Pegfilgrastim

Granulocyte colony-stimulating factor (human), 3-hydroxypropyl-\(N\)-methionyl-1-ether with \(\alpha\)-methyl-\(\Omega\)-hydroxypoly (oxy-1,2-ethanediyl)

\[
\begin{align*}
\text{TPLGPASSLP} & \quad \text{OSFLLKCLEQ} & \quad \text{VRKIOGDGAA} & \quad \text{LQEKLCAYK} \\
\text{LCHPEEVLIL} & \quad \text{GHSLGIPWAP} & \quad \text{LSSCPSQALQ} & \quad \text{LAGCLSLQHS} \\
\text{GLFLYQGLLQ} & \quad \text{ALEGISPELG} & \quad \text{PTLDTLQLDV} & \quad \text{ADFATTIWQQ} \\
\text{MEELGMAPAL} & \quad \text{OPTQGAMPAF} & \quad \text{ASAFQRAGGF} & \quad \text{VLVASHLQSF} \\
\text{LEVSYRVLRH} & \quad \text{LAQP}
\end{align*}
\]

\((C_2H_4O)_{n}C_8H_{133}N_{223}O_{253}S_9\)  \(\text{Mol. Wt. 39,000 Da}\)

Pegfilgrastim is a monoPEGylated form of filgrastim-recombinant human granulocyte colony-stimulating factor. It is a single chain, 175 amino acid, non-glycosylated polypeptide. It is prepared by coupling a linear polyethylene glycol (PEG) molecule, of an average molecular weight of 20 kDa, to the \(N\)-terminus of the filgrastim protein.

Pegfilgrastim contains \(N\)-terminally pegylated recombinant human methionyl granulocyte colony-stimulating factor, in a sterile solution. Pegfilgrastim contains not less than 80 per cent and not more than 125 per cent of the stated potency.

**Host cell derived proteins (HCP).** Not more than 100 ppm.

**Host cell or vector derived DNA.** Not more than 10 ng per dose.

**Category.** Haemopoietic growth factor

**Description.** A clear, colourless liquid.

**Identification**

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

B. Determine by Imaging capillary electrophoresis.

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 (a).

C. Determine by size-exclusion chromatography (2.4.16).

In the test for Impurities with molecular mass higher than that of pegfilgrastim, the retention time of the principal peak obtained with the test solution is similar to that of the principal peak obtained with the reference solution.
D. Determine by polyacrylamide gel electrophoresis under non-reducing conditions (2.4.12).

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 the test solution (a) is similar in position to the principal band in the electropherogram obtained with reference solution (b).

E. Determine by peptide mapping (2.3.47).

Solution (a). Dissolve $6.7 \text{ g of guanidium hydrochloride}$ and $0.36 \text{ g of tris hydrochloride}$ in 4 ml of water, adjust pH to 8.4 with hydrochloric acid and dilute to 10 ml with water.

Solution (b). Dissolve 10 µg of glutamyl endopeptidase (V8 protease) in 40 µl of 20mM tris hydrochloride pH 8.0.

Test solution (a). Dilute 100 µl of 10 mg per ml of preparation under examination in 900 µl of solution (a) in a polypropylene tube (1mg per ml of test solution), add 10 µl of 0.5 M β-mercaptoethanol and mix well. Cap the tube and incubate at 37° for 1 hour. Cool to ambient temperature, add 10 µl of 1M iodoacetamide and mix well. Incubate the tube at 37° for 1 hour in dark.

Test solution (b). Equilibrate a PD-10 column with 20mM tris hydrochloride pH 8.0. Add about 5 ml of 20mM tris hydrochloride pH 8.0 to the column and spin at 1000 g for 1 minute. Repeat 4-5 times. For elution, place the column outlet in a fresh tube. Load test solution (a) on the column, and spin at 1000 g for 1 minute. Measure the absorbance of eluted sample at 280 nm. Dilute the eluate using 20mM tris hydrochloride pH 8.0 to get an absorbance of 0.4-0.5.

Test solution (c). To 250 µl of test solution (b) add 20 µl of solution (b) and mix well. Incubate for 24 hour at 37° and stop the reaction by adding 10 µl of 10 per cent v/v of formic acid.

Reference solution. Prepare at the same time and in the same manner as for the test solution but use Pegfilgrastim RS instead of the preparation under examination.

