Edition: BP 2025 (Ph. Eur. 11.6 update)

Interferon Gamma-1b Concentrated Solution

General Notices

(Ph. Eur. monograph 1440)

$C_{734}H_{1166}N_{204}O_{216}S_5$ 16 465

Action and use

Cytokine.

Ph Eur

DEFINITION

Interferon gamma-1b concentrated solution is a solution of the *N*-terminal methionyl form of interferon gamma, a protein which is produced and secreted by human antigen-stimulated T lymphocytes in response to viral infections and various other inducers. It has specific immunomodulatory properties, such as potent phagocyte-activating effects. The protein consists of non-covalent dimers of two identical monomers. The formula of the monomer is as follows:

```
                                                  M
QDPYVKEAEN  LKKYFNAGHS  DVADNGTLFL  GILKNWKEES
DRKIMQSQIV  SFYFKLFKNF  KDDQSIQKSV  ETIKEDMNVK
FFNSNKKKRD  DFEKLTNYSV  TDLNVQRKAI  HELIQVMAEL
SPAAKTGKRK  RSQMLFRGR
```

The potency of interferon gamma-1b is not less than 20×10^6 IU per milligram of protein. Interferon gamma-1b concentrated solution contains not less than 30×10^6 IU of interferon gamma-1b per millilitre.

PRODUCTION

Interferon gamma-1b concentrated solution is produced by a method based on recombinant DNA technology, using bacteria as host-cells. It is produced under conditions designed to minimise microbial contamination.

Interferon gamma-1b concentrated solution complies with the following additional requirements.

Host-cell derived proteins

The limit is approved by the competent authority.

Host-cell- and vector-derived DNA

The limit is approved by the competent authority.

CHARACTERS

A clear, colourless or slightly yellowish liquid.

IDENTIFICATION

A. It shows the expected biological activity when tested as prescribed in the assay.
B. Examine the electropherograms obtained in the test for impurities of molecular masses differing from that of interferon gamma-1b. The principal bands in the electropherogram obtained with the test solution correspond in position to the principal bands in the electropherogram obtained with reference solution (a).
C. Examine by peptide mapping.

Solution A Prepare a solution containing 1.2 g/L of *tris(hydroxymethyl)aminomethane R*, 8.2 g/L of *anhydrous sodium acetate R*, 0.02 g/L of *calcium chloride R* and adjust to pH 8.3 with *dilute acetic acid R*. Add *polysorbate 20 R* to a concentration of 0.1 per cent *V/V*.

Test solution Desalt a volume of the preparation to be examined containing 1 mg of protein by a suitable procedure. For example, filter in a microcentrifuge tube and reconstitute with 500 µL of solution A. Add 10 µL of a freshly prepared 1 mg/mL solution of *trypsin for peptide mapping R* in *water R* and mix gently by inversion. Incubate at 30 °C to 37 °C for 24 h, add 100 µL of *phosphoric acid R* per millilitre of digested sample and mix by inversion.

Reference solution Dilute *interferon gamma-1b CRS* in *water R* to obtain a concentration of 1 mg/mL. Prepare as for the test solution, ensuring that all procedures are carried out simultaneously and under identical conditions.

Examine by liquid chromatography (*2.2.29*).

The chromatographic procedure may be carried out using:

— a stainless steel column, 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (10 µm),

— as mobile phase at a flow rate of 1.0 mL/min:

Mobile phase A (*0.05 M sodium phosphate buffer solution pH 3.3*). Solution I: dissolve 7.80 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent. Solution II: dilute 0.33 mL of *phosphoric acid R* to 100.0 mL with *water R*. Mix 920 mL of solution I and 80 mL of solution II. Adjust the pH if necessary,

Mobile phase B *Acetonitrile for chromatography R*, with the following elution conditions (if necessary, the gradient may be modified to improve the separation of the digest):

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 30	100 → 80	0 → 20
30 - 50	80 → 60	20 → 40
50 - 51	60 → 30	40 → 70
51 - 59	30	70

— as detector a spectrophotometer set at 214 nm,

maintaining the temperature of the column at 40 °C.

