolyzed technetium are 0.5- 1.0, 0.2 - 0.4 and 0 – 0.1 respectively. Calculate the per cent of activity present as ^{99m}Tc-ECD as follows:

$$\text{(per cent) RCP} = \frac{\text{Activity in the [}^{99m}\text{Tc] ECD zone} \times 100}{\text{Total Activity}}$$

The Radiochemical Purity should not be less than 90per cent.

Sterility (2.2.11) Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

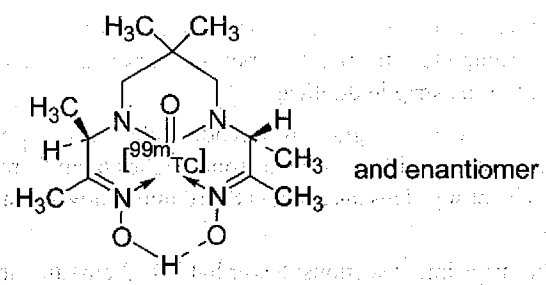
Bacterial endotoxins (2.2.3) Less than 175 per V EU per ml, V being the maximum recommended dose in ml, alternatively the preparation should pass the pyrogen test (2.2.8) in general chapter.

Storage. Store at room temperature with adequate shielding.

Labelling. The label states, in addition to the information specified under injection (1) the amount of ^{99m}Tc as labelled ECD expressed as total megabecquerels (or millicuries) and the radioactive concentration as megabecquerels per ml (or as millicuries per ml) on the date and time of reconstitution; (2) the expiration date and time; (3) the statement, "Caution — Radioactive Material".

Technetium (^{99m}Tc) Exametazime Injection

Technetium (^{99m}Tc) HMPAO Complex Injection



and enantiomer

Category. Diagnostic

Description. Technetium (^{99m}Tc) Exametazime Injection is a clear, colourless aqueous solution. It contains not less than 90.0 per cent and not more than 110.0 per cent of the content of technetium-99m stated on the label at the reference date and time. Not less than 80.0 per cent of the total technetium-99m radioactivity is present as lipophilic (^{99m}Tc) exametazime complex. Technetium (^{99m}Tc) Exametazime Injection should be used within 30 minutes from time of reconstitution.

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Radiopharmaceutical preparation. Technetium (^{99m}Tc) Exametazime Injection is prepared aseptically following instructions provided with the kit. The kit contains a mixture of *rac-N,N'*-bis [(1*R*, 2E)-2-(hydroxyimino)-1-methylpropyl]-2, 2-dimethylpropane-1, 3-diamine, reducing agents and may contain buffer, stabilizing agents and sodium chloride to make the solution isotonic with blood. *Sodium pertechnetate (^{99m}Tc) injection (fission or non-fission)* is used for preparation of Technetium (^{99m}Tc) Exametazime Injection.

Identification

A. Determine by gamma-ray spectrometry as described in general chapter on Radiopharmaceutical preparations. The most prominent gamma photon of technetium-99m has energy of 0.140 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of technetium (^{99m}Tc) exametazime.

Tests

pH (2.4.24). 5.0 to 10.0.

Radioactivity assay. Radioactivity of an aliquot of test solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparations. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Not less than 99.9 per cent due to Technetium-99m. Complies with the radionuclidic purity tests as described under monograph of *sodium pertechnetate (^{99m}Tc) injection (fission) or sodium pertechnetate (^{99m}Tc) injection (non-fission)* used for preparation of technetium (^{99m}Tc) exametazime.

Radiochemical purity. Determine by ascending paper chromatography (2.4.15).

Test solution. The injection under examination.

Mobile phase. Methyl ethyl ketone (system A), 0.9 per cent *sodium chloride* (system B) and a mixture of 50 volumes of *acetonitrile* and 50 volumes of *water* (system C).

Paper. Cellulose paper strips (system A and system B), silica gel impregnated glass fiber paper strips (system C).

Apply about 5µl of the injection to the suitable paper strip under examination, suitably diluted to give an optimum radioactive count rate. Allow the mobile phase to rise 15 cm and air dry the paper strip. Determine the distribution of radioactivity using a suitable method. In system (A), the secondary exametazime complex and reduced/hydrolysed technetium-99m have an R_f value of 0, and the lipophilic exametazime complex and the pertechnetate ion have an R_f value of 0.8 to 1.0. In system (B), reduced / hydrolysed technetium-99m has an R_f value of 0, and the lipophilic exametazime complex, the secondary exametazime complex and the pertechnetate ion have an R_f value of 0.8 to 1.0. In system (C), the pertechnetate ion has an R_f value of 0.8 to 1.0, and the lipophilic exametazime complex, the secondary exametazime complex and reduced / hydrolysed technetium-99m have an R_f value of 0. The sum of the percentages of radioactivity corresponding to the pertechnetate ion in system (C) and reduced / hydrolysed technetium-99m in system (B) is less than 10.0 per cent. Not less than 80.0 per cent of the total technetium-99m radioactivity is present as lipophilic exametazime complex.

Physiological distribution. To be done in laboratory animals as described in section 1.6 in general chapter on Radiopharmaceutical preparations using a set of three mice each weighing 20 g to 25 g. After 5 to 10 minutes of the injection, the activity in brain should not be less than 1.5 per cent of the injected radioactivity; the activity in the intestine should not be more than 20.0 per cent of the injected radioactivity and the activity in the liver should not be more than 15.0 per cent of the injected radioactivity.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparations. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in ml. Alternatively the preparation should pass the pyrogen test (2.2.8).

Storage. Store at a temperature between 2° to 8° with adequate shielding.

Labeling. The label states, in addition to the information specified under injections (1) the amount of ^{99m}Tc as labelled exametazime complex expressed as total MBq (mCi or µCi) and concentration as MBq (mCi or µCi) per ml at the time of calibration; (2) the expiration date and time; (3) the statement "Caution—Radioactive Material."

Technetium (^{99m}Tc) Glucoheptonate Injection

Technetium (^{99m}Tc) Gluceptate Injection; Technetium (^{99m}Tc) GHA Injection

Category. Diagnostic.

Description. A clear colourless solution. Technetium (^{99m}Tc) Gluceptate Injection is a sterile solution and contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of technetium 99m radioactivity at the time and date indicated on the label. Not less than 90 percent of the total technetium-99m is present as ^{99m}Tc-Glucoheptonate. Use the Technetium (^{99m}Tc) Gluceptate Injection within 6 hours from the date and time of preparation.

