Pegfilgrastim Injection

Pegfilgrastim Injection is sterile solution of a protein having primary structure of the granulocyte colony-stimulating factor (human), 3-hydroxypropyl-N-methionyl-1-ether with α-methyl-Ω-hydroxypoly (oxy-1,2-ethanediyl). Pegfilgrastim is a monopegylated form of Filgrastim- recombinant granulocute colony – stimulating factor.

It is a single chain, 175 amino acids, non-glycosylated polypeptide. It is prepared by coupling a liner polyethylene glycol (PEG) molecule, of an average molecular weight of 20kDa, to the N-terminus of the Filgrastim protein.

Pegfilgrastim contains N-terminally pegylated recombinant human methionyl granulocyte colony –stimulating factor with not less than 80 per cent and not more than 125 percent of the stated potency.

Usual strengths: 10.0mg/ml

Description: A clear, colourless liquid, Practically free from visible particles.

Identification

1. It shows the biological activity as described under Assay.

2. **Molecular size related method:** Perform method A or method B.

   Method A: Determination by size –exclusion chromatography (2.4.14)

   In the test for impurities with molecular mass higher than that of pegfilgrastim, the retention time of principal peak obtained with test solution is similar to that of the principal peak obtained with reference solution.

   Method B: Determine by polyacrylamide gel electrophoresis under non-reducing conditions

   In the test for impurities with molecular masses differing from that of pegfilgrastim under non-reducing conditions, the principal band in the electropherogram obtained with test solution is similar in position to the principal band in the electropherogram obtained with reference solution.

3. **Charge variant related method:** Perform method A or method B.

Method A: **Determine by isoelectric focusing (2.4.33).**

In the test for Impurities with charges different from that of pegfilgrastim, the principal band in the electropherogram obtained with the test solution is similar in position to the principal band in the electropherogram obtained with the reference solution

Method B: Determine by charge based Cation exchange chromatography

In the test for impurities with charges differing from that of pegfilgrastim, the retention time of principal peak obtained with test solution is similar to that of the principal peak obtained with reference solution.

4. Tests

pH: 4.00 ± 0.30

Osmolality: 320 ± 40 mOsm/kg

Impurities with molecular masses differing from that of Pegfilgrastim using

method A or Method B

Method A. Determine by size – exclusion chromatography (2.4.14).

*Formulation buffer pH 4.0***± 0.3**. Dissolve 5 g D-sorbitol in 40 ml of water and add 4.2 ml of 0.2 M acetic acid. Adjust the pH 4.0 with 0.2 M sodium acetate trihydrate. Add 330 µl of 10 mg per ml polysorbate 20 and sufficient water to produce 100 ml.

Test solution. Dilute the preparation under examination (if necessary) with formulation buffer to obtain a concentration of 1 mg per ml.

Reference solution (a). Dilute pegfilgrastim RS with formulation buffer to obtain a concentration of 1 mg per ml.

Reference solution (b). Incubate an appropriate amount of the reference solution (a) at 55° for 15 minutes in polypropylene tube. Cool it to room temperature after

incubation.

Chromatographic system

- a stainless steel column 30 cm x 7.8 mm, packed with hydrophilic silica gel, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10000 to 50000,

- mobile phase: a mixture of 6.8 ml of 85 per cent v/v solution of orthophosphoric acid in 800 volumes of water, adjust pH to 2.5 with 10 M sodium hydroxide, 50 volumes of ethanol and 150 volumes of water,

- flow rate: 1 ml per minute,

- spectrophotometer set at 214 nm,

- injection volume: 30 µl.

Inject reference solution (b). The test is not valid unless the per cent aggregate in reference solution (b) is not more than 5 per cent and the relative standard deviation for the per cent area of aggregate is not more than 10 per cent. Relative retention times with reference to pegfilgrastim monomer for aggregates is about 0.8 and for multi-pegylated is about 0.9.

Inject reference solution (a) for principal peak identification and test solution. Relative percent of all peaks in test solution eluting with retention times less than that of the principal peak is not more than 3 per cent.

Method B: Determine by electrophoresis (sodium dodecyl sulphate polyacrylamide gel electrophoresis) (SDS-PAGE) (2.4.12) under non-reducing conditions.

Resolving gel. 4-12 per cent polyacrylamide gradient bis-tris gel of 1 mm thickness.

Sample buffer (non- reducing conditions). Mix 60 mM Tris pH 6.8, 25 per cent glycerol, 2 per cent SDS and 0.2 per cent bromophenol blue (or equivalent).

Test stock solution. Dilute the preparation under examination with water to obtain a protein concentration of 1 mg per ml.

Test solution. Add 20 µl of test stock solution to 20 µl of non-reducing buffer

Reference solution (a). Dilute pegfilgrastim RS with water to obtain a concentration of 1 mg per ml.

Reference solution (b). Dilute pegfilgrastim RS with water to obtain a concentration of 0.1 mg per ml.

Reference solution (c). A solution of molecular markers suitable for calibrating SDS-polyacrylamide gels in the range 10-260 kDa.

