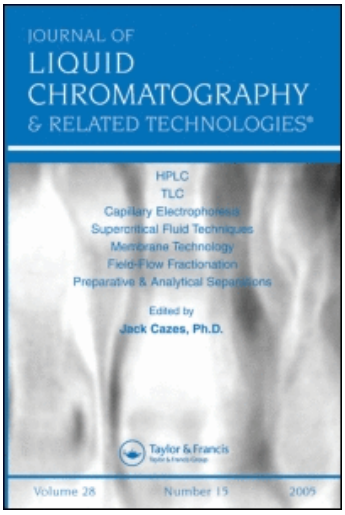

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Tonic-Ribarska, Jasmina , Brezovska, Katerina and Trajkovic-Jolevska, Suzana(2009) 'Development and Validation of an SEC-HPLC Method for the Analysis of *Lenogratsim* (rHuG-CSF) in Pharmaceutical Formulations', Journal of Liquid Chromatography & Related Technologies, 32: 17, 2545 — 2555
To link to this Article: DOI: 10.1080/10826070903249716
URL: http://dx.doi.org/10.1080/10826070903249716

Journal of Liquid Chromatography & Related Technologies®, 32: 2545–2555, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070903249716

Development and Validation of an SEC-HPLC Method for the Analysis of *Lenogratsim* (rHuG-CSF) in Pharmaceutical Formulations

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Abstract: A simple and sensitive size exclusion HPLC method was developed and validated for the analysis of lenograstim (rHuG-CSF) in pharmaceutical formulations. Analyses were performed on a Waters HPLC system employing a Fractogel® EMD BioSEC column and isocratic elution with phosphoric acid (pH 2.5; 0.1 M) containing 150 mmol L^{-1} NaCl at a flow rate of 2.0 mL min^{-1}. The validation results demonstrate that the proposed method is suitable for determination of lenogratsim in pharmaceutical formulations. Furthermore, the proposed SEC-HPLC method was able to separate and detect the aggregates and the intact protein.

Keywords: Aggregates, Lenograstim, Pharmaceutical formulations, Size-exclusion HPLC, Validation

INTRODUCTION

Human granulocyte colony–stimulating factor (HuG-CSF) is one of the hematopoietic growth factors which plays an important role in hematopoietic cell proliferation, differentiation of hemopoietic precursor cells,

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and activation of mature neutrophilic granulocytes (Herman, 1996). With the recent increased understanding of its biological properties *in vivo* together with available preparations of highly purified recombinant human G-CSF, this growth factor has become an essential agent for clinical applications. It is widely used after high dose chemotherapy regimes or bone marrow transplants, to correct low neutrophil levels in the blood and, thereby, reduces the duration and severity of the neutropenia.[1–3]

Two forms of rhG-CSF are currently commercially available for medical treatment. One is non-glycosylated protein, called *filgrastim*, expressed from engineered *Escherichia coli* cells. The other is glycosylated protein derived from Chinese hamster ovary (CHO) cells, called *lenograstim*. It is a 19.6 kDa glycoprotein consisting of 174 amino acids and possesses an *O*-linked carbohydrate chain attached to threonine-133 of the molecule. Lenograstim is indistinguishable in structure and activity from natural human (endogenous) G-CSF isolated from the CHU2 cell line.[1,4]

Glycosylation has been reported to confer many advantages over non-glycosylation, such as greater physical stability and higher biological and pharmacological potency. This has been shown in tests of the proliferation of bone marrow cells *in vitro*,[5] of neutrophil induction in laboratory animals[6] and of mobilization of stem cells in blood of humans.[7,8]

Unlike traditional synthetic pharmaceuticals, recombinant DNA derived pharmaceutical proteins are very difficult to be well characterized by ordinary analytical techniques used for small chemical molecules.[9,10]

SEC-HPLC was found to be an appropriate technique for the analysis of recombinant proteins. Also, it is one of the most sensitive and selective techniques that can separate and analyze aggregates and monomers.[11–13]

A number of published studies referring to analysis of filgrastim (non-glycosylated form of rHuG-CSF), as a first recombinant form of G-CSF, are available in the literature.[14–19]

The aim of our study was to develop a simple and sensitive SEC-HPLC method with UV detection for identification and determination of lenograstim (glycosylated form of rHuG-CSF) in pharmaceutical formulations and to validate the method following the ICH guidelines.[20]

EXPERIMENTAL

Chemicals and Reagents

The lenograstim (recombinant human granulocyte–colony stimulating factor derived from Chinese hamster ovary cells (rHuG-CSF)) reference

material was supplied by Ray Biotech (USA). Lenograstim sample preparation containing 263 μg per mL was obtained from commercial sources.