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Radiopharmaceutical preparation. Technetium (^{99m}Tc) Glucoheptonate Injection is prepared aseptically following manufacturer's instructions provided with the kit. The kit contains sodium gluceptate, stannous chloride and may contain antimicrobial agents and buffers. Sodium pertechnetate ^{99m}Tc (fission or non-fission), complying with specification given for it, is used with the kit to make the Technetium (^{99m}Tc) Glucoheptonate Injection.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon of Technetium (^{99m}Tc) has energy of 0.140 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of the Technetium (^{99m}Tc) Glucoheptonate.

Tests

pH (2.4.24). 5.0 to 8.0.

Radioactivity Assay. Radioactivity of an aliquot of Test Solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Not less than 99.9 per cent due to Technetium-99m.

Complies with the radionuclidic purity tests as described under monograph on Sodium Pertechnetate (^{99m}Tc) Injection (Fission) or Sodium Pertechnetate (^{99m}Tc) Injection (Non Fission) used for preparation of Technetium (^{99m}Tc) Gluceptate Injection.

Radiochemical purity. Determine by paper chromatography (2.4.15).

Support. Whatman chromatography paper No.1/No.3; 4 strips (3 x 23 cm)

Mobile phase. 85 per cent methanol in 0.9 per cent w/v of sodium chloride solution in WFI.

Spot about two to five microlitres of ^{99m}Tc-Glucoheptonate Injection with a capillary or a micropipette at the point of spotting marked at 3 cm from one end of the strip in duplicate.

Develop the chromatogram by ascending chromatography in both the solvents till the solvent front reaches 15 cm from the point of spotting. Allow the strips to dry. Scan the strips using a radiochromatogram scanner with collimator suitably adjusted to measure ^{99m}Tc or cut into one cm sections and count in a well type NaI(Tl) scintillation counter. Percentage of impurities viz. free pertechnetate and reduced hydrolysed technetium are determined in the solvents, viz. 85 per cent methanol (R_f 0.55– 0.75) and saline (R_f 0.0-0.1) respectively as follows:

$$\text{(per cent) free pertechnetate} = \frac{\text{(Activity in the [}^{99m}\text{Tc] pertechnetate zone} \times 100)}{\text{Total Activity}}$$

$$\text{(per cent) reduced hydrolysed technetium (RHT)} = \frac{\text{(Activity in RHT zone} \times 100)}{\text{Total Activity}}$$

per cent Radiochemical Purity = 100 - [(per cent) free pertechnetate + (per cent) reduced hydrolyzed technetium].

The Radiochemical Purity should not be less than 90per cent.

Physiological distribution. To be done in laboratory animals as described in section 1.6 in general chapter on radiopharmaceuticals. Activity in kidneys should not be less than 15per cent, Blood should not be more than 3 per cent, GIT should not be more than 15per cent, Liver should not be more than 5per cent injected dose in 2 out of 3 rats at 1 hour post injection.

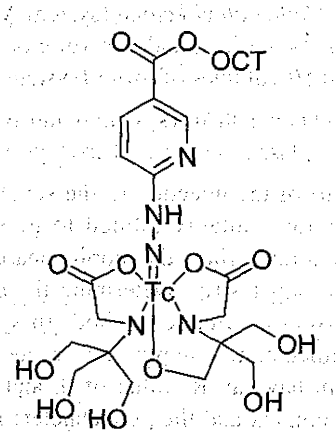
Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Not more than 175 per V EU per ml of the injection, in which V is the maximum recommended total dose in ml. Alternatively the preparation should pass the pyrogen test (2.2.8) in general chapter.

Storage. Store at room temperature with adequate shielding.

Labelling. The label states, in addition to the information specified under injection (1) the amount of ^{99m}Tc as labelled glucoheptonate expressed as total megabecquerels (or millicuries) and the radioactive concentration as megabecquerels per ml (or as millicuries per ml) on the date and time of reconstitution; (2) the expiration date and time; (3) the statement, "Caution — Radioactive Material".

Technetium (^{99m}Tc) HYNIC-TOC Injection



Category. Diagnostic.

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Description. Technetium (^{99m}Tc) HYNIC-TOC Injection is a clear, colourless sterile solution and contains not less than 90.0 per cent and not more than 110.0 per cent of the stated technetium-99m radioactivity at the date and time stated on the label. Not less than 90.0 per cent of the total technetium-99m is present as Technetium (^{99m}Tc) HYNIC-TOC. Technetium (^{99m}Tc) HYNIC-TOC Injection should be used within 6 hours from the time of preparation.

Radiopharmaceutical preparation. Technetium (^{99m}Tc) HYNIC-TOC Injection is prepared aseptically following manufacturer's instructions provided with the kit. The kit contains hydrazinonicotinyl-tyr^3-octreotide (HYNIC-TOC), stannous chloride and may contain coligands such as disodium ethylene diamine tetracetic acid, tricine, antimicrobial agents and buffers. *Sodium pertechnetate (^{99m}Tc) injection (fission or non-fission)* is used with the kit to make the (^{99m} Tc) HYNIC-TOC injection.

Identification

A. Determine by gamma-ray spectrometry as described in general chapter on Radiopharmaceutical preparations. The most prominent gamma photon of technetium-99m has energy of 0.140 MeV.

B. In the test for radiochemical purity, the chromatogram obtained contributes to the identification of the technetium (^{99m}Tc) HYNIC-TOC.

Tests

pH (2.4.24). 5.0 to 8.0.

Radioactivity assay. Radioactivity of an aliquot of test solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparations. It is expressed as MBq (mCi) in volume V ml at a particular time and date, V being the maximum recommended dose in ml.

Radionuclidic purity. Not less than 99.9 per cent due to technetium-99m.

Complies with the radionuclidic purity tests as described under monograph on *sodium pertechnetate (^{99m}Tc) injection (fission) or sodium pertechnetate (^{99m}Tc) injection (non-fission)* used for preparation of technetium (^{99m}Tc) HYNIC-TOC injection.

Radiochemical purity. Use method A or method B.

Method A. Determine by instant thin layer chromatography (2.4.17).

Mobile phase (a). *Methyl ethyl ketone.*

Mobile phase (b). A mixture of 50 volumes of *acetonitrile* and 50 volumes of *water.*

Test solution. The injection under examination.

(a) Apply to the plate 5 µl to 10 µl of the injection under examination. Allow the mobile phase (a) to rise 10 cm to 15 cm in about 10 minutes and dry the plate in air. Determine the distribution of radioactivity using a suitable detector. Pertechnetate ion migrates near to the solvent front. Technetium HYNIC-TOC complex and impurities of hydrolysed technetium in colloidal form remain at the point of spotting.