Chromatographic system

- a stainless steel column 25 cm x 4.6 mm, packed with butylsilyl silica gel (5µm),
- column temperature, 45±3°,
- mobile phase: A. a 0.1 per cent v/v solution of trifluoroacetic acid in water,
- B. a 0.1 per cent v/v solution of trifluoroacetic acid in 1000 ml of acetonitrile,
- flow rate: 0.2 ml per minute,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 215 nm,
- injection volume: 100 µl.

<table>
<thead>
<tr>
<th>Time (in min.)</th>
<th>Mobile phase A (per cent v/v)</th>
<th>Mobile phase B (per cent v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>98.2</td>
<td>1.8</td>
</tr>
<tr>
<td>2</td>
<td>98.2</td>
<td>1.8</td>
</tr>
</tbody>
</table>
Equilibrate the column at the initial conditions for at least 30 minutes.

Inject the test solution (c) and the reference solution. The profile of chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

**Tests**

**Impurities with molecular mass higher than that of pegfilgrastim.** Determine by size-exclusion chromatography (2.4.16).

*Test solution.* Dilute the preparation under examination (if required) with appropriate diluent to obtain a concentration of 10 mg per ml.

*Reference solution (a).* Dilute Pegfilgrastim RS with appropriate diluent to obtain a concentration of 10 mg per ml.  

*Reference solution (b).* Incubate an appropriate amount of the reference solution (a) at 55°C for 15 minutes in a 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 10,000 to 50,000,
- column temperature, 25±3°C,
- 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 N sodium hydroxide, 50 volumes of ethanol and 150 volumes of water,
- flow rate: 1 ml per minute,
- spectrophotometer set at 214 nm,
- injection volume: 3 μl (30 μg equivalent),

Inject reference solution (b). The test is valid if 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 between the triplicate injections is not more than 10 per cent. The retention time of the peak due to pegfilgrastim monomer in triplicate injections is not more than 0.2 minute. Relative retention times with reference to pegfilgrastim monomer: aggregates is about 0.8 and multi-pegylated is about 0.9.

Inject reference solution (a) and test solution. Relative percent of all peaks eluting with retention times less than that of the principal peak is not more than 3 per cent.
Impurities with molecular masses differing from that of pegfilgrastim. 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 solution.** Dilute the preparation under examination with water to obtain a protein concentration of 1 mg per ml.

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

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

**Reference solution (c).** A solution of molecular markers suitable for calibrating SDS-polyacrylamide gels in the range 15–100 kDa.

**Reference solution (d).** Add 10 µl of Reference solution (a).

**Reference solution (e).** Add 2 µl of Reference solution (b).

**Reference solution (f).** Add 1 µl of Reference solution (b).

Place the test solution, contained in a covered test tube, in boiling water bath for 5 minutes.

Apply reference solution (d), reference solution (e), reference solution (f), 5 µl of reference solution (c) and 10 µl of test solution to the stacking gel wells.

Detection: Commassie 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 (the range covering the product and its dimer or the product and its related impurities) 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 of 1 µl of reference solution 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 charge differing from pegfilgrastim. Determine by Imaging capillary electrophoresis.

All the solutions should be filtered through a 0.45 µm membrane filter before use.

**Test solution.** Dilute substance under examination to obtain a concentration of 0.5 mg per ml with 0.35 per cent methyl cellulose and 8 per cent ampholytes in water.
Reference solution (a). Dilute pegfilgrastim RS to obtain a concentration of 0.5 mg per ml with 0.35 per cent methyl cellulose and 8 per cent ampholytes in water.

Reference solution (b). Haemoglobin RS with carrier ampholytes in 0.35 per cent methyl cellulose.

Ampholyte solution. Three parts of ampholytes 3-10 mixed with one part of ampholyte 5-8.

Capillary system
- Image capillary electrophoresis analyzer system,
- fluorocarbon coated cIEF cartridge (100 µm ID × 5 cm)
- sample injection time: 200 seconds
- spectrophotometer set at 280 nm
- running conditions. 1 minute at 1500V, followed by 15 minute at 3000V
- pI markers range. 4.65 and 8.18 pI markers

Inject reference solution (b). The test is not valid unless the pI range is between 7.00 to 7.20 for first major peak in the electropherogram obtained with reference solution (b).

Inject reference solution (a) and test solution. In the electropherogram obtained with the test solution and reference solution (a), the pI of the major species is 6.0±0.1 and the profile of the electropherogram obtained with the test solution should also correspond to that of electropherogram obtained with reference solution. Relative percent area of main peak is not less than 90 per cent.