Equilibrate the column for at least 15 min at the initial elution composition. Carry out a blank run using the above-mentioned gradient.

Inject 100 µL of the test solution and 100 µL of the reference solution.

System suitability The chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of interferon gamma-1b digest supplied with *interferon gamma-1b CRS*.

Results The profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

D. Examine by *N*-terminal sequence analysis.
Use an automated solid-phase sequencer, operated in accordance with the manufacturer's instructions.

Equilibrate by a suitable procedure the equivalent of 100 µg of interferon gamma-1b in a 10 g/L solution of *ammonium hydrogen carbonate R*, pH 9.0.

Identify the phenylthiohydantoin (PTH)-amino acids released at each sequencing cycle by reverse-phase liquid chromatography. The procedure may be carried out using the column and reagents recommended by the manufacturer of the sequencing equipment for the separation of PTH-amino acids.

The separation procedure is calibrated using:

— the mixture of PTH-amino acids provided by the manufacturer, with the gradient conditions adjusted as indicated to achieve optimum resolution of all amino acids,

— a sample from a blank sequencing cycle, obtained as recommended by the equipment manufacturer.

The first fifteen amino acids are:

Met-Gln-Asp-Pro-Tyr-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr.

TESTS

Appearance

The preparation to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution Y_7 (*2.2.2, Method II*).

pH (*2.2.3*)

The pH of the preparation to be examined is 4.5 to 5.5.

Covalent dimers and oligomers

Not greater than 2 per cent, determined by size-exclusion chromatography (*2.2.30*).

Test solution Dilute the preparation to be examined with the mobile phase to a protein concentration of 0.1 mg/mL.

Reference solution (a) Dilute *interferon gamma-1b CRS* with the mobile phase to a protein concentration of 0.1 mg/mL.

Reference solution (b) Prepare a mixture of the following molecular mass standards: bovine albumin, ovalbumin, trypsinogen, lysozyme, at a concentration of 0.1 mg/mL to 0.2 mg/mL for each standard.

The chromatographic procedure may be carried out using:

— a stainless steel column 0.3 m long and 7.8 mm in internal diameter packed with *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins in the molecular weight range of 10 000 to 500 000 (5 µm),

— as mobile phase at a flow rate of 1.0 mL/min a mixture prepared as follows (0.2 M sodium phosphate buffer solution pH 6.8). Solution I: dissolve 31.2 g of *sodium dihydrogen phosphate R* and 1.0 g of *sodium dodecyl sulfate R* in *water R* and dilute to 1000.0 mL with the same solvent. Solution II: dissolve 28.4 g of *anhydrous disodium hydrogen phosphate R* and 1.0 g of *sodium dodecyl sulfate R* in *water R* and dilute to 1000.0 mL with the same solvent. Mix 450 mL of solution I and 550 mL of solution II. Adjust the pH if necessary,

— as detector a spectrophotometer set at 210 nm to 214 nm.

Inject 200 µL of each solution. The test is not valid unless: the molecular mass standards in reference solution (b) are well separated; the retention time of the principal peak in the chromatogram obtained with reference solution (a) is between the retention time of trypsinogen and lysozyme in the chromatogram obtained with reference solution (b).

Compare the chromatograms obtained with the test solution and with reference solution (a). There are no additional shoulders or peaks in the chromatogram obtained with the test solution compared with the chromatogram obtained with reference solution (a).

Calculate the percentage content of covalent dimers and oligomers.

Monomer and aggregates

Examine by size-exclusion chromatography (2.2.30). The content of monomer and aggregates is not greater than 2 per cent.

Solution A Prepare a solution of the following composition: 0.59 g/L of *succinic acid R* and 40 g/L of *mannitol R*, adjusted to pH 5.0 with *sodium hydroxide solution R*.

Test solution Dilute the preparation to be examined with solution A to a protein concentration of 1 mg/mL.

Reference solution Dilute *interferon gamma-1b CRS* with solution A to a protein concentration of 1 mg/mL.