(b) Apply to the plate 5 µl to 10 µl of the injection under examination. Allow the mobile phase (b) to rise 10 cm to 15 cm and dry the plate. Determine the distribution of radioactivity using a suitable detector. Technetium HYNIC-TOC complex and pertechnetate ion migrate near to the solvent front. Impurities of hydrolysed technetium in colloidal form remain at the point of spotting.

The sum of the percentages of radioactivity corresponding to impurities in the chromatograms obtained in test solutions (a) and (b) should not be more than 10.0 per cent.

Method B. Determine by liquid chromatography (2.4.14).

Test solution. The injection under examination.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to silica (5 µm),
- mobile phase: A. 0.1 per cent *trifluoroacetic acid* in *acetonitrile,*
  B. 0.1 per cent *trifluoroacetic acid* in *water,*
- a gradient programme using the conditions given below,
- flow rate: 1 ml per minute,
- radioactivity detector adjusted for 100 to 200 KeV energy,
- injection volume: 20 µl

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	0	100
3	0	100
5	30	70
18	30	70
22	50	50
24	50	50
25	50	50
30	0	100

The ^{99m}Tc-HYNIC-TOC shall have a retention time of 17-20 minutes. The sum of the percentage of radioactivity corresponding to impurities in the chromatograms obtained should not more than 10.0 per cent.

Physiological distribution. To be done in laboratory animals as described in section 1.6 in general chapter on

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Radiopharmaceutical preparations. After 4hour of the injection, the whole body radioactivity retention shall not be more than 10.0 per cent of the injected dose in 2 out of 3 swiss mice.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparations. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in ml. Alternatively the preparation should pass the pyrogen tests (2.2.8.).

Storage. Store at 2° to 8° with adequate shielding.

Labelling. The label states, in addition to the information specified under injection (1) the amount of ^{99m}Tc as labelled HYNIC-TOC expressed as total MBq (or mCi) and the radioactive concentration as MBq per ml (or as mCi per ml) on the date and time of reconstitution; (2) the expiration date and time; (3) the statement, "Caution - Radioactive Material."

Technetium (^{99m}Tc) Labelled Human Serum Albumin Nanocolloid Injection

(^{99m}Tc-HSA-NC Injection)

Category: Diagnostic.

Description. ^{99m}Tc-HSA-NC Injection is a clear, colourless sterile solution, containing not less than 90.0 per cent and not more than 110.0 per cent of the stated ^{99m}Tc-radioactivity at the date and time stated on the label. Not less than 90.0 per cent of the total technetium-99m is present as ^{99m}Tc-HSA-NC. ^{99m}Tc-HSA-NC injection should be used within 4 hours from the time of preparation.

Radiopharmaceutical preparation. ^{99m}Tc-HSA-NC Injection is prepared aseptically following the manufacturer's instructions provided with the kit. The kit contains *human albumin*, stannous chloride, Tween 20, ascorbic acid and buffer. ^{99m}Tc as *sodium pertechnetate (fission or non-fission)*, is used with the kit to make ^{99m}Tc-HSA-NC Injection.

Identification

A. Determine by gamma ray spectrometry using a gamma ray spectrometer. The prominent gamma photon of technetium-99m has energy of 0.140 MeV.

B. In the test for radiochemical purity, the chromatogram obtained contributes to the identification of ^{99m}Tc-HSA-NC.

Tests

pH (2.4.24). 5.0 to 6.0.

Radioactivity assay. Radioactivity of an aliquot of test solution is assayed using a pre calibrated dose calibrator. It is expressed as MBq (mCi) in volume V ml at a particular time and date, V being the maximum recommended dose in ml.

Radionuclidic purity. Not less than 99.9 per cent due to Technetium-99m.

Complies with the radionuclidic purity tests as described under monograph on *Sodium Pertechnetate (^{99m}Tc) Injection (Fission)* or *Sodium Pertechnetate (^{99m}Tc) Injection (Non Fission)* used for preparation of ^{99m}Tc-HSA-NC Injection.

Radiochemical purity. Determine by paper chromatography (2.4.15)

Support. Chromatography paper Whatman paper No. 1 or equivalent. (3 cm x 15 cm)

Mobile phase. 0.9 per cent sodium chloride solution.

Apply 5 to 10 µl of the injection under examination 3 cm from bottom end of the chromatography strip. Allow the mobile phase to rise 10 cm and dry the strip in air. Determine the distribution of radioactivity using a suitable radioactivity counting system. The free pertechnetate (Tc-99m) ion exhibits R_f in the range 0.8 to 1.0 whereas ^{99m}Tc-HSA-NC remains at the point of application (R_f 0.1). The percentage radioactivity (at R_f 0.8 to 1.0) should not more than 5 per cent.

Physiological distribution. To be done in laboratory animals as described in section 1.6 in General Chapter on Radiopharmaceutical preparations by using Wistar rats. The animals are administered 50 µl volume of the ^{99m}Tc-HSA-NC Injection subcutaneously to the footpad of the animal under anaesthesia. At 2 hour post injection the ^{99m}Tc-activity in relevant organs and tissues are measured using suitable radioactivity counter. ^{99m}Tc-activity in the sentinel node should not be less than 1.5 per cent and ^{99m}Tc-activity in popliteal node indicates sentinel lymph node accumulation and activity in upstream nodes (iliac, inguinal) and other regions indicates further spread of the preparation through the lymphatic stream. Not more than 1.0 per cent of injected dose should be present in blood, not more than 1 per cent of injected dose should be present in liver and not more than 1 per cent of injected dose should be present in kidney in two out of three rats.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparations. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in ml. Alternatively the preparation should pass the pyrogen tests (2.2.8.).

Storage: Injection may be stored at room temperature with adequate shielding.

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Labelling. The label states, in addition to the information specified under injection (1) the amount of ^{99m}Tc as labelled HSA-NC expressed as total megabecquerels (or millicuries) and the radioactive concentration as megabecquerels per ml (or as millicuries per ml) on the date and time of reconstitution; (2) the expiration date and time; (3) the statement, "Caution - Radioactive Material."

Technetium (^{99m}Tc) Macrosalb Injection

Category. Diagnostic

Description. Technetium (^{99m}Tc) Macrosalb Injection is a white, sterile suspension of *human albumin* in the form of irregular insoluble aggregates obtained by denaturing human albumin in aqueous solution. The particles are labelled with technetium-99m and have a typical diameter between 10 µm and 100 µm. Technetium (^{99m}Tc) Macrosalb Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated technetium-99m radioactivity at the date and time stated on the label. The human albumin employed complies with the requirements prescribed in the monograph *human albumin*. The Technetium (^{99m}Tc) Macrosalb Injection should be used within 4 hours from date and time of preparation.

