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Development and optimization of Assay method of Filgrastim injection by Reverse Phase- High Performance Liquid Chromatography

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ABSTRACT

Filgrastim is a human granulocyte colony stimulating factor which belongs to the family of cytokines. The goal of this study was to develop and validate a simple and rapid RP-HPLC method for determination of Filgrastim in protein formulations (Injection). The experiment was carried out by RP-HPLC using YMC-Pack C4 (150 X 4.6mm ID S-5 µm, 12nm column). The HPLC was operated at gradient mode using mobile phase composed of solvent A i.e. Water: Trifluoroacetic Acid (1000:1) and solvent B Water: Trifluoroacetic acid: ACN (100:1:899 and at 60°C and UV detection was set 215 nm. Under the proposed conditions the retention time of Filgrastim was about 21 minutes. Linearity was performed using six concentration of Filgrastim solutions in the range of 35-225 µg/ml and the value of correlation co-efficient was found to be R^2 =0.99 that shows good linearity curve. The RSD value for repeatability and reproducibility were 0.1%, 0.23% respectively. The obtained recovery value of 96.6 % indicates that the proposed method is quantitative and accurate.

INTRODUCTION

Filgrastim is granulocyte colony stimulating factor which belongs to the family of cytokines. It is used to treat neutropenia stimulating the bone marrow to increase production of neutrophils. Causes of neutropenia include chemotherapy and bone marrow transplantation. It may also be used to increase white blood cells for gathering during Leukapheresis. It is given either by injection into a vein or under the skin.

Chemical formula of Filgrastim:
$C_{845}H_{1343}N_{223}O_{243}S_9$

Molecular Weight: 18802.8 g/mol.

Since Filgrastim is used in different biologics, it is crucial for the purity of Filgrastim to be known. The main impurities present in Filgrastim are Oxidized and Reduced variants of the protein. It's an alternate method would enable substitution of a complex and time consuming biological assay with a Robust and Precise RP-HPLC method in many practical cases. **The objective of this work is to develop a Simple and Rapid RP-HPLC method as an alternate method for the estimation of Filgrastim in Filgrastim injection.**

Method Development

Chemicals and Reagents:

chemicals were procured from the supplier Trifluoroacetic acid (Sigma Aldrich), Water (HPLC Grade), Filgrastim Reference Standard

Instrumentation: HPLC shimadzu make 2010 C with LC solution software. Column employed in the method was YMC-Pack C_4 (150 X 4.6mm ID S-5 µm, 12nm). [The flow rate was selected 0.8 ml/min and detection was done at 215 nm. The column temperature and run time were 60° C and 40 minutes].

Glasswares: All the glassware used in the study was grade 'A' quality Borosil.

Table 1.0 Chromatographic Conditions

Parameters	Description	
Detector	UV-215nm	
Injection volume	20µl	
Flow Rate	0.8 ml/min	
Temperature	60° C	
Mobile Phase (Gradient Programming)	**Mobile Phase A**	**Mobile Phase B**
	Water : Trifluoroacetic Acid (1000:1)	Water : Trifluoroacetic Acid : ACN (100:1:899)
Diluent	Water	
Run Time	40.0 Minutes	

Gradient Programming

Time (Minutes)	Mobile Phase A	Mobile Phase B
0.01	60	40
30	20	80
35	60	40
40	60	40

Preparation of Standard Solution: Measure Approx. 397µl of 1.7mg/ml of Filgrastim RS and dilute to 3.0ml with diluent to obtained stock solution (Approx. 225ppm) of Filgrastim and dilute 670µl of this to 1.0ml with diluent (20µl of this solution injected into HPLC).

Preparation of Sample Solution: Sample solution was prepared in five replicates by diluting 0.5 ml to 1.0ml with diluent (20µl of this solution injected into HPLC and calculated the % RSD of assay of five replicates which was found to be1.8%).

NOTE: Used freshly prepared Standard and Sample Solutions.

