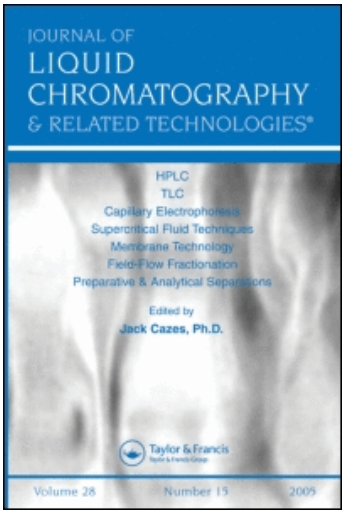

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Validation of an SEC-HPLC Method for the Analysis of rhG-CSF in Pharmaceutical Formulations

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ABSTRACT

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic cytokine produced by recombinant DNA technology and used clinically to treat neutropenia. An isocratic high performance liquid chromatography procedure was developed for the assay of filgrastim in pharmaceutical formulations. HPLC separation was carried out by size-exclusion chromatography on a TSK gel G2000 SW column (60 cm × 7.5 mm I.D.). The mobile phase was composed of phosphoric acid (pH 2.5;

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0.1 M), run at a flow rate of 1.0 mL min^{-1} and with UV detection at 214 nm. Method validation investigated parameters such as the range, linearity ($r^2 = 0.9998$), precision, accuracy, and robustness; the method yielded good results with a quantitation limit of 45 μg mL^{-1} and a detection limit of 12 μg mL^{-1}. The results demonstrate the validity of the SEC-HPLC method for the analysis of filgrastim.

Key Words: Recombinant granulocyte colony-stimulating factor; Size-exclusion chromatography; Validation; Quality control.

INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is a member of the hematopoietic colony-stimulating factors family.[1] Recombinant human G-CSF (rhG-CSF) is produced through genetic recombination in two forms: non-glycosylated and glycosylated. The non-glycosylated form of the hydrophobic protein derived from expression in *E. coli*, consists of 175 amino acids and contains an extra methionine at its N-terminus.[2,3] The protein has been used clinically to increase neutrophils at the time of bone marrow transplantation and to promote the recovery of neutrophil count in various conditions, including neutropenia due to anticancer chemotherapy and neutropenia associated with myelodysplastic syndrome or aplastic anemia.[4,5]

The biological activity of rhG-CSF can be assessed by an in vivo assay based on the evaluation of the leukocyte numbers after rhG-CSF administration to cyclophosphamide-treated mice.[6] The in vitro cell line assays with NFS-60 or GNFS-60 cells have been used to evaluate the proliferation-inducing activity of the G-CSF molecule.[7–9] Also, a specific enzyme-linked immunosorbent assay has been developed for the measurement of murine G-CSF in plasma and biological fluids, providing a valuable research tool.[10]

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. Among these techniques, high-pressure liquid chromatography (HPLC) has proven to be particularly useful.[4,11,12] Size-exclusion HPLC (SEC-HPLC) was found to be an appropriate technique since both covalent and non-covalent dimers are separated from monomers. The chromatographic behavior of some recombinantly produced proteins and aggregated species has been studied by SEC-HPLC, at neutral pH and below pH 5, to assess the suitability of the system in revealing protein degradation and aggregate formation, and to demonstrate the advantages of using eluent at low pH for stability studies.[13] In the case of G-CSF, SEC-HPLC has also been used to analyze the effect of pH on the polymerization of

intact and deglycosylated forms of the molecule, showing that the hG-CSF was rapidly inactivated at pH 7–8.[14] In addition, the stability of rhG-CSF during nebulization has been examined using SEC-HPLC at 4°C, with a mobile phase of sodium phosphate at pH 6.9.[15]

In this paper we report a sensitive SEC-HPLC determination method with UV detection, appropriate for the routine control of non-glycosylated G-CSF (filgrastim) in pharmaceutical formulations. The method was validated for linearity, range, precision, accuracy, robustness, limit of detection (LOD), and limit of quantitation (LOQ).

EXPERIMENTAL

Samples

The filgrastim reference material was an in-house reference preparation generously supplied by the National Institute for Biological Standards and Control (NIBSC), UK. Filgrastim sample preparations containing 300 μg of active substance per milliliter were obtained from commercial sources.

Reagents and Solvents

All chemicals used were of pharmaceutical or special analytical grade. HPLC-grade acetonitrile, sodium hydroxide, and orthophosphoric acid were purchased from Merck (Darmstadt, Germany) and bovine albumin was obtained from Sigma (Steinheim, Germany). For all analyses, double-distilled water filtered through a 0.45-μm membrane filter was used.