Radiopharmaceutical preparation. Technetium (^{99m}Tc) Macrosalb Injection is prepared aseptically following instructions provided with the kit. The kit contains *human albumin* and other excipients such as suitable buffer, reducing agents and an antimicrobial preservative. *Sodium pertechnetate (^{99m}Tc) injection (fission or non-fission)* is used with the kit to make technetium (^{99m}Tc) macrosalb injection.

Identification

A. Determine by gamma-ray spectrometry as described in general chapter on Radiopharmaceutical preparations. The most prominent gamma photon of technetium-99m has energy of 0.141 MeV.

B. The tests for non filterable radioactivity and particle size contribute to the identification of the preparation.

Tests

pH (2.4.24). 3.8 to 7.5.

Radioactivity assay. Radioactivity of an aliquot of test solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparations. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Specific radioactivity. Not less than 37 MBq of technetium-99m per milligram of aggregated albumin at the date and time of administration.

Non filterable radioactivity. Not less than 90.0 per cent of the total radioactivity.

Use a polycarbonate membrane filter 13 to 25 mm in diameter, 10 µm thick and with circular pores 3 µm in diameter. Fit the membrane into a suitable holder. Place 0.2 ml of the preparation to be examined on the membrane and filter, adding 20 ml of a 9 g per liter solution of *sodium chloride* during the filtration. Determine the radioactivity remaining on the membrane.

Particle size. Not more than 10 particles have a maximum dimension greater than 100 µm and no particle having a maximum dimension greater than 150 µm is present in a sample of at least 5000 particles.

Examine using a microscope. Dilute the preparation under examination if necessary so that the number of particles is just low enough for individual particles to be distinguished. Using a syringe fitted with a needle having a calibre not less than 0.35 mm, place a suitable volume in a suitable counting chamber such as a haemocytometer cell, taking care not to overfill the chamber. Allow the preparation to be examined to settle for 1 min and carefully add a cover slide without squeezing the sample. Scan an area corresponding to at least 5000 particles. The preparation may be released for use before completion of the test.

Physiological distribution. To be done in laboratory animals as described in section 1.6 in general chapter on Radiopharmaceutical preparations. Inject a volume not greater than 0.2 ml in to a caudal vein of each of 3 rats weighing 150-250 g. Euthanise the animals 15 minutes after the injection, remove liver, spleen and lungs. Measure the radioactivity in the organs using a gamma counter. Measure the radioactivity in the rest of the body, including the blood, after having removed the tail. Determine the percentage of radioactivity in the lungs, liver and spleen from the following expression:

$$\frac{A}{B} \times 100$$

Where, A = radioactivity of the organ concerned;
B = total radioactivity in the liver, spleen, lungs and rest of the body.

In not fewer than 2 of the 3 animals used, not less than 80.0 per cent of the radioactivity is found in the lungs and not more than a total of 5.0 per cent in the liver and spleen.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparations. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in millilitres. Alternatively the preparation should pass the pyrogen test (2.2.8).

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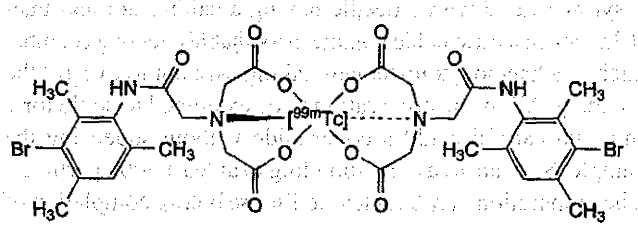
Storage. Store at a temperature between 2° to 8° with adequate shielding.

Labelling. The label states, in addition to the information specified under injections (1) the amount of ^{99m}Tc as labelled macrosalb expressed as total MBq (mCi or μCi) and concentration as MBq (mCi or μCi) per ml at the time of calibration; (2) the expiration date and time; (3) the statements "Caution—Radioactive Material", "shake well before use" and "the preparation is not to be used if after shaking, the suspension does not appear uniform".

Technetium (^{99m}Tc) Mebrofenin Injection



Category. Diagnostic.

Description. A clear colourless solution. Technetium (^{99m}Tc) Mebrofenin Injection is a sterile solution and contains not less than 90.0 per cent and not more than 110.0 per cent of the stated technetium-99m radioactivity, at the date and time stated on the label. Not less than 94 per cent of the total technetium-99m is present as ^{99m}Tc-Mebrofenin. Use the ^{99m}Tc-Mebrofenin injection within 4h from the date and time of preparation.

Radiopharmaceutical preparation. Technetium (^{99m}Tc) Mebrofenin Injection is prepared aseptically following manufacturer's instructions provided with the kit. The kit contains [[[(3-bromo-2,4,6-trimethylphenyl)carbamoyl]methyl]imino]diacetic acid (mebrofenin) and a stannous salt. The kit may have preadjusted pH and may contain reducing, chelating, stabilizing, filling and antioxidizing agents as well as antimicrobial preservatives and buffers. The injection may also be prepared under aseptic processing combined with sterilization by filtration. Sodium pertechnetate ^{99m}Tc (fission or non-fission), complying with specification given for it, is used with the kit to make the Technetium (^{99m}Tc) Mebrofenin Injection.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon of technetium-99m has energy of 0.140 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of ^{99m}Tc-Mebrofenin.

Tests

pH (2.4.24). 4.0 to 7.0.

Radioactivity Assay. Radioactivity of an aliquot of Test Solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Not less than 99.9 per cent due to Technetium-99m.

Comply with the radionuclidic purity tests as described under monograph on Sodium Pertechnetate (^{99m}Tc) Injection (Fission) or Sodium Pertechnetate (^{99m}Tc) Injection (Non Fission) used for preparation of Technetium (^{99m}Tc) Mebrofenin Injection.

Radiochemical purity. Not less than 94 per cent due to Technetium-99m as ^{99m}Tc Mebrofenin.

Impurity A (Tc-99m in colloidal form). Determine by Thin Layer Chromatography (2.4.17) coating the plate of 1 x 7.5 cm with Reverse Phase modified Silica Gel.

Mobile phase. a mixture of 55 volumes of 0.385 per cent solution of *ammonium acetate* and 45 volumes of *acetonitrile.*

Test solution. The preparation to be examined.

Determine the distribution of radioactivity using a suitable calibrated detector.

Retardation factor.

^{99m}Tc –Colloid	: 0 - 0.1
^{99m}Tc-Mebrofenin	: 0.4 - 0.6

per cent of radioactivity due to Impurity A is the per cent activity at R_f = 0 - 0.1.

Other Radiochemical impurities. Determine by liquid chromatography (2.4.14).

Test solution: The preparation under examination.

