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Research

Comparative Assessment of Filgrastim Bio-similars via vertical gel electrophoresis (SDS-PAGE) and HPLC

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Running title: Comparative Assessment of filgrastim bio-similars

Abstract

Background: Filgrastim bio-similar have emerged as cost-effective alternatives to the reference filgrastim, providing comparable efficacy and safety profiles. **Objective:** In this study, we aim to show physico-chemical similarity between filgrastim biosimilars using vertical gel electrophoresis & high-performance liquid chromatography. **Materials & Methods:** In the present study, we evaluate filgrastim biosimilars (grafeel, religrast, neukine, colstim, emigrast) via SDS-PAGE and HPLC thereby checking their molecular weights for similarity and their qualitative analysis. The same was compared with reference standard of filgrastim (Filgrastim, certified Reference Material). **Results:** In the scope of the data obtained, we found that the main band of filgrastim in filgrastim biosimilars has the same electrophoretic mobility as the reference standard under non-reduced circumstances in case of SDS-PAGE, the outcomes demonstrated that the position of the principal band in the electropherogram produced by the test solution and the principal band produced by the reference solution are similar. The retention time of the main peak obtained with the test solution and the peak obtained with the reference solution are similar. For HPLC, all the filgrastim biosimilars showed monomer peaks on the RP-HPLC chromatograms, which eluted at 12.4 minutes, matching the retention duration of the filgrastim reference standard. Conclusion: Filgrastim has transformed the management of neutropenia and has become an indispensable therapy in various clinical settings. The development and approval of filgrastim biosimilars have expanded treatment options and improved accessibility, offered cost-effective alternatives while maintained comparable safety and efficacy.

Keywords: Filgrastim, Bio-similar, Physicochemical, SDS-PAGE, HPLC.

Introduction

Biologics are a class of pharmaceutical medications made by biotechnology or from biological organism. Unlike traditional medicines with few molecules, they are created through biotechnology; biologics are large and complex molecules, typically proteins or nucleic acids. They are essential to modern medicine and have transformed the way that many diseases are treated [1]. They have transformed the treatment landscape for many diseases and have offered new therapeutic options to patients. With continued efforts to increase their

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effectiveness, safety, and accessibility, they continue to be a focus of considerable research and development [2].

Biosimilars can be defines as the biologic products which are like the originator or reference product, which is comparable to the existing authorized reference biologic therapeutic products. The creation of biosimilars guarantees that they are identical to the reference product in terms of quality, safety, and efficacy. It is important to note that while biosimilars undergo a robust approval process, they are not considered generic versions of biologics. Each biosimilar is developed and evaluated based on the specific reference product it aims to replicate [3]. Bio-similars are continue to gain acceptance worldwide, they play a significant role in improving patient access to effective and affordable biologic therapies while maintaining comparable safety and efficacy [4].

Filgrastim is a therapeutic protein, which belongs to the class of granulocyte colony-stimulating factors (G-CSF) [5]. Filgrastim, from Escherichia coli, sold under the brand name Neupogen®, and lenograstim, from Chinese hamster ovary cells, sold under the brand name Granocyte®, are two distinct types of rhG-CSF [6]. There are 175 amino acids in filgrastim, which is different from endogenous protein in terms of lacking O-glycosylation and, has an extra methionine group at its N-terminal [7]. It is a recombinant form of the natural human G-CSF, which is produced through genetic engineering techniques using bacteria or mammalian cells [8].

The primary function of filgrastim is to stimulate the production and maturation of neutrophils, a kind of white blood cell that participates in the immunological response of the body [9], [10]. Filgrastim is commonly used to manage neutropenia, a condition characterized by an abnormally decreased blood neutrophil concentration. Radiation or chemotherapy has the adverse effect of neutropenia where the destruction of rapidly dividing cells, including white blood cells, is a common occurrence. Filgrastim administration can help stimulate the production of neutrophils and reduce the risk of infections in individuals undergoing these treatments [11].

Additionally, filgrastim may be used to mobilize and collect stem cells from the peripheral blood of healthy donors or patients for hematopoietic stem cell transplantation. By increasing the release of stem cells from the bone marrow into the bloodstream, filgrastim facilitates their collection for subsequent transplantation procedures [12].The administration of filgrastim is typically done through subcutaneous injection, and the dosage and treatment duration depend upon the specific indication and patient's condition. Bone discomfort, headaches, lethargy, and minor allergic responses are frequent side effects [13], [14].