Apparatus and Chromatographic Conditions

A Shimadzu HPLC system (Shimadzu, Kyoto, Japan) was used, equipped with an SCL-10A_{VP} system controller, LC-10 AD_{VP} pump, DGU-14A degasser, SIL-10AD_{VP} autosampler, and an SPD-M10A_{VP} photodiode array (PDA) detector. The detector was set at 214 nm and peak areas were integrated automatically by computer, using a Shimadzu Class VP® software program. The experiments were carried out on a size exclusion TosoHaas (Montgomeryville, PA) TSK gel G2000 SW column (60 cm × 7.5 mm I.D., with a particle size of 10 μm and pore size of 125 Å). A TSK gel SW Guard column (7.5 cm × 7.5 mm I.D.) was used to protect the analytical column. The HPLC system was operated isocratically at ambient temperature, using a

mobile phase of phosphoric acid (pH 2.5; 0.1 M) adjusted by the addition of sodium hydroxide (10 N). This was filtered through a 0.45-μm membrane filter (Millipore), degassed with a DGU-14A degasser, and run at a flow rate of 1.0 mL min^{-1}. At the beginning of each experiment, 50 μL bovine albumin (1 mg mL^{-1}) was injected onto the column to reduce non-specific adsorption. The injection volume was 50 μL for both standards and samples, and all determinations were carried out in triplicate.

Procedure

Filgrastim Samples and Standard Solutions

Working standard and sample solutions of filgrastim were prepared daily by diluting to an appropriate concentration in water.

Validation of the Method

The method was validated by the determination of the following parameters: linearity, range, precision, accuracy, robustness, LOD, and LOQ, following the ICH guidelines.[16]

Linearity and Range

Linearity was determined by constructing three calibration curves. For the construction of each calibration curve, five standard concentrations of filgrastim in the range of 100–300 μg mL^{-1} were prepared. Before injection of the solutions, the column was equilibrated for atleast 45 min with the mobile phase flowing through the system. Triplicate 50 μL injections were made for the standard solution to verify the reproducibility of the detector response at each concentration.

The peak area of the chromatogram was plotted against the concentration of filgrastim to obtain the calibration curve. The five concentrations of the standard solution were subjected to regression analysis to calculate calibration equation and correlation coefficients.

Precision

The precision of the method was determined by repeatability and intermediate precision. Repeatability was examined by six determinations of

filgrastim samples at the same concentration, on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on two different days (inter-day) and also by another analyst performing the analysis in the same laboratory (between-analysts).

Accuracy

To confirm the accuracy of the proposed method, a total of nine determinations were performed with a minimum of three concentration levels covering the specified range.

Robustness

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters. The robustness of the method was assessed by altering experimental conditions, for example, by changing the flow rate to 0.6 and 1.2 mL min^{-1}, the pH of the mobile phase to 2.3 and 5.0, and the wavelength in the range of 200–280 nm. The higher molecular weight aggregates and dimers were evaluated in the samples after mechanical agitation lasting 5 min.

LOQ and LOD

The LOQ, taken as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy, and the LOD, taken as the lowest absolute concentration of analyte in a sample that can be detected but not necessarily quantified, were calculated based on the ICH guidelines.[16]

RESULTS AND DISCUSSION

The calibration curves for filgrastim were constructed by plotting the area of the peaks vs. concentration; this was found to be linear in the 100–300 $\mu g\ mL^{-1}$ range. This concentration range corresponds to approximately 50–150% of the nominal sample active concentration. A linear regression by the least squares method was then applied. The high value of the determination coefficient ($r^2 = 0.9998$) indicated good linearity of the calibration curve for the method.

The precision evaluated as the repeatability of the method, was studied by calculating the relative standard deviation (RSD) for six determinations at the same concentration, performed on the same day, and under the same experimental conditions. The RSD was 0.16%. The results of filgrastim determination in the pharmaceutical formulation are shown in Table 1.

Table 1. G-CSF concentrations obtained from six determinations by SEC-HPLC to show method repeatability.

Sample	Theoretical amount ($\mu g\,mL^{-1}$)	Experimental amount ($\mu g\,mL^{-1}$)	Purity[a] (%)	RSD (%)
1	300	309.1		
2	300	308.5		
3	300	309.5	103.1	0.16
4	300	309.8		
5	300	309.7		
6	300	308.9		

[a]Mean of six determinations.

The intermediate precision was assessed by analyzing two working solutions on two different days (inter-day, Table 2); the RSD values obtained were 0.41% and 0.54%, respectively. Between-analysts precision was determined by calculating the RSD for the analysis of two working solutions by two analysts; the values were found to be 0.56% and 0.96%, respectively (Table 3).

The accuracy was assessed from triplicate determinations of three different solutions containing 140, 200, and 260 $\mu g\,mL^{-1}$. The absolute means obtained were 103.1%, 102.7%, and 105.4% respectively, with a mean value of 103.73% and RSD of 0.28% as shown in Table 4.

The robustness of the method was determined by analyzing the same samples under a variety of conditions. The factors considered were: variations in the flow rate, in the pH of the mobile phase, and in wavelength. The experimental range of the selected variables is given in Table 5.