Chromatographic system

- a stainless steel column 25.0 cm x 4.6mm packed with octadecyl silane bonded to porous silica (5 μm),
- mobile phase: A. 0.385 per cent solution of *ammonium acetate*

B. *acetonitrile.*

- flow rate: 1 ml per minute,
- radioactivity detector connected in series,
- injection volume. 20 μl.

Time (in min.)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	70	30
20	40	60
30	40	60

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Retention time of ^{99m}Tc-Mebrofenin: 14-16 minutes and ^{99m}Tc-Pertechnetate ion: about 4 minutes.

Calculate the percentage of radioactivity due to ^{99m}Tc-Mebrofenin using the following expression:

$$(100 - A) \times T$$

In which A = per cent of radioactivity due to Impurity A determined in the test for Impurity A under radiochemical purity and T= proportion of the radioactivity in the peak due to ^{99m}Tc- Mebrofenin relative to the total eluted radioactivity in the chromatogram obtained with the test solution.

Not less than 94 per cent of ^{99m}Tc is present as ^{99m}Tc-Mebrofenin.

Physiological distribution. To be done in laboratory animals as described in section 1.6 in General Chapter on Radiopharmaceuticals. Not less than 70per cent of the injected radioactivity is present in the gallbladder and intestine not more than 10 per cent of the injected radioactivity is present in the kidneys, not more than 10per cent present in liver, not more than 5per cent of the radioactivity is found in the stomach and not more than 3per cent of the injected radioactivity is present in the blood in the 2 out of 3 mice at 30 minutes post injection.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in milliliters. Alternatively the preparation should pass the pyrogen test (2.2.8) in general chapter.

Storage. Store in a single-dose or in multiple-dose containers, at a temperature between 2° to 8°.

Labelling. The label states, in addition to the information specified for *injections* (1) the time and date of calibration; (2) the amount of ^{99m}Tc as labelled mebrofenin expressed as total megabecquerels (or millicuries) and the concentration as megabecquerels per ml (or as millicuries per ml) on the date and time of calibration; (3) the expiration date and time; (4) the statement, "Caution — Radioactive Material".

Technetium (^{99m}Tc) Medronate Complex Injection

Technetium (^{99m}Tc) MDP Complex Injection; (^{99m}Tc) Dihydrogen Methylene Diphosphonate (MDP) Complex Injection.

Category. Diagnostic.

Description. A clear colourless solution. Technetium (^{99m}Tc) Medronate (MDP) Complex Injection is a sterile solution and contains not less than 90.0 per cent and not more than 110.0 per cent of the stated technetium-99m radioactivity, at the date and time stated on the label. Not less than 95 percent of the total technetium-99m is present as ^{99m}Tc-MDP complex. Use the ^{99m}Tc-MDP complex injection within 6h from the date and time of preparation.

Radiopharmaceutical preparation. Technetium (^{99m}Tc) Medronate Complex Injection is prepared aseptically following manufacturer's instructions provided with the kit. The kit contains sodium methylene diphosphonate (sodium medronate) and a stannous salt. The kit may have preadjusted pH and may contain reducing, chelating, stabilizing, filling and antioxidizing agents as well as antimicrobial preservatives and buffers. The injection may also be prepared under aseptic processing combined with sterilization by filtration. Sodium pertechnetate ^{99m}Tc (fission or non-fission), complying with specification given for it, is used with the kit to make the (^{99m} Tc) MDP Injection.

Identification

A. Determine by gamma-ray spectrometry. The spectrum obtained corresponds to the spectrum obtained with reference solution of technetium-99m. The most prominent gamma photon of technetium-99m has energy of 0.140 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of ^{99m}Tc-Medronate.

Tests

pH (2.4.24). 5.0 to 8.0.

Radioactivity assay. Radioactivity of an aliquot of Test Solution is assayed using pre-calibrated radioactivity dose calibrator as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparation. It is expressed as MBq (mCi) in volume V ml at a particular time and date.

Radionuclidic purity. Not less than 99.9 per cent due to: Technetium-99m (^{99m}Tc).

Complies with the radionuclidic purity tests as described under monograph on Sodium pertechnetate (^{99m}Tc) injection (fission) or Sodium pertechnetate (^{99m})Tc injection (non-fission) used for preparation of Technetium (^{99m}Tc) MDP Complex Injection.

Radiochemical purity. Determine by ascending paper chromatography (2.4.15).

Support. Chromatography paper Whatman No.1/No3 strip (3 x 23 cm).

Mobile phase. 85 per cent *methanol* in distilled water, *0.5 M acetate buffer*, pH adjusted to 5.

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Spot about two to five micro liters of ^{99m}Tc-MDP with a capillary or a micropipette at the point of spotting marked at 3 cm from one end of the strip in duplicate.

Develop the chromatogram by ascending chromatography in both the mobile phase till the solvent front reaches 15 cm from the point of spotting. This takes about 90 min and about 60 min in 85 per cent *methanol* and *acetate buffer* respectively. Allow the strips to dry.

Scan the strips using a radiochromatogram scanner with collimator suitably adjusted to measure ^{99m}Tc or cut into one cm sections and count in a well type NaI(Tl) scintillation counter. Per cent of Impurities viz. free pertechnetate and reduced hydrolysed technetium are determined in the solvents, viz. 85 per cent methanol (R_f 0.55– 0.75) and acetate buffer (R_f 0.0-0.1) respectively as follows:

$$\text{(per cent) free pertechnetate} = \frac{\text{(Activity in the [}^{99m}\text{Tc] pertechnetate zone} \times 100)}{\text{Total Activity}}$$

$$\text{(per cent) reduced hydrolysed technetium (RHT)} = \frac{\text{(Activity in RHT zone} \times 100)}{\text{Total Activity}}$$

Percentage of each impurity should be not more than 5 per cent of the total activity.

The radiochemical purity of Tc-MDP = 100 – (per cent free pertechnetate + per cent reduced hydrolyzed technetium).

The Radiochemical Purity should not be less than 95per cent of the total activity.

Physiological distribution. To be done in laboratory animals as described in section 1.6 in General Chapter on Radiopharmaceuticals. Using a set of three Wistar rats, inject a volume less than 0.2 ml containing not more than 200 µg (0.2 mg) of MDP. Determine the radioactivity in the liver and kidney. Biodistribution pattern should not be less than 2 per cent in both femurs, not more than 5 per cent in liver and not more than 5 per cent in kidneys of Total Retained Dose (per cent TRD) in 2 out of 3 rats at 1 hour post injection.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparation. The injection may be released for use before completion of the test.