Related proteins. 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 per cent 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 Pegfilgrastim RS in 800 µl of diluent. Add 100 µl of 0.1M hydrogen peroxide and incubate at 37º 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 (5µm),
- column temperature. 45±2º,
- mobile phase: A. a 0.1 per cent v/v solution of trifluoroacetic acid in water,
  B. a 0.1 per cent v/v solution of trifluoroacetic acid in 1000 ml of acetonitrile,
- flow rate: 0.7 ml per minute,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 214 nm,
- injection volume: 20 μl.

<table>
<thead>
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<th>Time (in min.)</th>
<th>Mobile phase A (per cent v/v)</th>
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<tr>
<td>55</td>
<td>53</td>
<td>47</td>
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</tbody>
</table>

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 (retention time of about 27 minutes). Resolution between oxidized peak 1 and 2 is not less than 1.5 and resolution between oxidized peak 2 and main peak to be 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.

**Test for free mPEG.** Determine by electrophoresis (sodium dodecyl sulphate polyacrylamide gel electrophoresis) (SDS-PAGE) (2.4.12) under non-reducing condition.

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

*Sample buffer (5X).* A mixture of 60 mM *tris hydrochloride* pH 6.8, 25 per cent *glycerol*, 2 per cent *sodium dodecyl sulphate*, 0.2 per cent *bromophenol blue* (or equivalent).

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

**Reference solution (a).** Dilute *Pegfilgrastim RS* to obtain a concentration of 1 mg per ml in *water*.

**Reference solution (b).** Dilute 20 kDa PEG in water to obtain a concentration of 0.1 mg per ml.

**Reference solution (c).** Solution of molecular markers suitable for calibrating SDS-polyacrylamide gels in the range 14 to 100 kDa.

**Reference solution (d).** Mix 10µl of reference solution (a) with 4µl of reference solution (b)

**Reference solution (e).** Add 4µl of reference solution (b)

**Reference solution (f).** Add 2µl of reference solution (b)
**Sample treatment.** Heat the test solution and reference solutions at 70° for not more than 2 min after adding 1X sample buffer separately.

Load reference solution (d), reference solution (e), reference solution (f), 5 µl of reference solution (c) and 10µl of test solution to the stacking gel wells. Perform the electrophoresis under the conditions recommended by the manufacturer of the equipment.

Detection: Iodine staining.

After electrophoresis immerse the gel in 20 ml of 0.1M perchloric acid for 15 minutes. Remove the solution and add 5 ml of 5 per cent barium chloride solution followed by 2 ml of 0.1 g of resublimed iodine crystal dissolved in 2 ml of isopropanol. Incubate the gel for about 5 minutes at room temperature, allowing the bands to develop. After the bands have developed, rinse the gel with water to remove colloidal iodine particles and immediately scan the gel.

The test is not valid unless (1) the bands of all proteins with molecular weight markers between 6.5 and 200 kDa are visible and well resolved in the gel; (2) the principal band and the PEG band in the reference solution (d) do not show an overlap and (3) the principal PEG band in 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).

**Residual Filgrastim.** Determine by liquid chromatography (2.4.14)

**Sample dilution buffer.** 10 mM sodium acetate buffer solution pH 4.0, 5 per cent D-sorbitol and 0.004 per cent polysorbate 20.

**Test solution.** Dilute preparation under examination with sample dilution buffer to a concentration of 1.0 mg per ml.

**Reference solution (a).** Dilute Pegfilgrastim RS in suitable diluents to a concentration of 10.0 mg per ml.

**Reference solution (b).** Dilute filgrastim RS in suitable diluents to a concentration of 0.3 mg per ml.

**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 (5µm),
- column temperature. 60±2°,
- mobile phase: A. a 0.1 per cent v/v solution of trifluoroacetic acid in water,
  B. a 0.1 per cent v/v solution of trifluoroacetic acid in 1000 ml of acetonitrile,
- flow rate: 0.85 ml per minute,
- a linear gradient programme using the conditions given below,
- spectrophotometer set at 214 nm,
- injection volume: equivalent to 40 µg
<table>
<thead>
<tr>
<th>Time (in min.)</th>
<th>Mobile phase A (per cent v/v)</th>
<th>Mobile phase B (per cent v/v)</th>
</tr>
</thead>
<tbody>
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<td>55</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
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<td>32</td>
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<td>100</td>
</tr>
<tr>
<td>39</td>
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<td>55</td>
</tr>
<tr>
<td>44</td>
<td>45</td>
<td>55</td>
</tr>
</tbody>
</table>

Inject reference solution (c). The test is not valid unless, in the chromatogram obtained the resolution between main peak (pegfilgrastim) and main peak (filgrastim) is not less than 2 and relative standard deviation is not more than 10 per cent.