Resolution solution Prepare 500 µL of a mixture consisting of 0.04 mg/mL of *bovine albumin R* and 0.2 mg/mL of *interferon gamma-1b CRS* in solution A. Use this solution within 24 h of preparation.

The chromatographic procedure may be carried out using:

— a stainless steel column 0.3 m long and 7.8 mm in internal diameter packed with *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins in the molecular weight range of 10 000 - 300 000 (5 µm),

— as mobile phase at a flow rate of 0.8 mL/min a 89.5 g/L solution of *potassium chloride R* (1.2 M),

— as detector a spectrophotometer set at 214 nm.

Inject 20 µL of the resolution solution. In the chromatogram obtained, the retention time of the principal peak, corresponding to the native interferon gamma-1b dimer, is about 10 min. Bovine albumin elutes at a relative retention of about 0.85 with reference to the main peak. The test is not valid unless the resolution between the peaks due to bovine albumin and interferon gamma-1b is at least 1.5.

Inject 20 µL of the test solution and 20 µL of the reference solution. The chromatograms obtained show principal peaks with identical retention times. Calculate the percentage content of monomer and aggregates from the peak area of the monomer peak and of peaks which elute prior to the native interferon gamma-1b peak in the chromatogram obtained with the test solution, by the normalisation procedure, disregarding any peak due to the solvent.

Deamidated and oxidised forms and heterodimers

Examine by liquid chromatography (2.2.29). The content of deamidated and oxidised forms is not greater than 10 per cent. The content of heterodimers is not greater than 3 per cent.

Test solution Dilute the preparation to be examined with *water R* to a protein concentration of 1 mg/mL.

Reference solution (a) Dilute *interferon gamma-1b CRS* with *water R* to a protein concentration of 1 mg/mL.

Reference solution (b) Dissolve the contents of a vial of *interferon gamma-1b for system suitability CRS* in *water R* to obtain a protein concentration of about 1 mg/mL.

The chromatographic procedure may be carried out using:

— a stainless steel column 0.075 m long and 7.5 mm in internal diameter packed with an appropriate hydrophilic polymethacrylate, strong cation-exchange gel (10 µm, 100 nm),

— as mobile phase at a flow rate of 1.2 mL/min:

Mobile phase A (*0.05 M ammonium acetate buffer pH 6.5*). A 3.86 g/L solution of *ammonium acetate R*, adjusted to pH 6.5 with *dilute acetic acid R*,

Mobile phase B (*1.2 M ammonium acetate buffer pH 6.5*). A 92.5 g/L solution of *ammonium acetate R*, adjusted to pH 6.5 with *dilute acetic acid R*,

with the following elution conditions (if necessary, the slope of the gradient may be modified to improve the separation).

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 1	100	0
2 - 30	100 → 0	0 → 100

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
31 - 35	0	100

— as detector a spectrophotometer set at 280 nm,

maintaining the temperature of the column at 35 °C.

Inject 25 µL of reference solution (b). In the chromatogram obtained, the retention time of the principal peak is about 26 min. Deamidated and oxidised forms co-elute at a relative retention of about 0.95 with reference to the principal peak. The test is not valid unless the resolution, defined by the ratio of the height of the peak corresponding to the deamidated and oxidised forms to the height above the baseline of the valley separating the two peaks, is at least 1.2.

Inject 25 µL of the test solution and 25 µL of reference solution (a). The chromatograms obtained show principal peaks with identical retention times. Calculate the percentage content of deamidated and oxidised interferon gamma-1b as a percentage of the area of the main peak. Heterodimers have relative retentions of 0.7 and 0.85 with reference to the main peak. Calculate the percentage of heterodimers as a percentage of the sum of the areas of all peaks.

Impurities of molecular masses differing from that of interferon gamma-1b

Examine by polyacrylamide gel electrophoresis (*2.2.31*). The test is performed under both reducing and non-reducing conditions, using resolving gels of 15 per cent acrylamide and silver staining as the detection method.

Sample buffer (non-reducing conditions) Dissolve 3.78 g of *tris(hydroxymethyl)aminomethane R*, 10.0 g of *sodium dodecyl sulfate R* and 0.100 g of *bromophenol blue R* in *water R*. Add 50.0 mL of *glycerol R* and dilute to 80 mL with *water R*. Adjust the pH to 6.8 with *hydrochloric acid R* and dilute to 100 mL with *water R*.

