American Journal of Analytical Chemistry, 2021, 12, 333-346
https://www.scirp.org/journal/ajac
ISSN Online: 2156-8278
ISSN Print: 2156-8251

Analytical Method Development and Validation of Filgrastim by UV and RP-UFLC Methods

Husna Kanwal Qureshi, Ciddi Veeresham*, Chinta Srinivas

Department of Pharmaceutical Analysis, University College of Pharmaceutical Sciences, Kakatiya University, Warangal, India
Email: *ciddiveeresham@gmail.com

How to cite this paper: Qureshi, H.K., Veeresham, C. and Srinivas, C. (2021) Analytical Method Development and Validation of Filgrastim by UV and RP-UFLC Methods. *American Journal of Analytical Chemistry*, **12**, 333-346.
https://doi.org/10.4236/ajac.2021.1210021

Received: September 20, 2021
Accepted: October 22, 2021
Published: October 25, 2021

Abstract

The research work was carried out for establishing a new Ultra Violet (UV)—Visible spectroscopy and Reverse phase-Ultra Fast Liquid Chromatography (RP-UFLC) method for the analysis and quantification of a biosimilar drug, Filgrastim. Filgrastim or recombinant methionyl granulocyte colony stimulating factor (rGCSF) is a glycoprotein. It has a biological action essential for proliferation and differentiation of hematopoetic and progenitor cells. The UV and RP-UFLC work was carried on a Shimadzu UV1800 Spectrophotometer and Shimadzu Prominence LC-20AD UFLC systems, respectively. The λ_{max} of filgrastim was found to be 215 nm. The correlation coefficient by UV spectroscopy was found to be 0.9994 for the concentration range of 1 to 3 µg/ml in double distilled water. The Reverse phase UFLC was done by using Phenomenex C4 (25 cm × 0.46 cm internal diameter) 15 µ, 300 A° analytical column. The optimized mobile phase for binary elution was Acetonitrile and double distilled water (80:20) with a flow rate of 1 ml/min. The retention time of drug was at 3.2 min. It was observed that the response of the detector was linear in the range of 5 - 15 µg/ml with correlation coefficient value of 0.999. After developing the methods, it was assured for the intended use by validation of the analytical parameters like linearity, accuracy, precision, limit of detection, limit of quantitation, ruggedness and robustness. The results of all the parameters for both the methods were found to be within the acceptance criteria as per the International Council for Harmonisation (ICH) guidelines.

Keywords

Biosimilars, Filgastim, Method Development, RP-UFLC, UV Visible Spectroscopy, Validation

1. Introduction

Natural Human granulocyte colony-stimulating factor (hG-CSF) is a single

chain polypeptide containing 174 amino acid residues [1]. It helps in proliferation of leucocyte stem cell into mature granulocytes [2]. There are nearly 4 CSF's are known, namely Granulocyte-Macrophage; Granulocyte CSF (G-CSF); Macrophage CSF and Multi-CSF (or interleukin 3) [3]. Neutropenia is a condition that occurs when neutrophil count fall below the nominal range of approximately 1.5×10^9/L [4]. Granulocyte colony-stimulating factor (G-CSF) is primarily used in clinical practice for the prevention of chemotherapy-induced neutropenia, neutropenia due to bone marrow transplantation and neutropenia associated with the myelodysplastic syndrome or aplastic anemia [5], and for the mobilization of hematopoietic stem cells [6]. G-CSF can decrease the period of neutropenia or prevent it altogether [4]. It is also used in the treatment of hematopoietic recovery after Acute Myocardial Infarction, Neonatal infections and AIDS [7] [8] [9].

Recombinant human granulocyte colony-stimulating factor (rhG-CSF) is one in the family of hematopoietic growth factors [5]. The non-glycosylated form of the hG-CSF gene protein is produced in genetically engineered Escherichia coli. The hydrophobic protein produced is a 175 aminoacids chain which at its N-terminus contains an extra methionine. (Ex: Filgrastim) [5].