Filgrastim biosimilars is developed to be tremendously similar to the reference biologics. These biosimilars are supposed to be identical to the reference product in terms of quality, efficacy and safety in clinical studies [15]. Several filgrastim biosimilars have been approved by regulatory agencies, such as Central Drugs Standard Control Organisation (CDSCO) in India. These approvals are based on comprehensive analytical, non-clinical and clinical studies that establish the bio-similarity of the filgrastim biosimilar to the reference filgrastim product [16].

Comparative analytical studies assess the physicochemical and biological characteristics of the filgrastim biosimilar in comparison to the reference filgrastim product [17]. In the present study, we aim to show similarity between filgrastim biosimilars using vertical gel electrophoresis & high-performance liquid chromatography comparison to the reference filgrastim product.

Materials and Methods

Materials, reagents and chemicals

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HPLC grade acetonitrile and Ultra-pure water using Milli-Qs Surepro pf (Millipore) system were used. Well characterized Filgrastim biosmilars (grafeel-300 µg/ml, religrast 300 µg/0.5 ml, emgrast-300 µg/0.5 ml, neukine-300 µg/ml and colistim-300 µg/ml) samples and reference standard (1.6 mg/mL) were used to carry out the experiments. All the chemicals and solvents used for performing the analysis were of analytical grade.

Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis (SDS-PAGE)

Filgrastim biosimilars and Reference solution were prepared using non-reducing SDS-PAGE sample buffer (125 mM Tris–HCl, pH 6.8; 20% glycerol; 4% SDS; 0.1% bromophenol blue) to obtain a final concentration of 100µg per ml. Similarly dilute the preparation under examination with SDS-sample buffer for non-reducing conditions to obtain a protein concentration of 100µg per ml. SDS-PAGE was carried out under non-reducing conditions using 10 wells of 14% Bis acrylamide Gels in mini gel tank. Filgrastim samples were heated for 2 minutes at 95°C by mixing with SDS-PAGE sample buffer in 1: 2 ratio (1 part sample: 2 parts sample buffer). Thus, 2µg of sample concentration was loaded into each well. The protein ladder with the molecular mass marker-spectra multicolor broad range of 10-260kDa was utilized to perform the experiment. The running conditions were 100V for 1.5 hour in 1X SDS running buffer. Proteins were made visible utilizing Coomassie brilliant blue staining.

Reverse Phase Ultra-Performance Liquid chromatography (RP-HPLC)

The analytical method is performed on a high-performance liquid chromatography (Make & Model of instrument used is Thermo Fisher Scientific, Dionex-Ultimate 3000, Separation module with UV-Vis detector) equipped with a quaternary solvent delivery pump, degasser, Autosampler and column thermostat using Chromeleon 7 Software (Version: 7.2.10 ES). A C4 column (15cm X4.6mm), packed with octadecylsilane bonded to porous silica (3um) was used. The column temperature was set to be 65ºC. Mobile phase A is prepared using 1ml of trifluoroacetic acid to 500ml water, and add 499ml of acetonitrile, and mobile phase B is prepared using 1ml of trifluoroacetic acid to 950ml with acetonitrile and add 49ml of water. A linear gradient programme as described below was used to run the samples at flow rate of 1ml per minute and injection volume 50 µl. The absorption of samples is read at 215 nm. The filgrastim biosimlars and reference solution were prepared using solution A (0.1 mg/ml polysorbate 80 and 50 mg/ml sorbitol in 0.1 M sodium acetate buffer solution, pH 4.0) to obtain concentration of 0.3 mg/ml.