All chemicals were of HPLC grade or analytical grade. Orthophosphoric acid and sodium chloride were purchased from Alkaloid (Macedonia). For all analyses HPLC grade water was used.

Preparation of Standard and Sample Solutions of Lenograstim

The standard and sample solutions of lenograstim were prepared daily by dissolving in water with concentration of 50 μg mL^{-1}.

Preparation of Sample Solution with Induced Aggregates

The aggregates were artificially produced after incubation under conditions that do not differ greatly from physiological. The sample solution with induced aggregates was prepared by dissolving the lenograstim in phosphate buffered saline pH 6.9 at the same concentration as standard solution (50 μg mL^{-1}) and incubated 5 days at 37°C on a heating block.

Linearity and Range

The calibration curve was constructed with five standard concentrations of lenograstim in the range of 12.5–100 μg mL^{-1}.

Precision

Repeatability of the method was determined by performing the analytical procedure six times using the samples at 100% of the working concentration (50 μg mL^{-1}), on the same day, under the same experimental conditions (intra-day). The intermediate precision was assessed by comparing the results obtained from six quantitative determinations at the same concentration (50 μg mL^{-1}), on three different days (inter-day).

Accuracy

The accuracy of the proposed method was assessed by performing a total of nine determinations of three concentration levels covering the specified range (25 μg mL^{-1}, 50 μg mL^{-1}, and 75 μg mL^{-1}).

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

The LOQ and LOD were obtained from the slope of the calibration curve and the standard deviation of the response, determined by a linear regression line as defined by the ICH guideline.[20]

Robustness

The robustness of the SEC-HPLC method performance was evaluated after slightly varying the experimental conditions from optimized values (flow rate, composition of mobile phase, and wavelength).

System Suitability

To ensure the validity of the analytical procedure, data from six injections of the working standard solution containing 50 μg mL^{-1} lenogratsim were used for the evaluation of the system suitability parameters, such as symmetry factor, area, and retention time.

Apparatus and Chromatographic Conditions

A Waters HPLC system was used equipped with a Waters 600 pump, Waters 996 photodiode array detector and Millennium 32® software for data handling.

The experiments were carried out on size exclusion Fractogel® EMD BioSEC column, superformance 600 – 16 mm (Merck, Germany). The HPLC system was operated isocratically at ambient temperature using a mobile phase, which was composed of phosphoric acid (0.1 mol L^{-1}; pH 2.5) containing 150 mmol L^{-1} NaCl, run at a flow rate of 2 mL min^{-1}, and with UV detection at 215 nm. The mobile phase was filtered using a 0.45 μm filter and was degassed before use. Samples were injected through a Rheodyne injector valve with 200 μL sample loop.

RESULTS AND DISCUSSION

In this study, a SEC-HPLC method for identification and determination of lenograstim (glycosylated form of rHuG-CSF) in pharmaceutical formulations has been developed and validated.

In contrast to other modes of chromatography where the mobile phase is an active participant in the separation process, the mobile phase

in SEC is simply a carrier transporting analytes through the column. In principle, the mobile phase chemistry in SEC is designed to keep the protein in solution and, most important, to keep the protein in the appropriate (native) conformation.[12]

In order to establish the chromatographic conditions (composition and pH of mobile phase and flow rate) a number of literature data were reviewed.

The conformation and thermal unfolding of human (endogenous) G-CSF were examined at pH 2.0, 4.0, and 7.0 using circular dichroism (CD) and Fourier transform infrared spectroscopy (FTIR). The results have shown that G-CSF maintains both thermal stability and tertiary structure at pH 2.0.[21] The chromatographic behaviour of some recombinant proteins and aggregated species has been studied using size exclusion chromatography (SEC), at neutral pH and at pH below 5. At neutral pH values, monomeric proteins exhibited non-ideal behaviour while aggregated species were not eluted.[22] It was found that the most convenient elution conditions for the simultaneous chromatography of monomeric and aggregated proteins were low pH values.[16,22,23]

To avoid non-specific interactions between the proteins and the matrix the mobile phase should contain 50 mM to 300 mM NaCl. Very high concentrations of salt are not suitable since protein precipitation may occur.[12,24]

High performance SEC columns are usually operated at modest flow rates, e.g., 1 mL min^{-1}.[12] However, the mechanical stability of Fractogel® EMD BioSEC allows operation at higher flow rates, e.g., 2.6 mL min^{-1} (vendor's protocol), reducing the time of analysis without loss of resolution.