Reference solution (d). Add 20µl of Reference solution (a) to 20µl of non-reducing buffer

Reference solution (e) & (f): Add 20 µl of Reference solution (b) to 20µl of non-reducing buffer

Place the test solution, in boiling water bath for 5 minutes.

Apply 20 µl of reference solution (d), 4 µl of reference solution (e), 2 µl of reference solution (f), 5 µl of reference solution (c) and 20 µl of test solution to the stacking gel wells.

Detection: Silver staining.

The test is not valid unless (1) the proteins of the molecular weight marker are distributed along 80 per cent of the gel and over the required separation range and are clearly visible; (2) the principal band of test solution and reference solution appears between 45 to 66 kDa marker bands; (3) the principal band reference solution (f) is clearly visible.

In the electropherogram obtained with the test solution, no band other than the principal band is more intense than the principal band in the electropherogram obtained with reference solution (e).

Impurities with charges differing from that of Pegfilgrastim using Method A or Method B

Method A. Determine by isoelectric focusing (2.4.33).

Test solution. Dilute the preparation under examination to produce a solution containing 0.3 mg per ml.

*Reference solution (a).*A solution of Pegfilgrastim RS containing0.3 mg per ml.

*Reference solution (b).*A solution of Pegfilgrastim RS containing0.03 mg per ml.

Reference solution (c). Use an isoelectric point (pl) calibration solution, in the pl range of 3.5 - 9.5, prepared according to manufacturer's instructions.

Focusing:

-pH gradient: 4.5 - 8.0,

-catholyte: 1 M sodium hydroxide,

- anolyte: 0.04 M glutamic acid in a 0.0025 per cent v/v solution of orthophosphoric acid,

- application: 20 µl

Detection. Proceed as described in lsoelectric Focusing (2.4.33).

Detect the product and its related impurities.

In the electropherogram obtained with reference solution (c), relevant isoelectric point markers are distributed along the entire length of the gel. In the electropherogram obtained with reference solution (a), the pl of the principal band is 7.4 to 7.8.In the electropherogram obtained with test solution no band other than the principal band is more intense than the principal band in the electropherogram obtained with reference solution (b) (10 per cent).

Method B. Determine by Cation exchange chromatography

Test solution (a). Dilute test sample to 10.0 mg/ml with water.

Test solution (b). Dilute 100 µl of test solution (a) with 100 µl of water to get concentration of 5.0 mg/ml. If concentration of test sample is less, adjust the injection volume to get column load of 100 µg.

Reference solution (a): Dilute the Pegfilgrastim RS to 10.0 mg/ml with water.

Reference solution (b): Dilute filgrastim RS to 0.6 mg/ml with water.

Reference solution (c): Add 100 µl of reference solution (a) to 33 µl of reference solution (b). Add 67 µl of water and mix well by vortexing.

Reference solution (d): Dilute 100 µl of reference solution (a) with 100 µl of water to obtain concentration of 5 mg/ml and mix well.

Chromatographic system:

-Mobile phase: A. Dissolve 3.1 g of ammonium acetate in 1800 ml water and adjust pH to 5.00 ± 0.10 with Glacial acetic acid. Add 100 ml isopropanol. Make up the volume to 2000 ml with water.

Mobile phase B: Dissolve 1.6 g of ammonium acetate and 26.7 g of ammonium chloride in 900 ml of water. Adjust pH to 5.00 ± 0.10 with Glacial acetic acid and add 50 ml isopropanol. Make up the volume to 1000 ml with water.

1. PolySulfoethyl A 200 x 2.1 mm, 5 µm, 1000 Aor equivalent column ,
2. Column temperature. 30°C
3. Sample compartment. 5°C
4. Flow rate. 0.25 ml per minute.
5. spectrophotometer set at 280 nm
6. injection volume. 20 µl
7. Gradient program

Time (in min)	Mobile phase A (percent v/v)	Mobile phase B (percent v/v)
0	100	0
2	95	5
31	60	40
32	0	100
35	0	100
36	100	0
45	100	0

Inject reference solution (d), reference solution (c) and test solution (b).

System suitability requirements:

The test is not valid unless

1. Resolution between pegfilgrastim and filgrastim peaks in reference solution (c) should be > 8.

2. Percent RSD for pegfilgrastim main peak area percent observed between two reference solution (d) injections should not be more than 1.0 percent.

3. The average retention time for pegfilgrastim main peak in reference solution (d) should be within ± 0.5 min of the average retention time of its initial and bracketing reference solution (d) injections.

Acceptance criteria:

Pre-peaks - ≤ 3.5 %

Main peak- ≥ 95.5 %

Related impurities: Determine by liquid chromatography (2.4.14)

Sample buffer. A mixture of 10 mM sodium acetate buffer solution pH 4.0 5 per cent D-sorbitol and 0.004 percent polysorbate 20.

Test solution. Dilute preparation under examination in sample buffer to the concentration of 1.0 mg per ml.

*Reference solution (a).*Dilute pegfilgrastim RS in sample buffer to the concentration of 1.0 mg per ml.