Table 1. *Gradient Programme of RP-HPLC method.*

Time (in min)	Mobile phase a (per cent v/v)	Mobile phase b (per cent v/v)
0	92	8
4	92	8
19	72	28
19.1	0	100
21	0	100
21.1	92	8
25	92	8

Results

Identification by SDS-PAGE

The specific results of SDS-PAGE for filgrastim bio-similar would typically include the migration pattern of the protein on the gel, which can be compared to the reference product. The intensity and distribution of protein bands on gel can also provide information about the protein's purity and potential impurities

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The current study was conducted to compare the bio-similarity of filgrastim bio-similar with reference product in accordance with the monograph published in the Indian Pharmacopoeia 2022. To confirm the anticipated mass of approximately 18 kDa, the molecular weight of filgrastim was assessed by SDS-PAGE in a comparable manner. The main band of filgrastim in filgrastim biosimilars has the same electrophoretic mobility as the reference standard under non-reduced circumstances. The outcomes demonstrated that the position of the principal band in the electropherogram produced by the test solution and the principal band produced by the reference solution are similar (Figure 1).

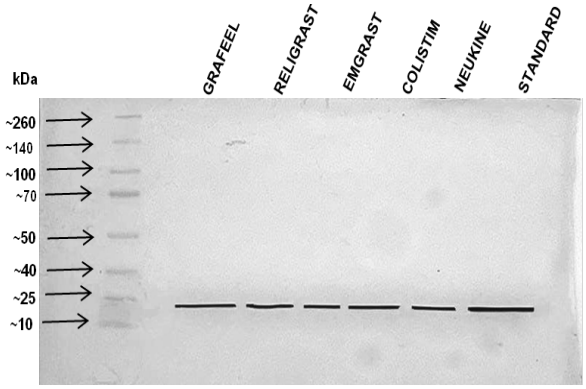


Figure 1. *Electropherogram of Filgrastim Bio-Similars and Reference Standard.*

Identification by RP-HPLC

The specific results of RP-HPLC for filgrastim biosimilars would typically include chromatograms, which show the elution profile of the protein. The protein's retention period, peak form, and peak area can be compared to those of the reference product. Any differences observed may indicate variations in the protein structure or impurities present.

It's important to note that the characterization and approval of biosimilars involve a comprehensive range of analytical techniques, including RP-HPLC, along with other assays, bioassays, and clinical studies to demonstrate similarity to the reference product in terms of quality, efficacy, and safety.

