New High Performance Liquid Chromatography Method For Analysis Of Filgrastim In Pharmaceutical Formulations

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Illustrations
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Abstract

Filgrastim is a human granulocyte colony-stimulating factor, which belongs to a family of cytokines. It is produced by recombinant DNA technology in genetically engineered Escherichia coli cells. Filgrastim has a biological action essential for proliferation and differentiation of hematopoietic and progenitor cells. It is used for decreasing the risk of infection in cancer patients, who receive chemotherapy or a bone marrow transplant.

The goal of this study was to develop and validate a simple and rapid RP-HPLC method for determination of filgrastim in protein formulations. The experiments were carried out by RP-HPLC using LiChrospher® WP 300 RP-18e, (150 x 4 mm i.d.) column. The HPLC system was operated at gradient mode using mobile phase composed of solvent A (0.1 % TFA in a mixture of water : acetonitril = 90:10 v/v) and solvent B (0.08 % TFA in a mixture of water: acetonitril = 10:90 v/v). The temperature was 50°C and UV detection was set at 215 nm. The method was validated according the ICH guidelines. Under the proposed chromatographic conditions, the retention time for Filgrastim was about 25 min. Good linearity of the method was confirmed by the high value of the correlation coefficient (r² = 0.9995), over a concentration range from 32 - 256 µg/ml. The RSD values for repeatability and inter-day precision were 0.45 % and 0.48 %, respectively. The obtained recovery values of 99.30 -101.38% indicate that the proposed method is quantitative and accurate. In conclusion, the RP-HPLC method used for determination of filgrastim complies with the requirements of the validation parameters, justifying its purpose for routine analysis.

Introduction

Filgrastim is a human granulocyte colony-stimulating factor (G-CSF), which belongs to a family of cytokines. Its essential action is to stimulate the production of neutrophils, thus range filgrastim among drugs called hematopoietic (blood-forming) agents and it is used for decreasing the risk of infection in cancer patients, who receive chemotherapy or a bone marrow transplant. [1-5]. Filgrastim is a 175 amino-acid polypeptide produced by recombinant DNA technology in genetically engineered Escherichia coli cells. Filgrastim differs from the natural hormone in that the former is not glycosylated and contains an additional methionine group at the N terminus, which is necessary for expression of the gene in E. coli [6,7]. Filgrastim contains four methionine residues: Met1, Met122, Met127 and Met138, which are susceptible to oxidation. Hydrogen peroxide induces oxidation of methionine residues to methionine sulfoxide, which leads to the inactivity of protein pharmaceuticals [8-18].

Experimental

Chemicals and reagents

Filgrastim was obtained from Roche products. All reagents used were of HPLC grade or analytical grade. Trifluoroacetic acid (TFA) p.a. and acetonitril were purchased from Merck, Germany. Hydrogen peroxide was purchased from Alkaloid, Skopje. HPLC grade water was used through the study.

Apparatus and chromatographic conditions

Varian HPLC system was used, equipped with Varian Prostar 240 pump, Varian 325 LC Detector and Galaxie1.9.301.220 software for data handling. The experiments were carried out on Lichrospher® WP 300 rp-18e, (150 x 4 mm i.d.) column. The HPLC system was operated at gradient mode using mobile phase composed of solvent A (0.1 % TFA in a mixture of water : acetonitril = 90:10) and solvent B (0.08 % TFA in a mixture of acetonitril : water = 90:10). The column was initially equilibrated with 40 % B to 60 % B over 10 minutes at a flow rate of 1 ml/min and from 60 % B to 70 % B over 20 minutes at a flow rate of 0.6 ml/min. Gradient mode is presented in Illustration 1.
The temperature was 50°C and the UV detection was set at 215 nm.

Preparation of standard and sample solutions of Filgrastim
The standard and sample solution of filgrastim were prepared by dissolving with water to the working concentration of 128 µg/ml.
The calibration curve was constructed with five standard concentrations of filgrastim in the range from 32-256 µg/ml.
The sample solution intended for induced oxidation was prepared by dissolving filgrastim with 0.1% hydrogen peroxide solution to the same concentration as a standard solution (128 µg/ml).

Results and discussion

A simple and rapid RP-HPLC method for determination of filgrastim was developed and validated. Good linearity, repeatability and accuracy were achieved.

Under the proposed chromatographic conditions, the retention time for filgrastim was about 25 min (Illustration 2a and 2b)

Validation parameters
Linearity was performed using five concentrations of filgrastim solution in the range from 32 - 256 µg/ml. The calibration curve was constructed by plotting the area of the peaks against concentration. A linear regression by the least squares method was applied. Good linearity of the method was confirmed by the high value of the correlation coefficient (a = 105.65; b = -913.42; r² = 0.9995).

Precision is confirmed as repeatability (intra-day precision) and inter-day precision. The repeatability is determined by six replicate injections of the same sample of standard solution of filgrastim in concentration of 128 µg/ml on the same day, under the same experimental condition. The RSD (%) of peak areas and also the RSD (%) of obtained concentrations were calculated (Illustration 3).

Inter-day precision of the RP-HPLC method was determined in six days by three replicate injections of standard solution of filgrastim in concentration of 128 µg/ml each day. The RSD values (%) for inter-day precision were 0.48% and 0.45% calculated through peak areas and concentrations(µg/ml), respectively (Illustration 4).

The accuracy of the method was determined by five concentration levels recovery study of the filgrastim, with repeatability of three injections at each concentration level. The obtained recovery of 99.30 -101.38% indicates that the proposed method is quantitative and accurate (Illustration 5).

Detection limit and quantification limit were calculated using the data from regression analyses of linear correlation between concentrations and areas under peaks [19-21]. The slope from calibration curve and residual standard deviation of a regression line were used to calculate LOD and LOQ.

Detection limit and quantification limit were found to be 8.03 µg/ml and 24.35 µg/ml, respectively.

Oxidation of Filgrastim
One of the main problems of protein drugs is their instability, both chemical and physical. Chemical instability of filgrastim, among others, is due to the presence of four methionine residues susceptible to oxidation and quality control of filgrastim always include investigation of the presence of oxidized forms. For that reason, the proposed RP-HPLC was applied for the analysis of samples of filgrastim with induced oxidation by adding 0.1 % hydrogen peroxide solution.

Representative chromatograms of filgrastim and filgrastim with specific oxidized forms occurred in definite critical time points were presented (Illustration 6. a-c). The chromatograms have shown that different oxidized forms are detected and satisfactorily separated, which allow us to assume that the method was shown to be suitable for following the oxidized forms of filgrastim. Further investigations are to be carried out in order to study the oxidation of methionine residues of Filgrastim in definite time intervals.

Conclusion

Although there are many HPLC methods applicable for determination of proteins in pharmaceutical preparations, in this study the RP-HPLC method which is used for both determination of filgrastim in protein formulations and separation and detection of oxidized forms of filgrastim in the beginning of their formation was developed.
The developed RP-HPLC method used for determination of filgrastim complies with the requirements of the validation parameters. It was shown to be simple and economical, which gives the opportunity for its use in quantitative determination in routine quality control of filgrastim preparations.

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