Filgrastim or recombinant methionyl granulocyte colony stimulating factor (rGCSF) (**Figure 1**) [10] is a glycoprotein (18.8 kDa) [3] [11] which is currently manufactured by Amgen Inc. as the drug Neupogen (United States) [12], which belongs to a family of cytokines [13]. Filgrastim has a biological action essential for proliferation and differentiation of hematopoetic [11] and progenitor cells. It promotes the maturation of myeloid cells and enhances the function of neutrophils and monocytes [12]. Filgrastim differs from the natural hormone (O glycosylated at Thr-133 [4] in that the former is not glycosylated as E.coli lacks the necessary enzyme for glycosylation [12]. It contains an additional methionine group at the N terminus [12] [14], which is necessary for expression of the gene in *E. coli* [13] [15]. The structure has a free cysteine at position 17, two intramolecular

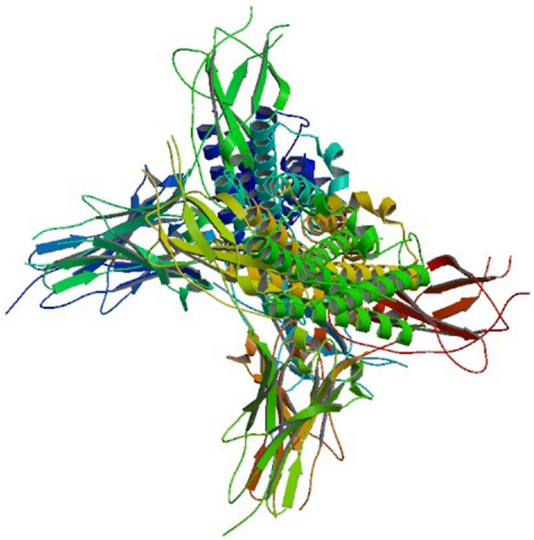

Figure 1. Structure of Filgrastim.

disulfide bonds (Cys36-Cys42 and Cys64-Cys74). These structural characteristics are required and are of importance to GCSF bioactivity and also for more efficient immune responses [11] [16].

Analytical characterization of proteins and recombinant proteins is necessary according to the ICH Q6B Guidelines [16]. A variety of analytical techniques, mostly based on physico-chemical properties, have been recommended for the assessment of identity, purity, and potency of recombinant proteins [6] [12] [17] [18] [19] [20].

Literature search revealed some reported methods for analyzing filgrastim and its formulation using Reverse Phase liquid Chromatography (RP-LC) and Size Exclusion Chromatography (SEC). RP-LC analysis used a Jupiter C4 column (250 mm × 4.6 mm) as the stationary phase whereas the mobile phase A consisted of water: acetonitrile (90:10, v/v) with 0.1% Tri fluoroacetic acid (TFA) and the mobile phase B was water: acetonitrile (20:80, v/v) with 0.1% TFA, run at a flow rate of 0.5 ml/min and detection at 280 nm [21]. Size-exclusion chromatography was carried out on a TSK gel G2000 SW column (60 cm × 7.5 mm). The mobile phase was composed of phosphoric acid (pH 2.5; 0.1 M), run at a flow rate of 1.0 ml·min^{-1} and with UV detection at 214 nm [22]. Another analysis was carried by high performance liquid chromatography for filgrastim in formulations. The analysis was done by using LiChrospherÒ WP 300 RP-18e, (150 × 4 mm) column. The HPLC system was operated at gradient mode with mobile phase composed of solvent A (0.1% TFA in a mixture of water: acetonitrile = 90:10 v/v) and solvent B (0.08% TFA in a mixture of water: acetonitrile = 10:90 v/v) at temperature of 50˚C and UV detection at 215 nm [13].

Capillary zone electrophoresis (CZE) was the other technique apart from RP-HPLC and SEC, which was used for validation and for the analysis of recombinant human granulocyte colony-stimulating factor (rhG-CSF) [5].