Validation of HPLC Method

Specificity: This parameter was performed to asses that the impurities, degraded products and diluents do not affect the sample analyzed. 20µl of diluent and standard solution were injected into the system and chromatogram recorded. The chromatogram **(Fig-2)** showed two peaks corresponding to oxidized Filgrastim 1 and Oxidized Filgrastim 2 that elute before the principal peak, (the second peak not being completely separated from the principal peak). The peak eluted after the principal peak is of the reduced Filgrastim which is well separated from the principal peak. Specificity of the method was demonstrated by calculating the resolution between two peaks eluted during the run and the tailing factor **(Table-2)**. It was found that the resolution between oxidized product-one and oxidized product two was 5.03, oxidized product two and Filgrastim was 1.73 and Filgrastim and reduced product was 1.99 respectively. The tailing factor for

components was also calculated and found to be less than 2.0.

Table 2.0 System suitability Parameters

S. No.	Components	Retention time (mint.)	rRT	Resolution	Tailing Factor
1	Oxidized Product-1	19.302	0.91	0	0.934
2	Oxidized Product-2	20.65	0.98	5.034	0.518
3	Filgrastim	21.19	1.0	1.727	1.045
4	Reduced Product	21.94	1.04	1.992	0.0

Chromatograms of Study

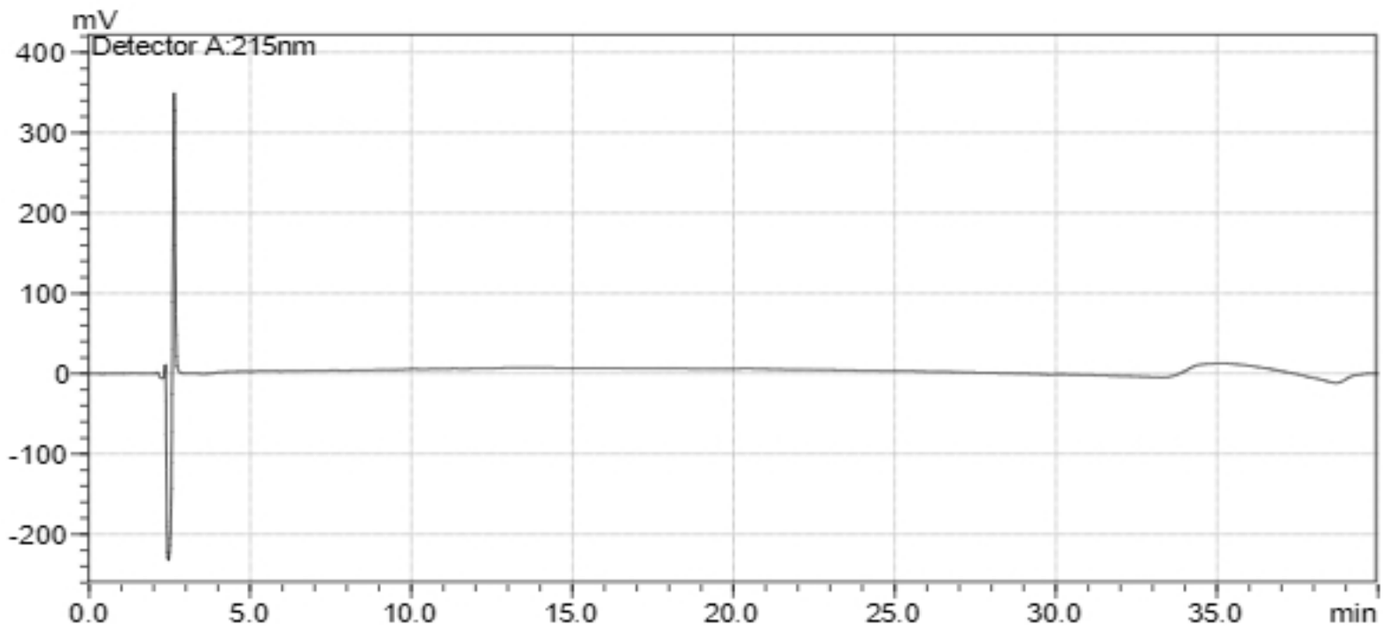


Fig No. -1-Blank Chromatogram

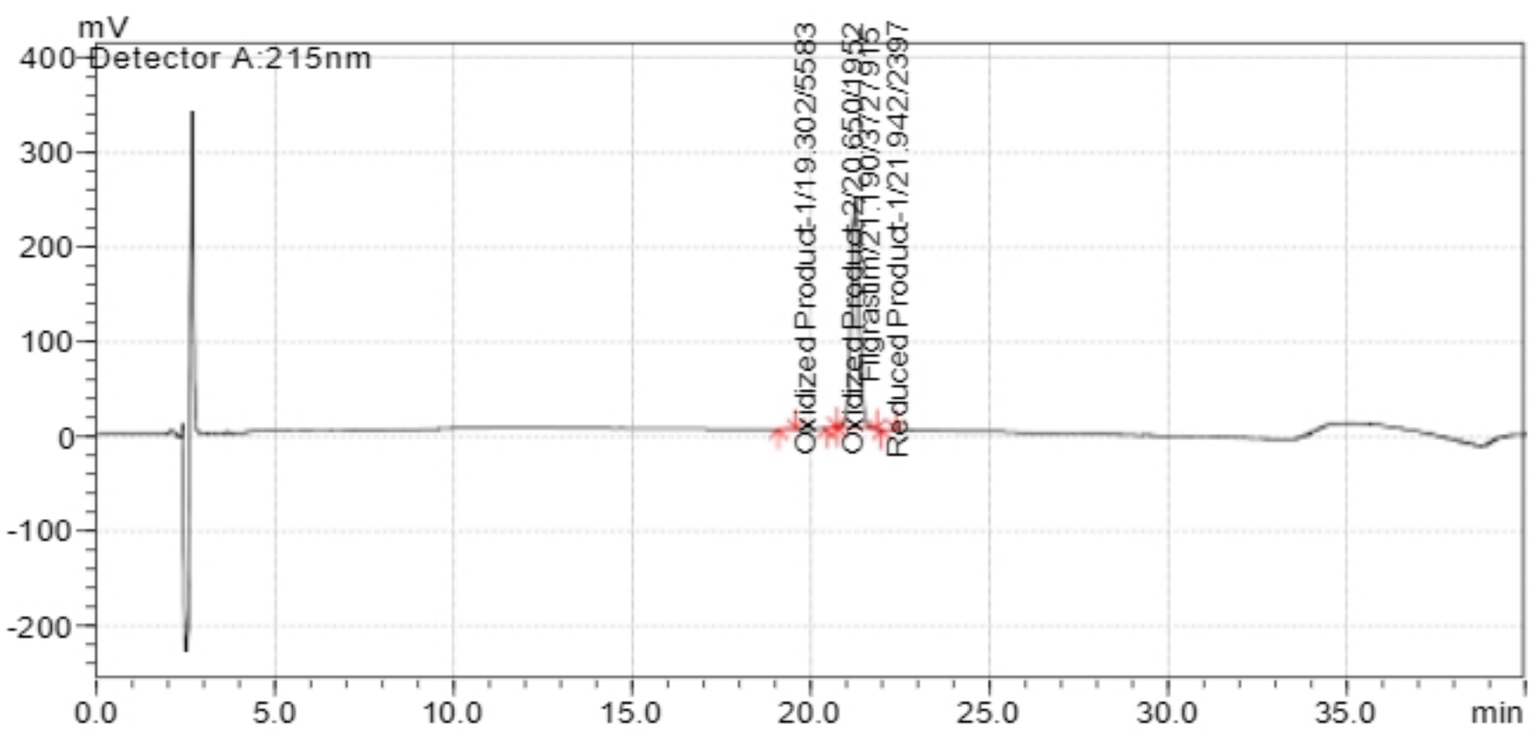


Fig No. -2- Filgrastim RS Chromatogram

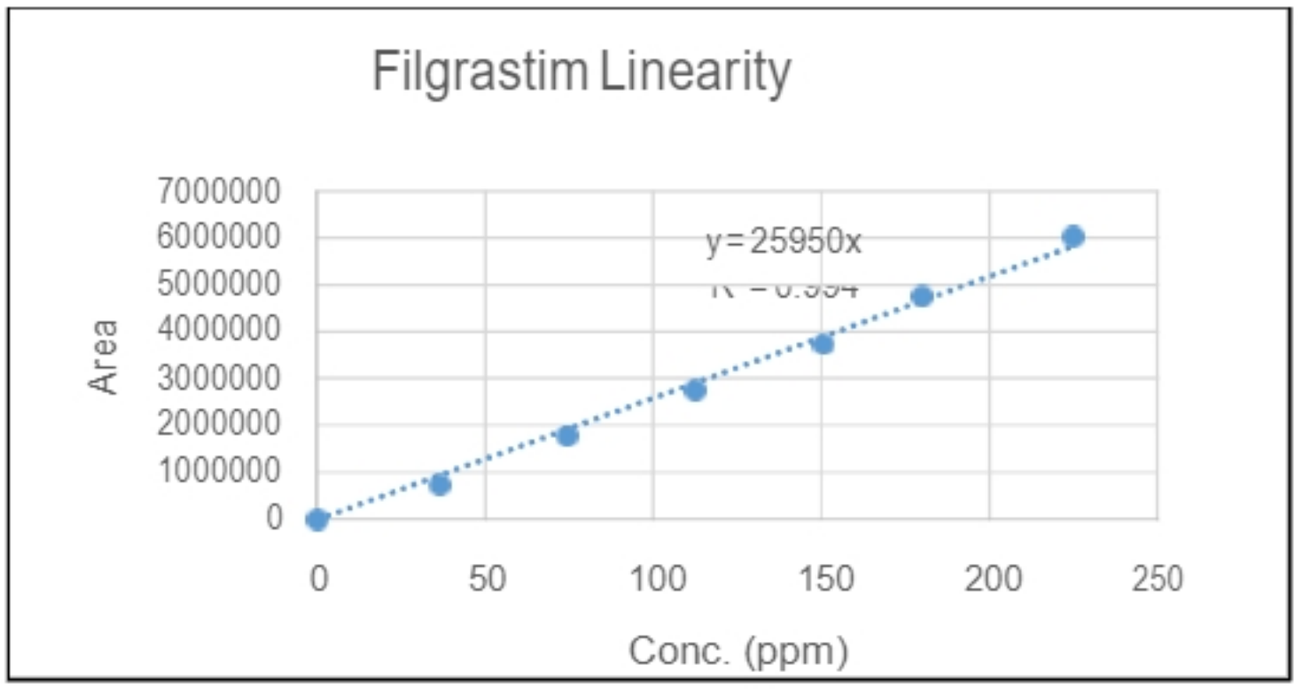
Linearity: The linearity of an analytical method is its ability (within a given range) to obtain test results that are directly proportional to the concentration of analyte in sample. The linearity of method was determined using different conc. of Filgrastim **(Table-3)**. Calibration graph was found to be linear from 35-225 µg/ml for Filgrastim.

Table 3.0 Linearity of Filgrastim

S. No.	Component	Value of x and Y variables							Correlation Coefficient
		Variables	1	2	3	4	5	6	
1.	Filgrastim	X (µg/ml)	36.47	74.24	112.48	150.72	179.97	224.96	
		Y (Area)	741587	1784074	2758075	3740174	4759062	6041656	0.99

X: Conc. of respective component in µg/ml.
Y: Y is the peak response of respective component in Area counts.

Conc.(ppm)	Area
0	0
36.47	741587
74.24	1784074
112.48	2758075
150.72	3740174
179.97	4759062
224.96	6041656



Precision: The precision of the method is the closeness of agreement between the series of measurements under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

The precision was measured on six injections of standard solutions containing Filgrastim i.e. System Precision and the precision of analytical method is the amount of scatter in the result obtained from multiple analysis of a sample is method precision.