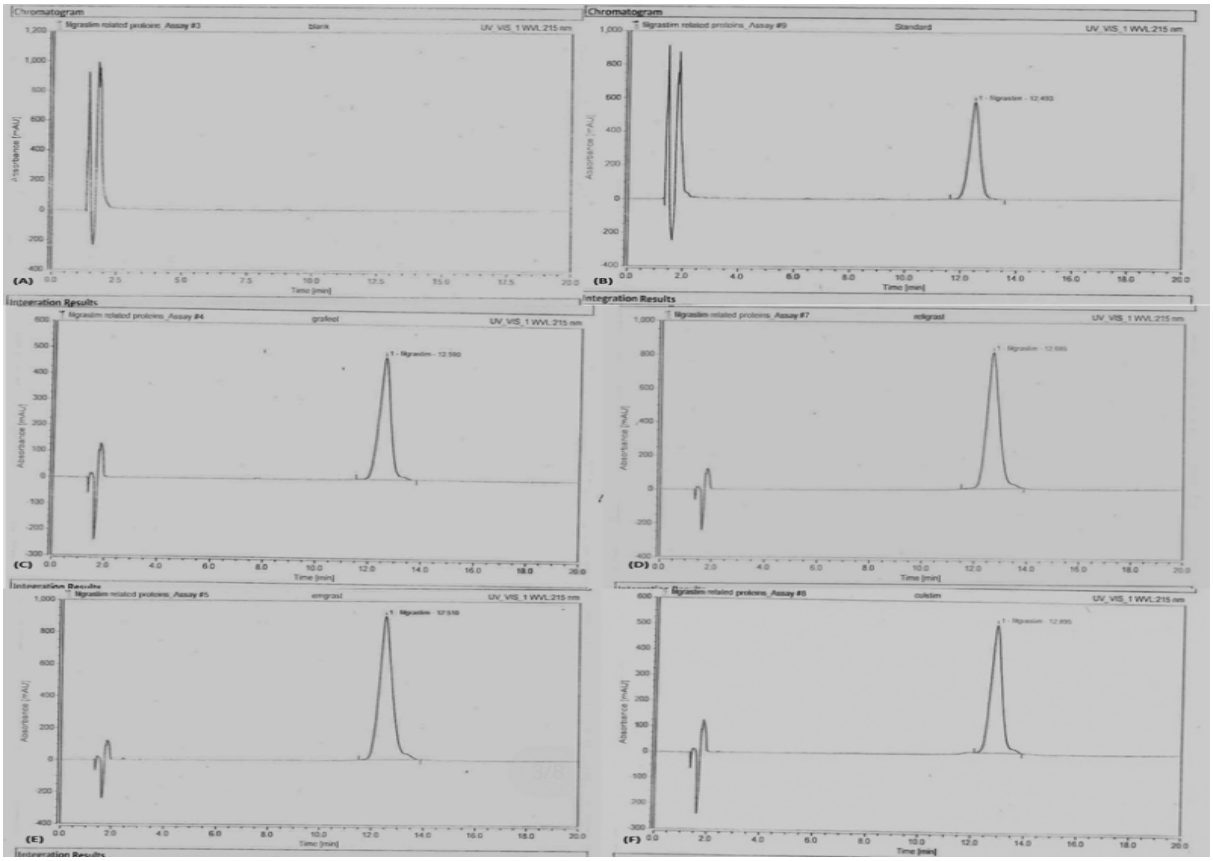


Figure 2. *HPLC Chromatogram of (A) Blank, (B) Reference Standard, (C) Grafeel, (D) Religrast, (E) Emgrast, (F) Colistim*

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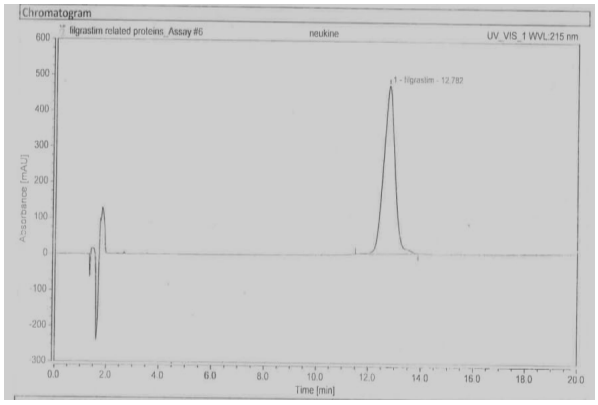


Figure 3. *HPLC Chromatogram of (G) Neukine*

All the filgrastim biosimilars showed monomer peaks on the RP-HPLC chromatograms, which eluted at 12.4 minutes, matching the retention duration of the filgrastim reference standard (Figure 2 A-G). The chromatograms of all the filgrastim bio-similars, including Grafeel, Religrast, Emgrast, Colstim, and Neukine are superimposed in Figure 3. The retention time of the main peak obtained with the test solution was found similar to the peak obtained with the reference solution.

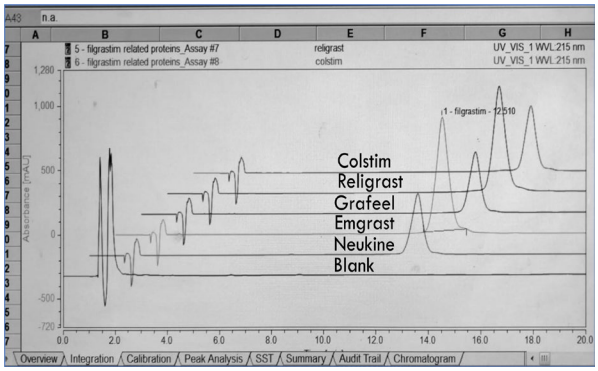


Figure 4. *Comparative Pattern of HPLC Chromatograms of Filgrastim Biosimilars*

Discussion

A comparative study using SDS-PAGE and HPLC for the analysis of filgrastim can provide valuable insights into the structural and quantitative aspects of the protein. Both techniques have their strengths and limitations, and combining their results can enhance the characterization of filgrastim.

SDS-PAGE is a method frequently used to evaluate the molecular weight, purity, and integrity of proteins. In the case of filgrastim, SDS-PAGE can help confirm the presence of the protein and assess its size compared to protein molecular weight markers. By subjecting filgrastim samples to electrophoresis, it is possible to visually observe the protein band on the gel. Any deviations from the expected molecular weight can indicate potential modifications or degradation [18]. However, SDS-PAGE alone cannot provide detailed information about the protein's structural integrity or post-translational modifications. It primarily provides a qualitative assessment of the protein's structural integrity or post-translational modifications. To obtain quantitative information and more detailed characterization, complementary techniques such as HPLC are often employed [19].