Ultra Violet (UV) spectroscopy is ascribed to be simple, easy and economical [23] for validating. Literature search unfolded that articles on method development and validation by UV spectroscopy have not been reported so far. Among various techniques, Reverse Phase Ultra-Fast liquid chromatography (RP-UFLC) has been evidenced to be particularly useful and reliable. For the analysis of Protein Biologics, prime step is analyzing by RP-HPLC. RP-HPLC/RP-UFLC technique is used for protein characterization, for estimating content, to establish purity, for determination of excipients, for analyzing disulphide bonds etc. [1] [2] [6] [12] [16] [17] [19].

Hence, for the research work, Reverse Phase-Ultra Fast Liquid Chromatography and UV techniques were selected because of their easy feasibility and applicability for routine analysis.

2. Materials and Method

2.1. Materials

Filgrastim reference standard was purchased from European Medicine Agency,

Strasbourg, France. Grafeel®, marketed formulation was obtained as gift sample from Dr. Reddy's Laboratories, Hyderabad. Double distilled water was obtained from Merck Millipore Direct Q UV water system. Acetonitrile was purchased from Merck.

2.2. Equipment

A Shimadzu UV1800 Spectrophotometer was used for the analysis. Quartz cells of 1 cm path length were used for measurement. UV Probe version 2.43 software was used for analysis. Shimadzu Prominence LC-20AD UFLC systems was used for RP-UFLC analysis with lab Solutions software. The mobile phase was degassed using Ultrasonic bath Sonicator (3.5 L) PCI analytics PVT LTD. The Mobile phases were filtered using Vacuum filtration unit with 0.22 µ membrane filter. Micro pipettes (Finnpipette from Thermo Fisher scientific) were also used.

3. Methodology

3.1. UV Visible Spectrophotometric conditions

3.1.1. Determination of Maximum absorbance λ_{max}

The diluted standard solution (50 µg/ml) was scanned at medium scanning speed (670 nm/min with 2 nm data interval) for a whole range of UV/VIS Spectrophotometer, the ranging from 800 - 200 nm with double distilled water as blank. After acquiring the spectrum, λ_{max} was identified. The above method was repeated thrice. The method was developed at room temperature (25˚C).

3.1.2. Preparation of Working Standard Drug Solution

Different Filgrastim standard concentrations were prepared like 1, 1.4, 1.8, 2.2, 2.6 and 3 µg/ml from the filgrastim reference standard by taking 14.7 µl, 20.5 µl, 26.4 µl, 32.3 µl, 38.2 µl and 44.1 µl and diluting to 5ml in volumetric flask with double distilled water, respectively.

3.1.3. Preparation of Calibration Curve

The Calibration curve was prepared by using 6 different dilutions prepared from Standard solution (1, 1.4, 1.8, 2.2, 2.6 and 3 µg/ml strength). An absorbance of every calibration standard was estimated at λ_{max} 215 nm using fixed wavelength measurement mode. The calibration curve representing concentration vs. absorbance was plotted utilizing Microsoft Excel 2013.

3.2. RP-UFLC Chromatographic Conditions

The reverse-phase chromatography was performed on Shimadzu Prominence LC-20AD UFLC with UV detection. The analysis was done by using Phenomenex C4 column (25 cm × 0.46 cm internal diameter) 15 µ, 300 Å analytical column containing Acetonitrile: water (80:20 v/v) as mobile phase in binary elution. The method was run at 1 ml/min at 215 nm UV detection.

Preparation of Filgrastim Working Standard Solutions

From filgrastim standard solutions, accurately measured volumes *i.e.*, 14.7 µl,

20.5 µl, 26.4 µl, 32.3 µl, 38.2 µl and 44.1 µl were taken and diluted to 1 ml with double distilled water and mixed thoroughly to get 5, 7, 9, 11, 13, 15 µg/ml standard dilute concentrations. After many experimental trials, the optimized conditions were noted and proceeded for validation as per ICH guidelines.