Both system precision and method precision were undergone the precision measured in six injection of standard solution in system precision and on six injection of sample solution in method precision.

Table 4.0 Calculation of Coefficient of variance for Filgrastim (Intra-day Precision)

S. No.	Analyte (µg/ml)	No. of Injections						(Average) $\bar{X}$	S.D.	%RSD
		1	2	3	4	5	6			
1.	Filgrastim (150ppm)	3715425	3717528	3713439	3710310	3709763	3708842	3712551	3475	0.1

X represent the average value of six replicate analysis.
SD the standard deviation calculated on the six replicates.
COV is coefficient of variation.

Table 5.0 Calculation of Coefficient of variance for Filgrastim (Inter-day Precision)

Days	Average area
Day-1	3712551
Day-2	3674464
Day-3	3661477
Average	3682831
Standard Deviation	26545
%RSD	0.7

% RSD for intra-day and inter-day precision was found to be 0.1 and 0.7 respectively. This shows that method is precise.

Ruggedness: The Ruggedness of an analytical method is a measure of its capacity to remain unaffected by different analyst on different day. Calculate the % RSD of six injections of a standard solution of Filgrastim.

Accuracy: The accuracy of an analytical method expresses the closeness of agreement between the value that is accepted either as conventional true value or an accepted reference value and the value found. The accuracy of method was determined by calculating percent recovery of known added amount of analyte.

Table-8.0 Ruggedness studies of Filgrastim

Injection	Area
1	3855310
2	3840600
3	3851309
4	3849404
5	3835709
6	3833160
Average	3844249
STDev	9033.01
%RSD	0.23

Table-6.0 Recovery studies of filgrastim at 100% Level

S. No.	Sample Taken	Dilution (ml)	Sample conc.(ppm)	Std. stock conc.(ppm)	Std. volume Spiked (ml)	Expected conc.
1	0.5	1	152	306	0.5	305
2	0.5	1	152	306	0.5	305
3	0.5	1	152	306	0.5	305

Table-7.0 % Recovery studies of Filgrastim at 100% Level

S. No.	Average Standard Area	Test Area	Total Area	Average Test Spiked Area	% Recovery
1	4066384	4035938	8102322	7806064	96.34
2	4066384	4035938	8102322	7829966	96.64
3	4066384	4035938	8102322	9831695	96.66

Average Recovery (%) : 96.55
Standard Deviation : 0.18
%RSD : 0.18

The percent recovery were found to be 96.34 - 96.66% which is well within acceptance criteria.

Limit if Detection (LOD) and limit of Quantitation (LOQ): The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not quantitated as an exact value.

The detection limit of Filgrastim was found to be 10µg/ml.

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

The Quantitation limit of Filgrastim was found to be 30µg/ml.

NOTE: % RSD of LOD & LOQ for filgrastim was found to be not more than 33.0 % and 10% respectively.

Discussion: A chromatographic parameters were fixed and HPLC system was studied for suitability of drug analysis. The developed method was performed for Specificity, linearity, Precision, Accuracy, Ruggedness, LOD & LOQ. The goal of this study to made an alternative method due to complexity and time consuming biological assay.

Conclusion: The present study was carried out to develop a sensitive, precise and accurate HPLC method for the analysis of Filgrastim in injectable. In order to develop a method under gradient conditions, solvent A i.e. Water: trifluoroacetic Acid (1000:1) and solvent B i.e. Water: trifluoroacetic acid: ACN (100:1:899) in different ratio were tested as mobile phase on YMC-Pack C4 (150 X 4.6mm ID S-5 µm, 12nm column. The retention time obtained for Filgrastim 21.19 minutes. In order to test the linearity of this method, six dilutions of standard solution in the range of 35 µg/ml to 225 µg/ml were prepared. The method was validated by evaluation of the required parameters. The Filgrastim content in the formulation was quantified by using the proposed analytical method. A low limit of % RSD indicates the reproducibility of the method in formulation. Based on these results a conclusion was made that RP-HPLC could be used as an alternate method for the estimation of Filgrastim injections samples.

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