According to the literature data and after a lot of experiments, the satisfactory results were obtained using a mobile phase composed of phosphoric acid (pH 2.5; 0.1 mol L^{-1}) containing 150 mmol L^{-1} NaCl, run at a flow rate of 2 mL min^{-1}.

The chromatograms of standard solution (a) and sample solution (b) of lenograstim obtained under the proposed chromatographic conditions are presented in Figure 1.

Once the chromatographic and the experimental conditions were established, the method was validated by the determination of the following parameters: linearity, range, precision, accuracy, limit of quantitation, limit of detection, and robustness following the ICH guidelines.[20]

The calibration curve was constructed by plotting the area of the peaks versus concentration. Linearity was observed in a concentration range from 12.5 μg mL^{-1} to 100 μg mL^{-1}. A linear regression by the least squares method was applied ($a = 10567.14$; $b = 6012.41$; $r^2 = 0.9992$). The value of the determination coefficient indicated significant linearity of the method.

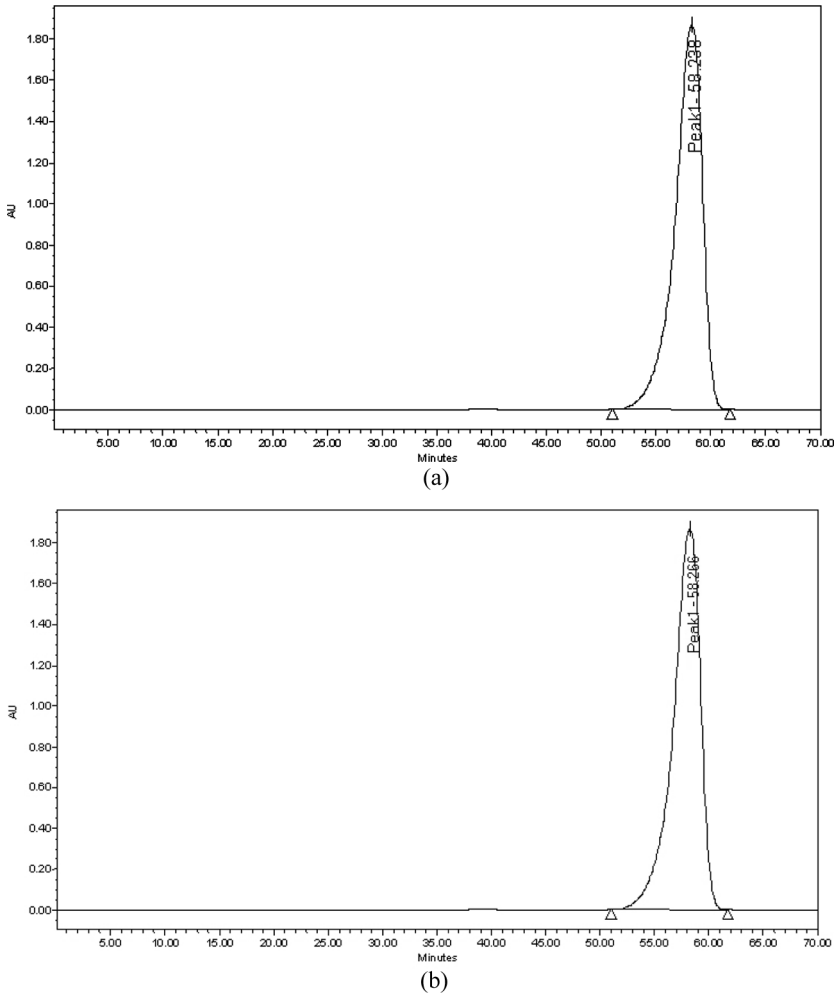

Figure 1. SEC-HPLC chromatograms of: (a) Standard solution of lenograstim (50 μg mL^{-1}); (b) Sample solution of lenograstim (50 μg mL^{-1}).

The precision of the analytical method was studied as the repeatability (intra-day precision) and the intermediate precision (inter-day precision).

The obtained RSD value for repeatability was 0.50% and for intermediate precision was 0.54% as shown in Table 1.

The accuracy was confirmed from triplicate determinations of three different solutions containing 25, 50, and 75 μg mL^{-1} lenograstim. The results are shown in Table 2.

Table 1. Repeatability and inter-day precision data of SEC-HPLC for lenogratsim

	Theoretical ($\mu g\,mL^{-1}$)	Determined ($\mu g\,mL^{-1}$)	SD	RSD (%)	P = 95%
Intra-day (n = 6)	263	263.43	1.32	0.50	263.43 ± 1.39%
Inter-day (n = 18)	263	264.52	1.45	0.54	264.52 ± 3.59%

Table 2. Accuracy of SEC-HPLC for lenogratsim

Theoretical ($\mu g\,mL^{-1}$)	Determined ($\mu g\,mL^{-1}$)	Determined* ($\mu g\,mL^{-1}$)	Recovery (%)
25	25.45	267.73	101.80
50	50.08	263.42	100.16
75	74.01	259.53	98.68

n = 3.
*expressed as nominal concentration.