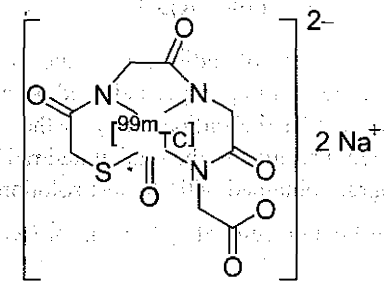
Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in ml. Alternatively the preparation should pass the pyrogen test (2.2.8) in general chapter.

Storage. Store at room temperature with adequate shielding.

Labelling. The label states, in addition to the information specified for injections (1) the time and date of calibration; (2) the amount of ^{99m}Tc as labelled mebrofenin expressed as total megabecquerels (or millicuries) and the concentration as megabecquerels per ml (or as millicuries per ml) on the date and time of calibration; (3) the expiration date and time; (4) the statement, "Caution — Radioactive Material".

Technetium (^{99m}Tc) Mertiatide Injection

Technetium (^{99m}Tc) MAG_3 Injection



Category. Diagnostic

Description. Technetium (^{99m}Tc) Mertiatide Injection is a sterile, clear, colourless aqueous solution. The content of technetium-99m is not less than 90.0 per cent and not more than 110.0 per cent of the stated technetium-99m radioactivity at the date and time stated on the label. Not less than 94.0 per cent of the total technetium-99m radioactivity is present as technetium (^{99m}Tc) mertiatide complex. Technetium (^{99m}Tc) Mertiatide Injection should be used within 6 hours from the time of preparation.

Radiopharmaceutical preparation. Technetium (^{99m}Tc) Mertiatide Injection is prepared aseptically following instructions provided with the kit. The kit contains S-benzoylmercaptoacetyltriglycine, a chelating agent such as tartrate, and a stannous salt. *Sodium pertechnetate (^{99m}Tc) injection (fission or non-fission)* is used with the kit to make the Technetium (^{99m}Tc) Mertiatide Complex Injection. The injection may have the pH adjusted and may contain reducing, chelating, stabilizing, filling and antioxidizing agents as well as antimicrobial preservatives, buffers and sodium chloride to make the solution isotonic with blood.

Identification

A. Determine by gamma-ray spectrometry as described in general chapter on Radiopharmaceutical preparations. The most prominent gamma photon of technetium-99m has energy of 0.140 MeV.

B. In the test for radiochemical purity, the chromatogram obtained contributes to the identification of technetium (^{99m}Tc) mertiatide.

Tests

pH (2.4.24). 4.0 to 7.5.

Radioactivity assay. Radioactivity of an aliquot of test solution is assayed using precalibrated radioactivity dose calibrator

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as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparations. It is expressed as MBq (mCi) in volume V ml at a particular time and date, V being the maximum recommended dose in mililitres.

Radionuclidic purity. Not less than 99.9 per cent due to technetium-99m. Complies with the radionuclidic purity tests as described under monograph of *sodium pertechnetate (^{99m}Tc) injection (fission) or sodium pertechnetate (^{99m}Tc) injection (non-fission)* used for preparation of technetium (^{99m}Tc) mertiatide.

Radiochemical Purity. Determine by thin layer chromatography (2.4.17), using *silica gel* coated plates or ready to use silica gel strips.

Mobile phase: a mixture of 45 volumes of *methanol*, 55 volumes of 0.9 per cent w/v solution of *sodium chloride* and 1 volume of *acetic acid*.

Apply 5 µl of the injection under examination to the chromatographic plate and allow the mobile phase to rise 15 cm and dry the plate in air. Determine the distribution of radioactivity using a gamma counter. The R_f value of Technetium (^{99m}Tc) mertiatide complex about 0.45 to 0.55, the pertechnetate ion about 0.9 to 1.0 and reduced / hydrolysed technetium-99m about 0 to 0.1. Not less than 94.0 per cent of the total radioactivity is in the spot corresponding to the technetium (^{99m}Tc) mertiatide complex.

Physiological distribution. To be done as described in section 1.6 in general chapter on Radiopharmaceutical preparations using a set of three mice or rats. After 1 hour of the injection, the activity in urine should not be less than 80.0 per cent of the injected radioactivity; the activity in the kidneys should not be more than 5.0 per cent of the injected radioactivity and the activity in the liver should not be less than 1.0 per cent of the injected radioactivity.

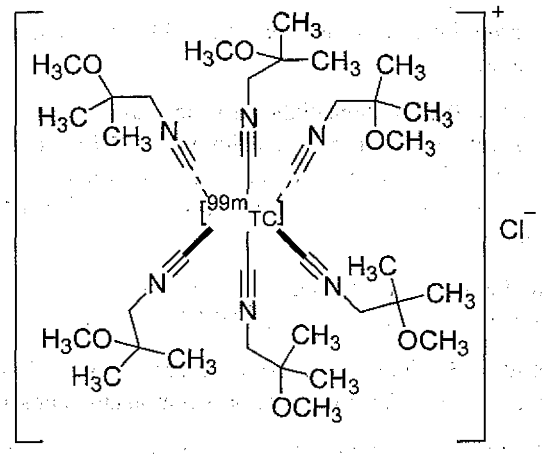
Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparations. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in mililiters. Alternatively the preparation should pass the pyrogen test (2.2.8).

Storage. Store at a temperature between 2° to 8° with adequate shielding.

Labelling. The label states, in addition to the information specified under injections (1) the time and date of calibration; (2) the amount of ^{99m}Tc as labelled mertiatide expressed as total MBq (mCi or µCi) and the concentration as MBq per ml (mCi or µCi per ml) on the date and time of calibration; (3) the expiration date and time and; (4) statement, "Caution-Radioactive Material."

Technetium (^{99m}Tc) MIBI Injection



Category. Diagnostic.

Description. A clear colourless or a very slightly yellow solution. Technetium (^{99m}Tc) Sestamibi Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated technetium-99m radioactivity at the date and time stated on the label. Not less than 90 percent of the total technetium-99m is present as ^{99m}Tc-MIBI. Use the Technetium (^{99m}Tc) MIBI Injection within 4 hours from the date and time of preparation.

Radiopharmaceutical preparation. Technetium (^{99m}Tc) MIBI Injection is prepared aseptically following manufacturer's instructions provided with the kit. The kit contains a mixture of [tetrakis(2-methoxy-2-methylpropyl-1-isocyanide) copper (1+)] tetrafluoroborate, a weak chelating agent, a stannous salt. Technetium (^{99m}Tc) MIBI Injection ((*OC*-6-11)-hexakis[1-(isocyano-κ*C*)-2-methoxy-2-methyl-propane] [^{99m}Tc]technetium(I) chloride) may be prepared by adding Sodium pertechnetate ^{99m}Tc (fission or non-fission), complying with specification given for it, to the kit and heating it as instructed.