Sample buffer (reducing conditions) Dissolve 3.78 g of *tris(hydroxymethyl)aminomethane R*, 10.0 g of *sodium dodecyl sulfate R* and 0.100 g of *bromophenol blue R* in *water R*. Add 50.0 mL of *glycerol R* and dilute to 80 mL with *water R*. Adjust the pH to 6.8 with *hydrochloric acid R* and dilute to 100 mL with *water R*. Immediately before use, add *dithiothreitol R* to a final concentration of 250 mM.

Test solution Dilute the preparation to be examined in *water R* to a protein concentration of 1 mg/mL. Dilute 150 µL of the solution with 38 µL of sample buffer.

Reference solution (a) Prepare in the same manner as for the test solution, but using *interferon gamma-1b CRS* instead of the preparation to be examined.

Reference solution (b) (5 ng control) Mix 50 µL of a 0.01 mg/mL solution of *bovine albumin R* with 2000 µL of *water R* and 450 µL of sample buffer.

Reference solution (c) (2 ng control) Mix 20 µL of a 0.01 mg/mL solution of *bovine albumin R* with 2000 µL of *water R* and 450 µL of sample buffer.

Reference solution (d) Use a solution of molecular mass standards suitable for calibrating SDS-polyacrylamide gels in the range of 10 kDa to 70 kDa.

Leave each solution, contained in a test tube, at ambient temperature for 15 min, then store on ice.

Apply 25 µL of each solution to the stacking gel wells. Perform the electrophoresis under the conditions recommended by the manufacturer of the equipment. Detect proteins in the gel by silver staining.

The test is not valid unless: the validation criteria are met (*2.2.31*); a band is seen in the electropherograms obtained with reference solutions (b) and (c).

The principal band in the electropherogram obtained with the test solution is similar in intensity to the principal band in the electropherogram obtained with reference solution (a). In the electropherogram obtained with the test solution, no significant bands are observed that are not present in the electropherogram obtained with reference solution (a) (0.01 per cent). A significant band is defined as any band whose intensity is greater than or equal to that of the band in the electropherogram obtained with reference solution (c).

Norleucine

Not more than 0.2 mole of norleucine per mole of interferon gamma-1b, determined by amino acid analysis.

Test solution Add 2.5 mL of the preparation to be examined onto a column suitable for the desalting of proteins previously equilibrated with 25 mL of a 10 per cent *V/V* solution of *acetic acid R*. Elute the sample with another 2.5 mL of a 10 per

cent *V/V* solution of *acetic acid R*. Determine the protein content by measuring the absorbance of this solution as described under Protein, in the Assay section. Pipette a volume containing the equivalent of 100 µg of interferon gamma-1b into each of three reaction vials. Evaporate to dryness under reduced pressure.

Perform the hydrolysis of the three samples as follows. Add to each reaction vial 200 µL of a 50 per cent *V/V* solution of *hydrochloric acid R* containing 1 per cent *V/V* of *phenol R*, evacuate the samples, purge with nitrogen and hydrolyse in the gas phase. Heat the reaction vials at 110 °C for 22 h. After hydrolysis evaporate to dryness under reduced pressure.

Perform the derivatisation of the samples as follows. Prepare immediately before use a mixture consisting of two volumes of *ethanol R*, one volume of *water R* and one volume of *triethylamine R*. Add 50 µL of this solution to each reaction vial and shake lightly. Evaporate to dryness under reduced pressure. Add to each vial 50 µL of a mixture consisting of 7 volumes of *ethanol R*, one volume of *water R*, one volume of *triethylamine R* and one volume of *phenyl isothiocyanate R*. Shake lightly and allow to stand at room temperature for about 15 min. Evaporate to dryness under reduced pressure. Reconstitute the samples in 250 µL of mobile phase A.

Norleucine stock solution Prepare a 250 nmol/mL solution of *DL-norleucine R* in *0.01 M hydrochloric acid*. *This solution may be kept for two months at 4 °C*.