4. Method Validation

As per ICH guidelines [6] [7] the checked validation parameters for spectroscopic (UV-Vis) and chromatographic (RP-UFLC) methods were accuracy, precision, linearity, Limit of Detection (LOD), Limit of Quantitation (LOQ), robustness and ruggedness [8].

4.1. System Suitability

For evaluating the suitability of UFLC system and procedure, the Filgrastim standard solution of 5 µg/ml concentration was prepared and about 20 µl was injected into the UFLC system. Then the chromatogram was recorded.

4.2. Linearity and Range

Linearity data for the spectrophotometric method was obtained at an absorption maxima of 215 nm (**Figure 2**). Calibration curve was obtained by plotting absorbance of six different standard dilution against concentration. The dilutions prepared from standard solution were 1, 1.4, 1.8, 2.2, 2.6 and 3 µg/mL.

Linearity data for the chromatographic method was obtained by using six concentrations within the range of 5 - 15 µg/ml. Calibration curve was obtained plotting peak area against concentration. Both the methods were studied using three replicates of each sample concentrations.

4.3. Accuracy

In order to determine the method's accuracy, the drug was spiked at 80%, 100% and 120% levels for both the methods. The absorbances for spectroscopic method and chromatograms with peak areas were recorded. From this, the average recovery of analyte was calculated.

4.4. Precision

Intra-day assay and inter-day precision were evaluated to determine UV and RP-UFLC method's precision.

4.4.1. Intra-Day Precision

To evaluate the intra-day precision by spectroscopic method, 2.2, 2.6, 3.0 µg/ml concentration solutions were analyzed at 215 nm. Relative Standard Deviation (%RSD) was calculated in order to assess precision. Each sample was analyzed in triplicate.

For Chromatographic method, 9, 11, 13 µg/ml concentration was injected for six times under unchanged conditions within a short period of time. The peak areas for the six replicate injections were collected and calculated the % Relative standard deviation.

4.4.2. Inter-Day Precision

To evaluate the Inter-day precision, for spectroscopic method, the samples were analyzed on three consecutive days. % RSD was estimated for establishing precision.

For chromatographic method, the sample was analyzed on different days under unchanged conditions. Peak areas were collected and % Relative standard deviation was calculated.

4.5. Robustness

Robustness of both the methods was studied using six replicates of the sample at a concentration level of 13 µg/ml (for UFLC) and 3 µg/ml (for UV).

For UV spectroscopic method, robustness was evaluated by analyzing at different temperature rather than optimized room temperature.

Robustness was evaluated by slightly modifying the chromatographic conditions which includes change in percent organic solvent and flow rate of the chromatographic method. The filgrastim solution was analyzed by slightly varying the percent organic solvent *i.e.*, Acetonitrile: Double distilled water as 79:21 and 81:19 ratios rather than 80:20 v/v and also by slightly changing flow rate, at 0.9 ml/min and 1.1 ml/min rather than optimized flow rate of 1.0 ml/min. Chromatograms were recorded for juxtaposing with optimized chromatographic conditions.

4.6. Ruggedness

For both the methods ruggedness was calculated by analyzing on different instruments.

4.7. Limit of Detection

Following formula was used for calculating LOD.

LOD = 3.3 × standard deviation of response/slope of the calibration curve.

4.8. Limit of Quantitation

The formula used to calculate the quantitation limit was:

LOQ = 10 × standard deviation of response/slope of the calibration curve.

5. Preparation of Sample Solution (Marketed Formulation)

For spectroscopic analysis and for chromatographic analysis, Grafeel® pre filled injections were taken and a concentration equivalent to 2 µg/ml and 10 µg/ml were prepared, respectively. The sample was analyzed in triplicate. % assay was calculated.