Inject reference solution (b) and test solution. Determine per cent area of filgrastim to estimate the per cent purity of the test solution. In the chromatogram obtained with the test solution, the per cent free filgrastim is not more than 0.5 per cent.

**Bacterial endotoxin** (2.2.3). Not more than 350 EU amount equivalents to per dose of drug product.

**Microbial contamination** (2.2.9). Carry out the test using suitable selective media. The total aerobic viable count is not more than 1 cfu per ml.

**Assay**

**Potency.** The potency of the preparation is determined by comparison of the dilutions of preparation under examination with the dilutions of *pegfilgrastim RS* or with dilutions of reference standard calibrated with international standard of pegfilgrastim.

Determine the potency using a filgrastim responsive cell line (e.g., NFS-60 or its variant, MNFS-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 RS*. 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) for assessing the proliferation of cells.

**Medium A. RPMI 1640** (Roswell Park Memorial Institute 1640) with 2 mM glutamine, 10 mM HEPES, 2 g per litre of sodium bicarbonate, 4.5 g per litre of glucose, 1 mM sodium pyruvate, 0.05 mM mercaptoethanol, 10 per cent fetal bovine serum (FBS), and 20 ng per ml mouse IL3 (Interleukin 3) or 62 ng per ml of M-CSF (human recombinant macrophage colony stimulating factor).
Medium B. RPMI 1640 with 2 mM glutamine, 10 mM HEPES, 2 g per litre of sodium bicarbonate, 4.5 g per litre of glucose, 1 mM sodium pyruvate, 0.05 mM mercaptoethanol, 10 per cent FBS, and 1 ng per ml of filgrastim

Medium C. RPMI 1640 with 2 mM glutamine, 10 mM HEPES, 2 g per litre of sodium bicarbonate, 4.5 g per litre of glucose, 1 mM sodium pyruvate and 1 per cent FBS.

Cell line. Filgrastim adapted M-NFS 60.

Cell adaptation to filgrastim. Revive and passage M-NFS-60 cells in Medium A until passage No. 2, and then transfer cells to Medium B. Passage the cultures until passage no. 11 and after passage 11, the cells are defined as filgrastim adapted and can be used.

Cell suspension. 1.0 × 10⁵ M-NFS-60 cells per ml suspensions in medium C with 0.05 mM β-mercaptoethanol (Cells should be in a uniform suspension during addition).

Prepare an intermediate stock of 3 X 10⁴ pg per ml from stock of test and reference in medium C and then prepare dilution series as shown below

<table>
<thead>
<tr>
<th>Dilution step</th>
<th>Stock (pg per ml)</th>
<th>Dilution factor</th>
<th>Stock (µl)</th>
<th>Diluent (µl)</th>
<th>Concentration before adding cells (pg per ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3 x 10⁴</td>
<td>4X</td>
<td>250</td>
<td>750</td>
<td>7500</td>
</tr>
<tr>
<td>2</td>
<td>7500</td>
<td>5X</td>
<td>250</td>
<td>1000</td>
<td>1500</td>
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<tr>
<td>3</td>
<td>1500</td>
<td>2.5X</td>
<td>250</td>
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</tr>
<tr>
<td>4</td>
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<td>2.5X</td>
<td>250</td>
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<tr>
<td>9</td>
<td>6.144</td>
<td>5X</td>
<td>250</td>
<td>1000</td>
<td>1.2</td>
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</tbody>
</table>

To a suitable microtitre plate, transfer 100 µl each of reference 1–9 and test 1–9 to designated wells for the reference and test. Transfer 100 µl of medium C to designated cell control wells. To each standard, sample, and cell control well, add 50 µl of cell suspension. Transfer 150 µl of medium C to the designated medium control wells.

Incubate the plate at 37 ± 1° for a period of 42 ± 2 hours in a humidified incubator using 5 per cent CO₂. Remove the plate from the incubator, and add 20 µl of pre-warmed resazurin (Sodium) to each well. Gently agitate the plate, and incubate for an additional 7± 1 h at the initial conditions. Remove the plates from the incubator, and allow them to cool to room temperature by placing on a plate shaker for 10 to 15 minutes. Measure the fluorescence using a suitable 96 well plate reader using an excitation wavelength of 530 -560 nm and emission wavelength of 590 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 $\geq 2$.

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

**Storage.** Store at 2° to 8° in an airtight container.

**Labelling.** The label of the sealed container states (1) the name (2) content in mg per ml, and (3) potency of the drug substance.