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Determination of pegfilgrastim aggregates by size-exclusion high-performance liquid chromatography on a methacrylate-based column

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ABSTRACT

A size-exclusion high-performance liquid chromatographic method using a methacrylate-based column was developed, validated and implemented for the determination of pegfilgrastim aggregates. The samples were directly injected into a TSKgel G4000PWXL column (7.5 mm × 300 mm, 10 μm, <500 A°) with a mobile phase of 100 mM phosphate, pH 2.5. Detection was made at 215 nm and analyses were run at a flow-rate of 0.6 ml/min at 10 °C. Vortex-mixing of samples produced oligomers, however, very high molecular weight aggregates were formed at high temperatures. The method exhibited linearity over the concentration range of 0.1–14 mg/ml for pegfilgrastim monomer and high molecular weight aggregates with a correlation coefficient of greater than 0.99. The method was specific and sensitive, with a lower quantification limit of 0.1 mg/ml and a detection limit of 0.02 mg/ml. Over 1200 samples were analyzed by the present method without significant change in the column performance.

1. Introduction

Pegfilgrastim is a long-acting form of recombinant methionyl human granulocyte colony-stimulating factor (rh-Met-G-CSF, filgrastim) which requires only once-per-cycle administration for the management of chemotherapy-induced neutropenia [1]. The covalent attachment of 20 kDa polyethylene glycol (PEG) to the N-terminal amine group of the parent molecule is attained using site-directed reductive alkylation [2,3].

One of the most troubling and challenging tasks in the production of protein pharmaceuticals is to deal with their physical instabilities. The most common physical instability is protein aggregation, which can be occurred during purification, formulation, packaging and storage processes [4]. Size-exclusion high-performance liquid chromatography (SE-HPLC) is widely used and generally accepted as the most appropriate analytical approach for monitoring protein aggregation [5]. The main advantage of SE-HPLC is the mild mobile phase conditions which permits the characterization of proteins with minimal impact on the conformational structure and local structure [6,7]. However, developing a robust SE-HPLC method for the analysis of pegfilgrastim proved challenging [8]. PEG and sometimes PEGylated proteins are sticky and non-specific column interactions with silica-based stationary phases using a typical SE-HPLC mobile phase containing a phosphate buffer and sodium chloride at neutral pH may lead to rapid loss of resolution [9]. The addition of ethanol to the mobile phase has been suggested to decrease deleterious column interactions with PEGylated proteins [10]. This strategy has been adapted and used for pegfilgrastim [8,11–14]. However, in our experience on several G3000SWXL columns (silica-based from TOSOH Biosciences) with ethanol containing mobile phase, the columns lost their resolution after 150–200 injections.

Other options for SE-HPLC of PEGylated proteins are agarose and methacrylate-based columns [9]. Hydroxylated polymethacrylate-based columns (e.g. G4000PWXL from TOSOH Biosciences) have been designed for aqueous, SE-HPLC of proteins, polysaccharides, oligosaccharides, PEGs, DNA, and RNA. The column packing materials are porous, hydrophilic, and rigid. They exhibit excellent chemical and mechanical stability, have been used for the pH 2.0 to 12.0, and can be cleaned with 0.5 M sodium hydroxide [15].

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In the present work for the first time, we investigated the chromatographic performance of a hydroxylated polymethacrylate-based column for SE-HPLC of pegfilgrastim. Our aim was to develop a robust chromatographic method which could tolerate a minimum of 1000 injections. The method was implemented for in-process quality control and product comparability studies.

2. Materials and methods

2.1. Reagents

Filgrastim as well as pegfilgrastim were produced in a pharmaceutical grade environment in AryaTinaGene biopharmaceutical Co. (Gorgan, Iran). Filgrastim was biosynthesized by *Escherichia coli* bacteria into which has been inserted the human granulocyte colony-stimulating factor gene. Pegfilgrastim was prepared by attaching a 20 kDa PEG propionaldehyde to the N-terminal amino acid of filgrastim. Following PEGylation, monopegylated filgrastim was purified, formulated, and finally concentrated to prepare the formulated bulk solution of pegfilgrastim at a concentration about 14 mg/ml. The pegfilgrastim internal reference standard (IRS, 1 mg/ml) was prepared from the bulk solutions in formulation buffer consisting of 10 mM acetate (pH 4), 50 mg/ml sorbitol and 40 μg/ml polysorbate 20. The filgrastim IRS formulation (1 mg/ml) was similar to pegfilgrastim IRS except for the used surfactant (i.e., polysorbate 80). HPLC grade ethanol, acetonitrile and analytical grade sodium dihydrogen phosphate monohydrate, sodium hydroxide, sodium acetate, 85% phosphoric acid, acetic acid, polysorbate 20 and polysorbate 80 were obtained from E. Merck (Darmstadt, Germany). Water for injection (Shiraz Serum Co., Shiraz, Iran) was used throughout the study. Sorbitol was purchased from Samchun Pure Chemical Co. (Gyeonggi-do, Korea).