6. Results and Discussions

6.1. Method Development

For Spectroscopic method development, identification of wavelength of maxi-

mum absorbance is the first step for quantitative UV analysis. Determination of maximum wavelength for filgrastim (50 µg/mL) was carried out using full scan mode of spectrophotometer (Figure 2). The λ_{max} was identified with the help of software to be 215 nm.

For the method development by RP-UFLC, various ratios of mobile phases, different stationary phases and flow rates were tried to elute the drug with good peak parameters with good performance in assay. Finally, the best separation was achieved on phenomenex C4 column (250 mm × 4.6 mm id, 15 µm, 300 A˚) comprising mobile phase of ACN: Double distilled water [80:20 v/v] with binary elution. The method was run at a flow rate of 1.0 ml/min and the analyte was detected at 3.2 min by UV detector at 215 nm. The chromatogram showed the peak with good shape, more theoretical plates and the tailing factor was also found to be within the limits (Figure 3) with absorption maximum at 215 nm.

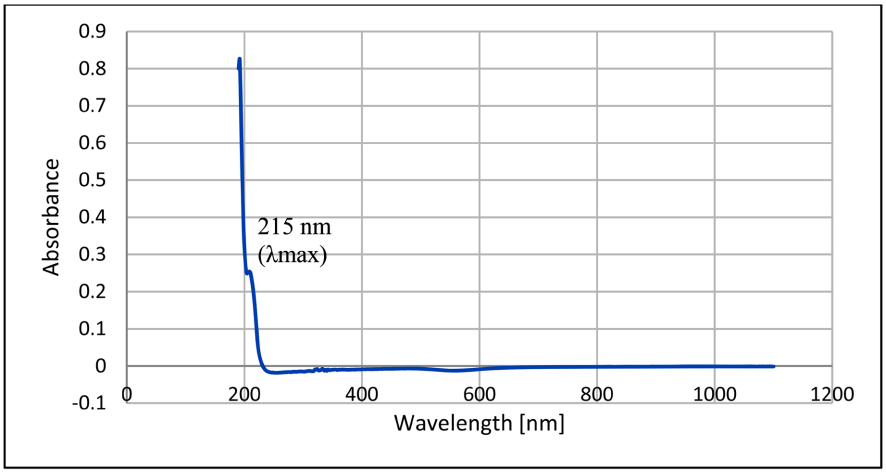

Figure 2. Absorption maximum of Filgrastim.

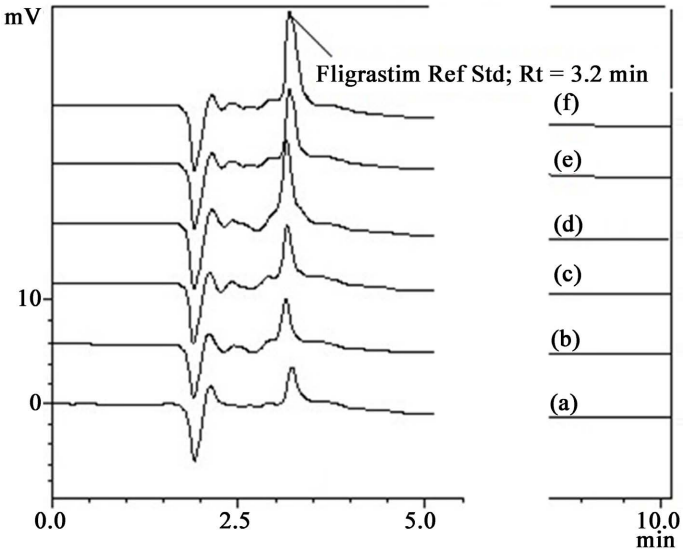

Figure 3. Chromatogram of Filgrastim reference standard at optimized conditions ((a)—5 µg/ml; (b)—7 µg/ml; (c)—9 µg/ml; (d)—11 µg/ml; (e)—13 µg/ml; (f)—15 µg/ml).