Table 2. Between-day precision data of SEC-HPLC for filgrastim in samples of pharmaceutical formulations.

Sample	Day	Purity[a] (%)	Mean[b]	RSD (%)
1	1	103.8	103.5	0.41
	2	103.2		
2	1	102.9	103.3	0.54
	2	104.0		

[a]Mean of three replicates.
[b]Mean of two days.

Table 3. Between-analyst precision data of SEC-HPLC for filgrastim in samples of pharmaceutical formulations.

Sample	Analyst	Purity[a] (%)	Mean[b]	RSD (%)
1	A	104.2	103.8	0.56
	B	103.4		
2	A	103.8	103.1	0.96
	B	102.4		

[a]Mean of three replicates.
[b]Mean of two analysts.

The higher molecular aggregates and dimeric forms were analyzed as shown in Fig. 1, demonstrating that the method is able to detect and separate the dimers, related substances of higher molecular mass, and the intact protein.

There were no significant changes in the chromatographic pattern when the above modifications were made in experimental conditions, thus showing the method to be robust.

The LOD and LOQ were obtained from the slope and the standard deviation of the intercept from three calibration curves, determined by a linear regression line as defined by ICH. The LOD and LOQ were found to be 12 and 45 $\mu g\,mL^{-1}$, respectively.

Table 4. Accuracy of SEC-HPLC for filgrastim.

Theoretical amount ($\mu g\,mL^{-1}$)	Experimental amount[a] ($\mu g\,mL^{-1}$)	Accuracy[b] (%)	RSD (%)
140	144.34		
200	205.40	103.73	0.28
260	274.04		

[a]Mean of three replicate analyses.
[b]Accuracy: obtained concentration expressed as % of the nominal concentration.

Table 5. Chromatographic conditions and range investigated during robustness testing.

Variable	Optimized value	Range investigated
Flow rate ($mL\,min^{-1}$)	1.0	0.6–1.2
pH	2.5	2.3–5.0
Wavelength (nm)	214	200–280

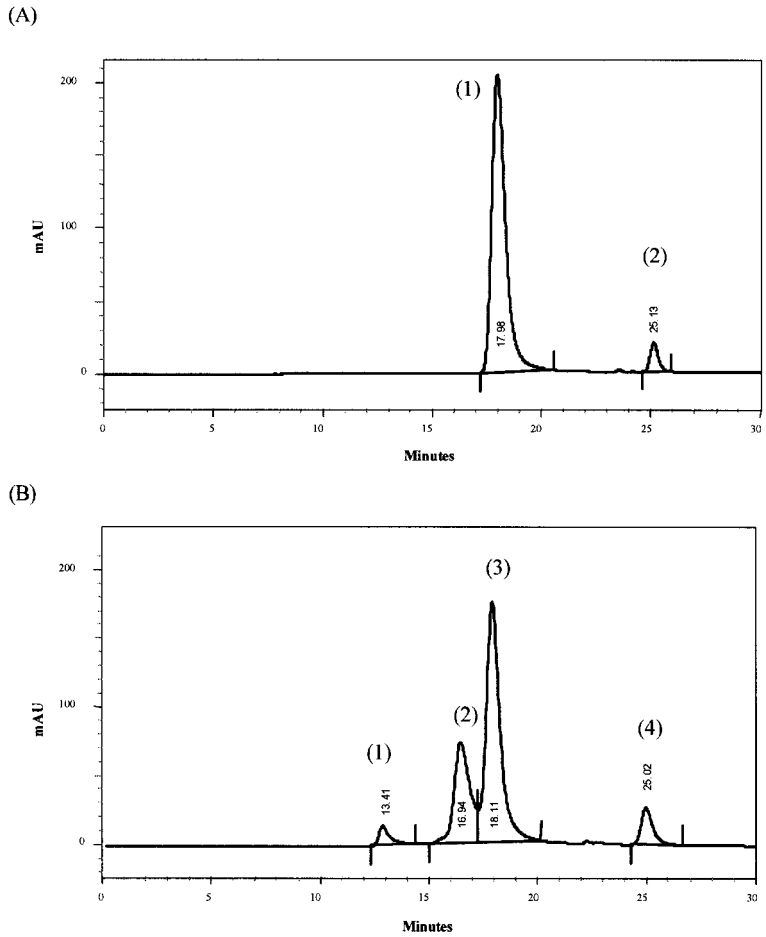

Figure 1. SEC-HPLC chromatograms of filgrastim: (A) Reference substance (peak 1: monomer and peak 2: excipient). (B) Degraded pharmaceutical sample (peak 1: aggregates, peak 2: dimer, peak 3: monomer, and peak 4: excipient).

CONCLUSION

The data validation shows that the proposed method is accurate and robust and possesses excellent linearity and precision characteristics. This SEC-HPLC method has been successfully used on a routine basis and allows the quantification of the drug in pharmaceutical formulations.

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