Leucine stock solution Prepare a 250 nmol/mL solution of *leucine R* in *0.01 M hydrochloric acid*. *This solution may be kept at 4 °C for two months*.

Reference solution Mix 10 µL of norleucine stock solution with 100 µL of leucine stock solution in each of the three reaction vials. Evaporate to dryness under reduced pressure. Perform the derivatisation of the samples as described for the preparation of the test solution.

Examine by liquid chromatography (*2.2.29*).

The chromatographic procedure may be carried out using:

— a stainless steel column 0.15 m long and 3.9 mm in diameter packed with *octadecylsilyl silica gel for chromatography R* (4 µm),

— as mobile phase at a flow rate of 1.0 mL/min:

Mobile phase A Mix 70 volumes of a 19 g/L solution of *sodium acetate R* containing 0.05 per cent *V/V* of *triethylamine R* and adjusted to pH 6.4 with *dilute acetic acid R* and 30 volumes of mobile phase B,

Mobile phase B Mix 40 volumes of *water R* and 60 volumes of *acetonitrile R*,

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)	Comment
0 - 7	100	0	isocratic
7 - 7.1	100 → 0	0 → 100	linear gradient
7.1 - 10	0	100	washing step
10 - 10.1	0 → 100	100 → 0	linear gradient
10.1 - 15	100	0	re-equilibration

— as detector a spectrophotometer set at 254 nm,

maintaining the temperature of the column at 43 °C.

Inject 50 µL of each solution.

In the chromatograms obtained with the test solution, identify the peaks corresponding to leucine and norleucine. The retention time of norleucine is 6.2 min to 7 min.

Calculate the content of norleucine (in moles of norleucine per mole of interferon gamma-1b) from the peak areas of leucine and norleucine in the chromatograms obtained with the reference and test solutions, considering that there are 10 moles of leucine per mole of interferon gamma-1b.

Bacterial endotoxins (*2.6.14*)

Less than 5 IU in the volume that contains 20×10^6 IU of interferon gamma-1b.

ASSAY

Protein (*2.2.25*)

Dilute the substance to be examined in *water R* to obtain a concentration of 1 mg/mL. Record the absorbance spectrum between 220 nm and 340 nm. Measure the value at the absorbance maximum of 280 nm, after correction for any light scattering due to turbidity measured at 316 nm. Calculate the concentration of interferon gamma-1b using a specific absorbance value of 7.5.

Potency

The potency of interferon gamma-1b is estimated by evaluating the increase of the expression of human-leukocyte-antigen-DR (HLA-DR) due to the interferon gamma-1b present in test solutions during cultivation of the cells, and comparing this increase with the same effect of the appropriate International Standard of human recombinant interferon gamma or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Carry out the assay by a suitable method, based on the following design.

Use COLO 205 cells under standard culture conditions. Trypsinise a 3- to 5-day-old flask of COLO 205 cells and prepare a cell suspension at a concentration of 1.0×10^6 cells/mL.

Add 100 µL of the dilution medium to all wells of a 96-well microplate. Add an additional 100 µL of this solution to the wells designed for the blanks. Add 100 µL of each solution to be tested onto the plate and carry out a series of twofold dilution steps in order to obtain a standard curve. Then add 100 µL of the cell suspension to all wells and incubate the plate under appropriate conditions for cell cultivation.

After cultivation remove the growth medium and wash and fix cells to the plate. Add an antibody able to detect HLA-DR expressed due to the presence of interferon gamma-1b and incubate under appropriate conditions. After washing the plate, incubate with an antibody conjugated to a marker enzyme which is able to detect the anti-HLA-DR antibody. After this incubation step, wash the plate and add an appropriate substrate solution. Stop the reaction. Measure the absorbance of the solution and calculate the potency of the preparation to be examined by the usual statistical methods.

The estimated specific activity is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits (P = 0.95) are not less than 70 per cent and not more than 140 per cent of the estimated potency.

STORAGE

Store in an airtight container, protected from light and at a temperature of -70 °C.

Ph Eur