6.2. Analytical Method Validation

6.2.1. System Suitability

As per ICH guidelines, the theoretical plate number (greater than 2000; *i.e.* 3727 (mean)), Tailing factor (<2; 1.08 (mean)) and percentage relative standard deviation (≤2%; from 0.1 to 1.15% RSD) obtained were within the acceptance criteria and demonstrated that the method can generate the accurate and precise results with optimized conditions (Figure 3).

6.2.2. Linearity and Range

The linearity of filgrastim employing UV method was constructed by considering concentration (µg/ml) on X-axis and Absorbance on Y-axis. The regression coefficient was considered to be 0.9994 (linear) over a concentration range of 1 to 3 µg/ml. The representative linearity equation was found to be y = 0.182x + 0.0811 (Figure 4).

The linearity of proposed filgrastim employing UFLC method was constructed by considering concentration (µg/ml) on X-axis and peak area on Y-axis (Figure 3). The regression coefficient was considered to be 0.999 over a concentration range of 5 - 15 µg/ml. The representative linearity equation was found to be y = 4090.8x − 536.95 (Figure 5).

For both the methods the % RSD was found to be within the ICH acceptable theoretical limits of ≤2%.

6.2.3. Accuracy

It was found that the average recovery at 80%, 100%, and 120% were found to be within the limits which indicated the methods accuracy. The measured results are shown in Table 1.

6.2.4. Precision

The % RSD obtained was within the limits indicating the methods precision. Table 2 and Table 3 represents the results of intraday precision and interday precision of spectroscopic and chromatographic methods, respectively.

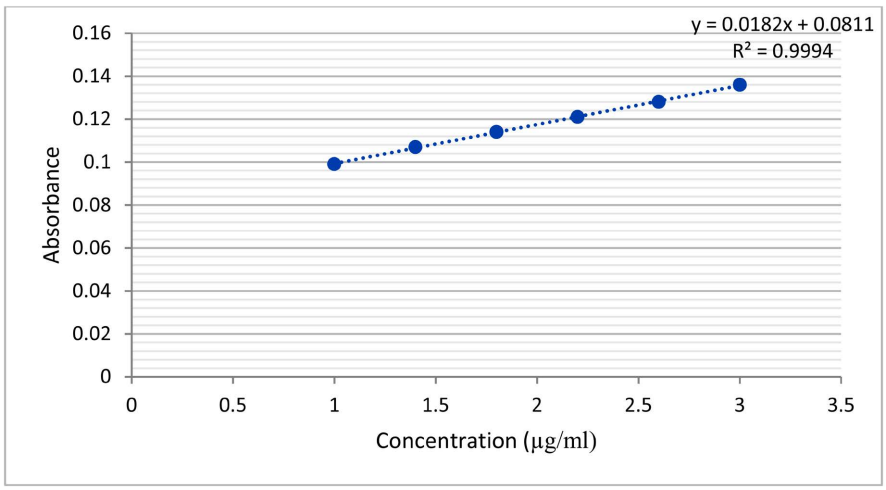

Figure 4. Calibration curve of Filgrastim reference standard by UV spectroscopy.

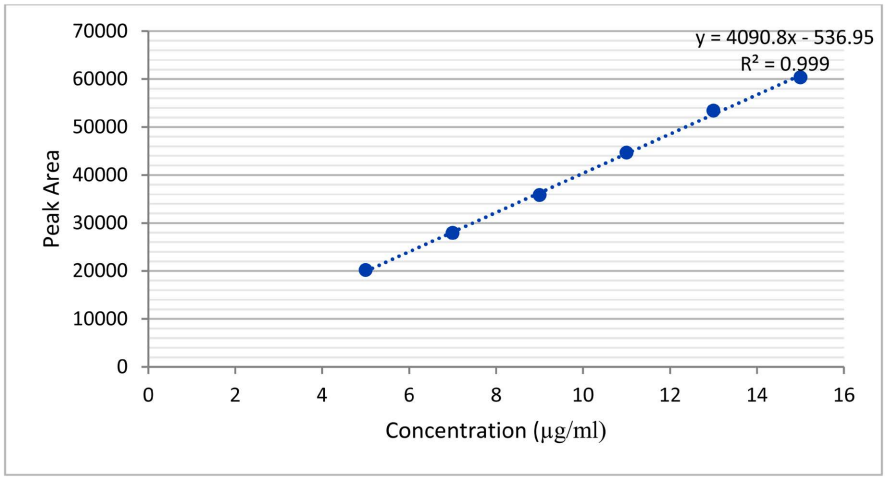

Figure 5. Calibration curve of Filgrastim reference standard by RP-UFLC.