HPLC is a powerful analytical technique that can provide quantitative data on the concentration of filgrastim and detect impurities or modifications. Reverse-phase HPLC, in particular, is commonly used for the separation and analysis of protein-based therapeutics like filgrastim. It relies on differences in hydrophobicity and molecular interactions to separate and quantify protein components in a sample

The combination of SDS-PAGE & HPLC in a comparative study allows for a more comprehensive analysis of filgrastim. SDS-PAGE confirms the presence and approximate molecular weight of filgrastim, while HPLC provides quantitative data on its concentration and can detect potential modifications or impurities. By comparing the results from both techniques, researchers can assess the similarity and consistency of different filgrastim samples, including biosimilars, in terms of purity, structural integrity and concentration [20].

It is important to note that the specific experimental conditions, methodologies, and instruments used for SDS-PAGE & HPLC analysis may vary among studies. Therefore, careful optimization and validation of the methods are essential to ensure reliable and accurate results. Additionally, the interpretation of the data

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should consider the established reference standards and regulatory guidelines for filgrastim and similar biologic products [21].

The data furnished here suggested that filgrastim biosimilar is similar to the reference solution i.e. standard in terms of identity. There are few studies available which substantiates the findings of the present study. There are few previously published physicochemical and biological comparative studies assessing the similarity of biosimilar products, which have been approved in India, Europe and United States [22-24]. The same principle used for establishing comparability of originator or innovator biologics after changes in manufacturing process. Bio-similarity assessment begins with standard quality control testing, including comparison of primary structure and higher-order structure to existing specifications, post-translational modifications, glycosylation and bioactivity. The stability of proteins under stress conditions is important to demonstrate comparable behavior. The study published by Halim et al., provide a head-to-head comparative study on the quality of biosimilar and copy filgrastim products with the innovator product. The author suggested except for the deviating specific activities of the two copy filgrastim products, there was no substantial differences in product quality between the filgrastim products [1]. Similar findings are reported in the present study when we compared filgrastim biosimilars (Grafeel, Religrast, Emgrast, Neukine and Colstim) with reference standard. Further, another study demonstrated the similarity of a filgrastim manufactured by Eurofarma (Fiprima®) and one manufactured by Amgen Inc. (commercialized by Hoffman-La Roche Ltd, Granulokine®) in terms of identity, structure, purity, and bioactivity. The author compared both the products using peptide mapping, reduced and non-reduced polyacrylamide electrophoresis, bi-dimensional electrophoresis, Western blotting, reverse-phase HPLC, size-exclusion HPLC, far and near circular dichroism, fluorescence emission, X-ray crystallography, liquid chromatography–tandem mass spectrometry (LC-MS), matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), receptor binding, and potency by in vitro cell proliferation [25-29]. Bio-similarity to Granulokine was demonstrated in terms of identity, structure, purity, and bioactivity. To assure the safety and efficacy of filgrastim biosimilars a study carried out a rigorous analytical workflow for chemical characterization and clinical trials to evaluate the efficacy and safety is required to demonstrate their high similarities to the reference drug. Thus, implicate the necessity of quality comparison of filgrastim biosimilars.

Conclusion

The study reports a head-to-head comparative quality study of filgrastim biosimilars approved in India. Using sodium dodecyl sulphate polyacrylamide gel electrophoresis and high performance liquid chromatography, the study demonstrates that there are no significant differences in identity between the filgrastim biosimilars. The retention time of the principal peak obtained with the test solution is like that of the principal peak obtained with the reference solution. This comparative study corroborates the previous findings that the similar nature of the biosimilars, strengthens the scientific justification for reducing the extent of non-clinical and clinical variations. This underscores the importance of consistent regulatory standards and rigorous evaluation processes in ensuring the efficacy and safety of biosimilar products. These findings not only reinforce the scientific basis for regulatory guidelines but also emphasize the potential of biosimilars to offer safe and effective treatment options. Moving forward continued research and stringent evaluation will be crucial in maintaining confidence in the quality and therapeutic equivalence of biosimilar medicines.

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Conflicts of interest

There are no conflicts of interest.

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