Reference solution (b). Dilute 100 µl of 10 mg per ml pegfilgrastim RS in 800 p.1 of diluent. Add 100 µl of 0.1Mhydrogen peroxide and incubate at 37° for 5 hours. Dissolve 30 mg of L-methionine to quench the reaction.

Reference solution (c). Mix equal volumes of reference solution (a) and reference solution (b).

Chromatographic system

- a stainless steel column 15 cm x 4.6 mm, packed with polystyrene and divinylbenzene (5 µm),

- column temperature: 45°,

- Mobile phase A. a 0.1 per cent v/v solution of trifluroacetic acid in water,
- Mobile phase B. a 0.1 per cent v/v solution of trifluroacetic acid in 1000 ml of acetonitrile,
- a gradient programme using the conditions given below,
- flow rate: 0.7 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 20 µl.

Time (in min)	Mobile phase A (per cent v/v)	Mobile phase B (per cent v/v)
0	53	47
7	53	47
43	44	56
43.1	0	100
46	0	100
47	53	47
55	53	47

Inject reference solution (c), the profile of the chromatogram obtained shows two oxidized peaks corresponding to oxidized peak 1 with relative retention time of about 0.9 and oxidized peak 2 with relative retention time of about 0.95 with respect to the pegfilgrastim main peak. The test is not valid unless resolution between oxidized peak 1 and 2 is not less than 1.5, and resolution between oxidized peak 2 and main peak is not less than 0.9.

Inject reference solution (a) and test solution. In the chromatogram obtained with the test solution, the sum of the areas of all the peaks other than the principal peak is not more than 5.0 per cent of the total area of all the peaks.

Bacterial endotoxin: Not more than 33.3 EU/mg dose of pegfilgrastim.

Sterility: Complies

Assay

A. Protein. Determine by liquid chromatography (2.4.14) as described under the test for related proteins.

Calculate the content of pegfilgrastim from the declared content of *pegfilgrastim IPRS*.

B. *Potency.* The potency of the preparation is determined by comparison of the dilutions of preparation under examination with the dilutions of *pegfilgrastim IPRS* using below described method.

Determine the potency using a filgrastim responsive cell line (e.g., NFS-60 or its variant, M-NFS-60) in a cell-based proliferation assay with a suitable read out. Perform a comparison of a dilution series of preparation under examination with a dilution series of *pegfilgrastim IPRS*. The reference and test solution should be diluted based on estimated protein concentration obtained using appropriate procedure. The reference and test concentrations should be adjusted so that the fluorescence values are normalized. Use a validated protein estimation procedure.

Determination of the biological activity of pegfilgrastim solution is based on its property of stimulation of proliferation of M-NFS-60 cells (ATCC No.CRL 1838). The following method uses the resazurin (sodium) or any other suitable staining method for assessing the proliferation of cells.

The following method uses the conversion of tetrazoliumbromide (MTT) or any other suitable dye as a staining method. Alternative methods based on luminescence or fluroscence detection may also be used as the assay readout, subject to appropriate validation.

M-NFS-60 (ATCC No. CRL-1838) or any other suitable Myeloblastic cell line are incubated with varying dilution of test and reference preparations of pegfilgrastim. They are then incubated with a solution of MTS, MTT or any other suitable dye. They are then incubated with a solution of MTS, MTT or any other suitable dye. The potency of test preparation is determined by comparison of the dilutions of the test preparation with the dilutions of appropriate International Standard of pegfilgrastim or with a reference preparation calibrated in International Units, which yield the same response.

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.

Add 100 µl of the dilution medium to all wells of a 96-well microtitre plate. Add an additional 150 µl of this solution to the blank wells. Add 100 µl of each solution under examination in triplicate (test preparation and reference preparation at a concentration of about 10 ng per ml, plus a series of two fold dilutions to obtain a standard curve). Prepare a suspension of M-NFS-60 or any other suitable Myeloblastic cell line containing 5 x 105cells per ml immediately before use, add 50 ul of prepared cell suspension to each well, maintaining the cells in a uniform suspension during addition.

Incubate the plate at 37° ± 1° for a minimum of 46 ± 2 hours in a humidified incubator using 6 ± 1 per cent carbon dioxide. Add 20 ul of suitable dye to each well and re-incubate for 4 hours. Estimate the quantity of formazan produced using a microtitre well plate reader at 490 nm.

Assess the slope, and parallelism for each sample compared to the standard using a validated parallel line assay data analysis software or alternate equivalent software. If system suitability criteria are met and sample passes parallelism criteria to reference standard, calculate the relative potency of the sample.

Alternative methods of quantifying cell proliferation, such as measurement of intracellular ATP by luciferase bioluminescence may be used as the assay readout, subjected to appropriate development and validation. The assay conditions such as cell concentration, incubation time and dilution are then adapted accordingly.

The ratio between the relative fluorescence units of highest and lowest concentrations should be ≥ 2.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency.

Storage: Store protected from light in refrigerator (2° to 8°)

Labeling: The label of the sealed container states (1) the name (2) content in mg per ml,