Table 1. Accuracy data of filgrastim by UV Spectroscopy and RP-UFLC.

S. No.	Concentration (%)	By UV Spectroscopy			By RP-UFLC		
		% Recovery	Mean % Recovery	% RSD	% Recovery	Mean % Recovery	% RSD
1.	80	98.82			99.89		
2.	80	100.34	99.32	0.22	100.15	99.99	0.15
3.	80	98.82			99.93		
4.	100	100.42			98.67		
5.	100	101.64	100.53	0.31	99.19	98.73	0.45
6.	100	99.51			98.33		
7.	120	101.38			100.15		
8.	120	100.46	100.45	0.25	99.56	100.20	0.67
9.	120	99.53			100.89		

Table 2. Intraday and interday precision of Filgrastim by UV Spectroscopy.

Intraday precision									
S. No	Conc. (µg/ml)	Morning			Afternoon			Evening	
		Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1.	2.2	0.1206	98.63	0.48	0.1214	100.45	0.48	0.1204	98.13	0.96
2.	2.6	0.1277	98.46	0.45	0.1280	99.84	0.78	0.1287	100.38	0.45
3.	3.0	0.1360	100.34	0.74	0.1356	99.67	0.85	0.1350	98.70	0.74
Interday precision									
S. No	Conc. (µg/ml)	Day 1			Day 2			Day 3	
		Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1.	2.2	0.1213	100.36	0.48	0.1206	98.63	0.48	0.1204	98.13	0.48
2.	2.6	0.1277	98.46	0.45	0.1283	99.73	0.45	0.1276	98.23	1.2
3.	3.0	0.1364	101.26	0.43	0.1353	99.26	0.43	0.1350	98.7	0.74

Table 3. Intraday and interday precision of Filgrastim by RP-UFLC.

Intraday precision										
S. No.	Conc. (µg/ml)	Morning			Afternoon			Evening		
		Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1.	7	27,871.34	99.20	0.54	27,782.67	98.88	0.56	27,762	98.81	0.65
2.	9	35,846.34	98.81	0.33	35,822	98.75	0.86	35,823.34	98.75	1.15
3.	11	44,526.67	100.13	0.57	44,488	100.05	1.02	44,855	100.87	0.52
Interday Precision										
S. No.	Conc. (µg/ml)	Day 1			Day 2			Day 3		
		Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1.	7	27,841	99.10	0.21	27,725	98.68	0.18	27,682	98.54	0.23
2.	9	35,946.34	99.09	0.2	35,751	98.56	0.22	35,716	98.47	0.1
3.	11	44,493.34	100.06	0.34	44,526	100.13	0.14	44,539.3	100.16	0.15

Table 4. Robustness of data at different temperatures by UV Spectroscopy.

S. No	Conc. (µg/ml)	Temperature	Absorbance	% RSD
1.	3	25˚C	0.1353	0.43
2.	3	28˚C	0.1350	0.74

Table 5. Robustness of data at different mobile phase and flow rate by RP-UFLC.

S. No	Concentration (µg/ml)	Flow rate (ml/min)	Mobile Phase ratio (ACN:Water)	Rt	Peak area (Mean)	% RSD
1.	13	1	79:21	3.2	53,580	0.3
2.	13	1	81:19	3.2	53,525	0.16
3.	13	0.9	80:20	3.2	53,484	0.1
4.	13	1.1	80:20	3.2	53,564	0.16

6.2.5. Robustness

Upon slight changes in the temperature, flow rate and percent organic solvent, the results confirmed the robustness of the method. Results were presented in Table 4 and Table 5.