The pegfilgrastim prefilled syringes containing 6 mg in a fill volume of 0.6 ml were from F. Hoffmann-La Roche Ltd. (Neulastim®, Basel, Switzerland) and AryaTinaGene biopharmaceutical Co. (Tinapeg®). The original brand, Neulastim was obtained from the Indian market.

2.2. Instrumentation

For SE-HPLC, a biocompatible Azura HPLC system equipped with a P 6.1L solvent delivery pump, 2.1L ultraviolet detector, CT 2.1 column heater/cooler (Knauer, Berlin, Germany), Clarity Lite HPLC software (Data Apex, Petrzilkova, Czech Republic), and Rheodyne 9725i loop injector (USA). The chromatographic column TSKgel G4000PWXL (7.5 mm × 300 mm, 10 μm, <500 A°) and the guard column TSKgel G4000PWXL (6 mm × 40 mm, 12 μm, <500 A°) were from Tosoh, Japan.

2.3. Determination of pegfilgrastim monomer and aggregates

To determine the monomer and aggregates, a volume of 10 μl of sample was directly injected into the chromatographic system. The mobile phase comprised of 100 mM phosphoric acid, adjusted to pH 2.5 with 10 M sodium hydroxide. Analyses were run at a flow rate of 0.6 ml/min at 10 °C and UV detection was carried out at 215 nm. The standard concentrations were prepared in the formulation buffer in the concentration range of 0.1–14 mg/ml. Calibration curves for pegfilgrastim were constructed by plotting peak area (y) of pegfilgrastim versus its concentration (x) and a linear regression was used for quantitation. Aggregates were expressed as percent values and obtained by dividing their peak area to the total peak areas of monomer and aggregates.

2.4. Induction of aggregate formation

For filgrastim, its IRS (1 mg/ml) was mixed with 100 mM sodium acetate (adjusted to pH 4 with acetic acid) to reach a final concentration of 0.4 mg/ml. Then, the sample was vortex-mixed for 1 min [15].

For pegfilgrastim, a volume of 50 μl of its IRS (1 mg/ml) in 1.5-ml polypropylene microtube was subjected to incubation at different temperatures or vortex-mixing at 2600 rpm. Each experiment was carried out in a minimum of 3 replicates. The analysis was performed immediately after stress for the following time periods: 30, 60, 90 and 120 min.

2.5. The addition of sodium dodecyl sulfate (SDS) to the heat-stressed samples of pegfilgrastim

A volume of 50 μl of pegfilgrastim IRS was incubated at 60 °C for 120 min to complete conversion to aggregates. Then, the samples were immediately mixed with 50 μl of different concentrations of SDS in formulation buffer to reach the final SDS concentrations of 0.1, 0.2, 0.5, 1 and 2% and then injected to the chromatograph.

3. Results and discussions

3.1. Method development

We started from a neutral mobile phase containing 50 mM ammonium hydrogen carbonate and a column temperature of 30 °C [16]. When aggregated filgrastim was injected, the acetate and aggregates peaks came before and after monomer peak, respectively. A relation between the resolution of peaks and the temperature as well as flow rate was observed. Moreover, the addition of ethanol had a negative impact on separation (Fig. 1). Elution of the acetate as a small anion before the protein could be probably explained by the column negative charges due to the small amount of residual carboxyl groups in methacrylate-based packing leading to the ion-exclusion properties for the column. Due to the pI value of 6.1, filgrastim carries a negative charge in pH 7, moreover the aggregates are probably less negative in comparison to the monomers and thus elute lately. The addition of sodium chloride and arginine to the neutral mobile phase was tried to eliminate ionic interactions with column packing material, but it lead to complete binding of pegfilgrastim and filgrastim to the column.

In the next step, we started from a 100 mM phosphate (pH 2.5) mobile phase and a column temperature of 20 °C [17]. Although pegfilgrastim gave a sharp peak, nevertheless the filgrastim peak had to tail. We evaluated the mobile phases containing 50 or 200 mM phosphate. As determined by the peak area and the number of theoretical plates, the best column efficiency for filgrastim and pegfilgrastim was achieved at a phosphate concentration of 100 mM, as proposed for other proteins [18]. Substitution of phosphate with 100 mM of trifluoroacetate and perchlorate, pH 2.5 decreased the column efficiency. Similar to the neutral mobile phase, adding sodium chloride or arginine to the acidic mobile phase resulted in complete binding of filgrastim and pegfilgrastim to the column. We noticed that the chromatography in low temperatures improves the peak sharpness for filgrastim and pegfilgrastim. This may be explained by our observation in which an increase in temperature could increases the hydrophobicity of filgrastim and pegfilgrastim leading to an increased retention during reversed-phase chromatography (unpublished data). As a result, SE-HPLC of pegfilgrastim in low temperature may decrease the need for the addition of ethanol to the mobile phase. However, filgrastim gave a tailed peak in this condition. To improve peak