Identification

A. Determine by gamma-ray spectrometry. The most prominent gamma photon of Technetium 99m has energy of 0.140 MeV.

B. In the test for Radiochemical purity, the chromatogram obtained contributes to the identification of the Technetium (^{99m}Tc) MIBI.

Tests

pH (2.4.24). 5.0 to 7.0.

Radioactivity assay. Radioactivity of an aliquot of Test Solution is assayed using precalibrated radioactivity dose calibrator

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cent and not more than 110.0 per cent of the stated technetium-99m radioactivity at the date and time stated on the label. Not less than 90.0 per cent of the total technetium-99m is present as Technetium (^{99m}Tc) TRODAT-1. Technetium (^{99m}Tc) TRODAT-1 Injection should be used within 4 hours from the date and time of preparation.

Radiopharmaceutical preparation. Technetium (^{99m}Tc) TRODAT-1 Injection is prepared aseptically following manufacturer's instructions provided with the kit. The kit contains (2β(N,N'-bis(2-mercaptoethyl) ethylenediamino-methyl)-3β-(4-chlorophenyl)tropane) (TRODAT-1), stannous chloride and may contain transchelating agents such as disodium ethylene diamine tetracetic acid, sodium glucoheptonate (GHA) other than antimicrobial agents and buffers. *Sodium pertechnetate (^{99m}Tc) injection (fission or non-fission)* is used with the kit to make the (^{99m}Tc) TRODAT-1 Injection.

Identification

A. Determine by gamma-ray spectrometry as described in general chapter on Radiopharmaceutical preparations. The most prominent gamma photon of technetium-99m has energy of 0.140 MeV.

B. In the test for radiochemical purity, the chromatogram obtained contributes to the identification of the Technetium (^{99m}Tc) TRODAT-1.

Tests

pH (2.4.24). 5.0 to 8.0.

Radioactivity assay. Radioactivity of an aliquot of test solution is assayed using precalibrated radioactivity dose calibrator as described in measurement of radioactivity in general chapter on Radiopharmaceutical preparations. It is expressed as MBq (mCi) in volume V ml at a particular time and date, V being the maximum recommended dose in ml.

Radionuclidic purity. Not less than 99.9 per cent due to Technetium-99m.

Complies with the radionuclidic purity tests as described under monograph on *sodium pertechnetate (^{99m}Tc) injection (fission)* or *sodium pertechnetate (^{99m}Tc) injection (non-fission)* used for preparation of technetium (^{99m}Tc) TRODAT-1 injection.

Radiochemical purity. Use method A or method B

Method A. Determine by thin layer chromatography (2.4.17), coating the plate with polyethylene terephthalate (PET) foil precoated Polyamide 6 or equivalent.

Use a plate such that during development the mobile phase migrates over a distance of 10 cm to 15 cm in about 10 minutes.

Mobile phase. A mixture of 80 volumes of *acetonitrile* and 20 volumes of 0.005 M aqueous solution of *dimethyl glutaric acid.*

Test solution. The injection under examination.

Apply to the plate 5 µl to 10 µl of the injection under examination. Allow the mobile phase to rise 10 cm to 15 cm and dry the plate in air. Determine the distribution of radioactivity using a suitable detector. Pertechnetate ion exhibits R_f between 0.3-0.5, impurities in colloidal form remain at the point of spotting, (^{99m}Tc) GHA impurity has an R_f of 0.5-0.6, whereas (^{99m}Tc) TRODAT-1 complex migrates near to the solvent front (R_f of 0.7-1.0). The percentage of activity in each peak should be calculated and the sum of the percentages of radioactivity corresponding to all impurities in the chromatograms obtained should not more than 10.0 per cent.

Method B. Determine by liquid chromatography (2.4.14).

Test sample. The injection under examination.

Chromatographic system

- a stainless steel column 25 cm x 4.1mm, packed with octadecylsilane bonded to silica (3 µm),
- mobile phase: a mixture of 80 volumes of *acetonitrile* and 20 volumes of 0.005 M aqueous solution of *dimethyl glutaric acid*
- flow rate: 1 ml per minute,
- radioactivity detector adjusted for 100 to 200 keV energy,
- injection volume: 20 µl

The retention time of (^{99m}Tc) TRODAT-1 shall have 13 minutes to 14 minutes.

The sum of the percentages of radioactivity corresponding to all impurities in the chromatograms obtained in test should not more than 10.0 per cent.

Physiological distribution. To be done in laboratory animals as described in section 1.6 in general chapter on Radiopharmaceutical preparations using three wister rats. Three wister rats weighing 150-200 g should be intravenously injected with equivalent to 20 MBq of (^{99m}Tc) TRODAT-1. At 1 hour post injection rats should be sacrificed and the per cent radioactivity in brain should be measured or alternately the rats could be imaged in an animal SPECT camera and the per cent activity in the brain can be calculated. The per cent radioactivity in the brain should be not less than 0.4 per cent of injected dose in at least 2 out of 3 rats.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparations. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in ml. Alternatively the preparation should pass the pyrogen tests (2.2.8.).

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Storage. Store at room temperature with adequate shielding.

Labelling. The label states, in addition to the information specified under injection (1) the amount of ^{99m}Tc as labelled TRODAT-1 expressed as total MBq (or mCi) and the radioactive concentration as MBq per ml (or as mCi per ml) on the date and time of reconstitution; (2) the expiration date and time; (3) the statement, "Caution - Radioactive Material."

Thallous (^{201}Tl) Chloride Injection

Category. Diagnostic

Description. Thallous (^{201}Tl) chloride injection is a sterile, clear, colourless, aqueous solution of thallium-201 as thallous chloride. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the content of thallium-201 stated on the label at the reference date and time stated on the label. Thallous (^{201}Tl) chloride injection may contain sodium chloride to make the solution isotonic with blood as well as suitable antimicrobial preservatives and stabilizing agents. The specific radioactivity is not less than 3.7 gigabecquerel (100 millicuries) of thallium-201 per milligram of thallium at the reference date and time stated on the label. Thallous (^{201}Tl) chloride injection should be used within 7 days from the date of preparation.

Identification

A. Determine by Gamma-ray and X-ray spectrometry using a gamma ray and X-ray spectrometer. The prominent gamma photons of thallium-201 have energies of 0.135 MeV, 0.166 MeV and 0.167 MeV and the X-rays have energies of 0.069 MeV to 0.083 MeV.

B. In the test for radiochemical purity, the distribution of radioactivity contributes to the identification of the preparation.

Tests

pH (2.4.24). 4.0 to 7.0.