6.2.6. Ruggedness

Ruggedness data is represented in Table 6 for UV and RP-UFLC method.

6.2.7. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The calculated LOD and LOQ values of filgrastim by UV spectroscopy are 0.1813 µg/ml and 0.5494 µg/ml respectively. By chromatographic method, LOD and LOQ values were found to be 0.1346 and 0.4080 µg/ml respectively (Table 7).

6.2.8. Assay Determination of Filgrastim Marketed Formulation (Grafeel®)

The % purity of Filgrastim present in the marketed formulation was found to be 99.328% and 99.54% by UV and RP-UFLC methods respectively. The assay results

Table 6. Ruggedness data for Filgrastim.

S. No	Conc. (µg/ml)	Instrument Model & Make (UV spectroscopy)		Absorbance	% RSD
1.	3	UV 1800 Shimadzu spectrophotometer		0.1356	0.85
2.	3	Elico Double beam SL 210 UV VIS spectrophotometer		0.1350	0.74
S. No	**Conc. (µg/ml)**	**Instrument Model & Make (RP-UFLC)**	**Rt**	**Peak Area (Mean)**	**% RSD**
1.	5	Shimadzu Prominence LC-20AD UFLC system	3.2	20349	0.9
2.	5	Shimadzu LC 20 AD UFLC, Diode array detector	3.19	20299	0.91

Table 7. LOD and LOQ data of Filgrastim by UV spectrometer and RP UFLC system.

S. No.	Limit of Detection/Quantitation	UV	RP-UFLC
1.	LOD	0.1813 µg/ml	0.1346 µg/ml
2.	LOQ	0.5494 µg/ml	0.4080 µg/ml

of marketed formulation of filgrastim were found to be within specified limits as per ICH guidelines.

7. Discussion

Biosimilar analysis requires employment of advanced analytical techniques not only for validation but also for comparing innovator biotherapeutic with its newly introduced biologics. Some of the intricate reported works on filgrastim include assessment by thermal stress testing [1], Comparison of biosimilars in terms of protein characterization, pharmacokinetics, pharmacodynamics with innovator [6] [12] [17] [18] [19] [20], analyzing by capillary electrophoresis [5], carrying out bioactivity assays for comparing structure and function [12], characterization by Fourier transform Infra-red spectroscopy [7] etc. Previous literature unfolded the analysis of filgrastim (rhG-CSF) by SEC-HPLC [22] and by RP-LC [21] methods. In comparison to earlier methods, the present work deals with reliable and economic analysis of filgrastim using UV spectroscopy and RP-UFLC. The UV spectroscopic method can be easily used for quantitative estimation. Furthermore, this technique is ascribed to be simple, rapid and economical. The optimized RP-UFLC method is evinced to be less time consuming (Rt—3.2 min with binary elution) in collation to already reported works where the retention time is ten folds more than developed method. Binary elution was employed for the analysis in comparison to gradient time programmed elution as in reported articles. The LOD and LOQ data was much better in comparison to SEC-HPLC and RP-LC methods. LOD and LOQ for the optimized RP-UFLC method was found to be 0.1346 and 0.4080 µg/ml. The lower values of LOD and LOQ indicate accuracy, sensitivity and precision of the developed method.

Hence, the developed methods by UV and RP-UFLC can be used to analyze protein biologics from different manufacturers. The optimized method was found to be accurate, sensitive, specific and prudent. All the results were in ac-

cordance with ICH guidelines. These methods can be used for routine analysis of Filgrastim.

Acknowledgements

The authors are grateful to Dr. Reddy's laboratories, Hyderabad, for providing the Grafeel® pre filled syringes (marketed formulation) of Filgrastim. The authors are thankful to Phenomenex for providing us with Phenomenex C4 sample column.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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