The obtained recovery values of 101.80%, 100.16%, and 98.68%, respectively, indicate that the proposed SEC-HPLC method is quantitative and accurate within the investigated range.

The LOQ and LOD were found to be 10.03 $\mu g\ mL^{-1}$ and 3.31 $\mu g\ mL^{-1}$, respectively, indicating that the method is sensitive.

The robustness of the method was determined by analyzing the same samples under slightly varying the experimental conditions from optimized values (Table 3).

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

Results of system suitability tests are given in Table 4, showing that the system is suitable for the analysis to be performed.

A current concern in the administration of recombinant derived proteins is that the presence of aggregates may lead to loss of efficacy and

Table 3. Robustness testing

Variable	Optimized value	Investigated value
Flow rate mL min^{-1}	2.0	1.8–2.2
Composition of mobile phase	0.1 M H_3PO_4 + 150 mM NaCl	0.1 M H_3PO_4 + 100 mM NaCl; 0.1 M H_3PO_4 + 150 mM NaCl
Wavelength nm	215	210–220

Table 4. Results of the system suitability test

Parameter	Results	RSD (%)
Symmetry factor	0.93	0.32
Retention time	58.23	0.03
Area	316957.41	0.49

n = 6.

may cause adverse effects within patients.[15,25] The presence of aggregates of any type (soluble/insoluble, covalent/noncovalent, and reversible/irreversible) is typically considered to be undesirable. However, the levels of soluble aggregates, such as dimers and trimers that are acceptable are not well defined and depend on case to case.[26]

The possibility of the proposed SEC-HPLC method for determination of lenograstim to be applied for separation and detection of aggregates from intact protein was examined. The aggregates in the samples were induced after incubation during 5 days under physiological conditions (phosphate buffered saline pH 6.9 at 37°C).[23]

For the determination of the aggregates any peak from the chromatographic run, which was eluted before the intact monomer is defined as aggregates.[11,12]

The chromatogram of sample solution with induced aggregates presented in Figure 2, has shown, that under the proposed chromatographic conditions monomeric protein and aggregate are completely separated one from each other in accordance with their molecular masses. The

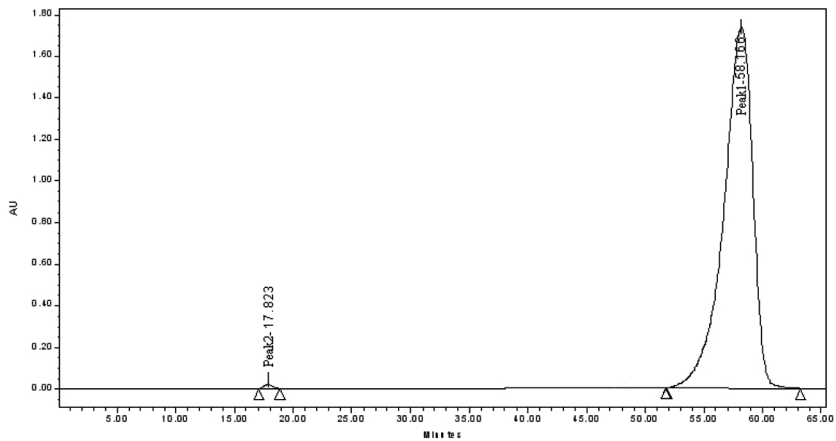

Figure 2. SEC-HPLC chromatogram sample solution of lenograstim with induced aggregates (peak 1–intact protein, monomer; peak 2-aggregate).

obtained retention times were approximately 58.17 min for intact protein and 17.82 min for aggregate.

The results demonstrated that the proposed SEC-HPLC method was able to separate and detect the aggregates and the intact protein and can be used for the investigation of the purity of lenograstim in pharmaceutical formulations.

CONCLUSION

In this study, a simple and sensitive SEC-HPLC method for identification and quantification of lenograstim in pharmaceutical formulations was developed. The validation data shows that the proposed method is linear, precise, accurate, and robust, and can readily be used in the routine analysis of lenograstim in pharmaceutical formulations.

The method was successfully applied to separate and detect the aggregates and the intact protein–lenograstim.

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Received April 8, 2009
Accepted April 16, 2009
Manuscript 6520