Radioactivity assay. Radioactivity of an aliquot of Thallous (^{201}Tl) chloride test solution is assayed using precalibrated radioactivity dose calibrator. It is expressed as megabecquerels (millicuries) per ml at a particular time and date.

Radionuclidic purity. Determine by gamma-ray and X-ray spectrometry the relative amounts of thallium-201, thallium-202 and other radionuclidic impurities that may be present. Not less than 97.0 per cent of the total radioactivity is due to thallium-201. Not more than 2.0 per cent of the total radioactivity is due to thallium-202.

Radiochemical purity. Determine by Zone electrophoresis (2.4.12)

Test solution. a mixture of 50 volumes of the preparation under examination and the 50 volumes of the *0.05 M Disodium Edetate* solution.

Soak a 2.5 cm x 15.0 cm cellulose polyacetate strip in *0.05 M Disodium Edetate* solution for 45 to 60 minutes. Remove the strip with forceps, taking care to handle the outer edges only. Place the strip between 2 absorbent pads, and blot to remove excess solution. Apply not less than 5 µl of the test solution to the centre of the blotted strip, and mark the point of application. Attach the strip to the support bridge of an electrophoresis chamber containing equal portions of *0.05 M Disodium Edetate* in each side of the chamber. Ensure that each end of the strip is in contact with the electrolyte solution. Attach the chamber cover and perform the electrophoresis at 250 volts for 30 minutes. Remove the strip from the chamber and allow to air dry without blotting. Determine the radioactivity distribution using suitable method. Not less than 95.0 per cent of the radioactivity on the strip migrates towards the cathode as a single peak.

Chemical Purity

Thallium. Transfer 1.0 ml of the Thallous (^{201}Tl) chloride injection and 1.0 ml of *thallium standard solution (2 ppm Tl)* individually to two 10 ml screw cap test tubes. To each tube, add the following five solutions (A, B, C, D and E) and mix after each addition: solution A. 2 drops of a solution prepared by carefully mixing 18 ml of *nitric acid* (~100 per cent w/v) and 82 ml of *hydrochloric acid* (~25 per cent w/v); solution B. 1.0 ml of *sulfosalicylic acid* (0.1 mol/l); solution C. 2 drops of *hydrochloric acid* (~25 per cent w/v); solution D. 4 drops of a solution prepared by dissolving 50 mg of *rhodamine B* in *hydrochloric acid* (~25 per cent w/v) and diluting to 100.0 ml; solution E. 1.0 ml of *diisopropyl ether*.

Screw the caps tightly, shake the tubes for 1 minute, releasing any pressure build-up by loosening the caps slightly. Recap the tubes and allow the phases to separate. The colour of the ether layer obtained from the injection is not more intensely coloured than that from the *thallium standard solution (2 ppm Tl)*.

Sterility (2.2.11). Complies with the test for sterility as described in general chapter on Radiopharmaceutical preparations. The injection may be released for use before completion of the test.

Bacterial Endotoxins (2.2.3). Less than 175 per V EU per ml, V being the maximum recommended dose in ml. Alternatively the preparation should pass the pyrogen test (2.2.8).

Storage. Store in single dose or multiple dose containers at a temperature between 2° to 8°.

Labeling. The label states, in addition to the information specified under injections (1) the time and date of calibration;

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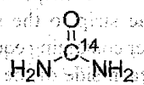
(2) the amount of ^{201}Tl as labeled thallous chloride expressed as total megabecquerels (millicuries or microcuries) and concentration as megabecquerels (millicuries or microcuries) per ml at the time of calibration; (3) the expiration date and time and; (4) the statement "Caution- Radioactive Material."

Urea (^{14}C) Capsules



Category. Diagnostic

Description. Urea (^{14}C) Capsules contains $^{14}CH_4N_2O$ in which a portion of the molecules are labelled with radioactive ^{14}C to provide not more than 37 KBq (1 μCi) of radioactivity per capsule. It contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of ^{14}C expressed as KBq (μCi) at the date stated on label. C-14 Urea Capsules containing carbon-14 in the form of urea on a solid support. Use within 6 months from the date of determination of radiochemical purity.

Identification

Determine by using liquid scintillation counter as described in general chapter on Radiopharmaceutical preparations. A solution of 1 or more capsules in 1 N *hydrochloric acid* when tested using a liquid scintillation counter produces beta emission having mean energy of 0.049 MeV and maximum energy of 0.156 MeV.

Tests

Dissolution (2.5.2).

Apparatus No. 1 (Basket),

Medium. A capsule is added to 10 ml of distilled water at 37° and mix it throughly.

Speed and time. 50 rpm and 10 minutes.

Determine the background levels of ^{14}C with a 1 ml portion of the filtered solution under test using a liquid scintillation counter.

D. Not less than 80.0 per cent of the stated amount of ^{14}C.

Uniformity of content. Comply with the tests stated under Capsules.

Radioactivity assay. Prepare a solution of a capsule in 10 ml of warm water and mix well. Using a precalibrated liquid scintillation counter, determine the radioactivity in KBq (μCi) per ml at a particular date.

Radionuclidic purity. Determine by liquid scintillation counter. Dissolve a capsule in 10 ml of warm water and take the beta spectra of an aliquot of test solution and ^{14}C standard. Beta spectra of preparation and of ^{14}C standard are identical.

Radiochemical purity. Determine by thin layer chromatography (2.4.17), coating the plate with 0.25 mm layer of silica gel or using ready to use silica gel impregnated strips.

Mobile phase. A mixture of 57 volumes of *n-butanol*, 14 volumes of *acetic acid* and 29 volumes of *water*.

Test solution. Open 2 capsules and place them in a suitable container, add 4 ml of *methanol* or *water* mix well and keep it for one hour.

Reference solution. A 4.0 per cent w/v solution of urea in *water*.

Apply to the plate 20 μl of the clear test solution and 4 μl of the reference solution. Allow the mobile phase to rise 8 to 10 cm. Locate the spots on the plate by spraying with *ehrlich's reagent (Dimethylaminobenzaldehyde reagent)*. Determine the radioactivity distribution with a suitable radiation detector, and obtain the R_f value. The R_f value of the principal spot from the test solution corresponds to that obtained from the reference solution, and the radioactivity of the ^{14}C band is not less than 90.0 per cent of the total radioactivity.

Storage. Preserve in air tight containers, and store at a dry place and under controlled room temperature.

Labelling. Label it to include, (1) the amount of ^{14}C expressed in KBq (μCi) per capsule at the date of calibration; (2) The expiration date; (3) The total radioactivity per container, and number of capsules per container; (4) The statement, "Caution-Radioactive material."