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Rapid formulation assessment of filgrastim therapeutics by a thermal stress test

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ABSTRACT

The biosimilar versions of recombinant methionyl human granulocyte colony-stimulating factor (rh-Met-G-CSF, filgrastim) are now widely available. Because changes to the formulation often lead to subtle differences, there is a critical need to define techniques to test and insure the quality of these products. The present study was designed to compare formulation and thermal stress stability of filgrastim products. The formulation ingredients including acetate, polysorbate 80, and sorbitol were determined using state-of-the-art validated analytical methods. The formulation pH and osmolality were also measured. Moreover, the stability profiles of 8 filgrastim products using thermal stress at 57 °C for 4 h were assessed by size-exclusion high-performance liquid chromatography (SE-HPLC) and in vitro biological assay. The products had different stability profiles. More stable products were within the specification for formulation and less stable products were beyond the specification limits. Altogether, the results suggest that a short-time stress study at 57 °C and analysis of filgrastim by SE-HPLC could unveil formulation problems and is potentially useful for comparability studies.

1. Introduction

Human granulocyte colony-stimulating factor (hG-CSF), a single chain polypeptide containing 174 amino acid residues, is central to neutrophil-based immune defenses due to its regulatory role in the growth, differentiation, survival, and activation of neutrophils and their precursors [1].

Filgrastim, an 18.8 kDa recombinant protein, is the methionyl hG-CSF produced by fermentation of a genetically modified *Escherichia coli* strain [2]. This cytokine derivative received FDA approval in 1991 (Neupogen®, Amgen Inc.) for the treatment of neutropenia caused by myelosuppressive radio- and chemotherapy, and for the prevention of the associated infections. Currently, filgrastim is recommended in acute myeloid leukemia, bone marrow transplantations, severe chronic neutropenia, aplastic anemia and myelodysplastic syndromes [3].

Filgrastim produced by Amgen, had its patent expired in 2006 and now many of its biosimilars are available. Regulatory approval is provided on the basis of comparable quality, safety, and efficacy to the innovator (reference) product, Neupogen. The biosimilars offer potential benefits to patients, physicians, and healthcare providers by reducing healthcare costs [4,5]. Biosimilars are similar, but not necessarily identical to the innovator product. The production methods of the therapeutic protein products are complex and it is essential to amass sufficient data to ensure the safety and efficacy of filgrastim therapeutics from different manufacturers [6–9].

One of the major concerns about the filgrastim therapeutics is the physical stability. At physiological temperature and pH, filgrastim aggregates within days to form insoluble nonreversible aggregate that is to some extent covalently cross-linked due to the presence of a free cysteine thiol [10–13]. This has negative consequences on the practical application of filgrastim as it implies the requirement for stringent formulation [14].

The innovator product Neupogen was first formulated at pH 4.0 with 10 mM acetate, 5% mannitol and 0.004% polysorbate 80, and

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was stable for greater than 24 months at 2–8 °C. Since mannitol crystallizes upon freezing, Neupogen is now formulated in 5% sorbitol [15]. Besides being a tonicity modifier, sorbitol stabilizes filgrastim in the formulation mainly by its exclusion from the protein surface, hence leading to preferential hydration of the protein [16]. The presence of non-ionic surfactant polysorbate 80 in the filgrastim formulation is necessary to prevent agitation-induced aggregation [17], but its higher concentrations are associated with increased aggregate formation [18]. Acetate buffer is used to adjust the pH and increase the solubility of filgrastim in the formulation, but at high concentration (i.e. 100 mM), it reduces unfolding temperature of filgrastim [19]. Therefore, it is crucial to evaluate the amount of polysorbate 80 and acetate to be used in the formulation.

The filgrastim biosimilars should demonstrate the effectiveness of their formulation for the prevention of physical instabilities. Long-term and accelerated stability studies are necessary and provide useful information, but are time-consuming. However, stress studies such as agitation [20] and temperature [21] rapidly provide useful information regarding the stability of therapeutic proteins. We hypothesized that a suitable stress test may not only provide a rapid way for comparing the stability of filgrastim therapeutics from different manufacturers, but also indirectly reveal their possible deviations from the formulation of innovator product.

In the present work, the formulation variables of 8 filgrastim products were determined. Moreover, a thermal stress test was developed to compare the stability of filgrastim products by size-exclusion (SE) HPLC and biological assay. The relationship between the determined formulation variables and observed stability in thermal stress was investigated.

2. Materials and methods

2.1. Therapeutic products

The filgrastim therapeutics containing 300 μg in fill volumes of 0.5 ml were from F. Hoffmann-La Roche Ltd. (Neupogen, Basel, Switzerland), AryaTinaGene Co. (Tinagrast, Gorgan, Iran), Varian Darou Co. (Tehran, Iran), Poyesh Darou Co. (Tehran, Iran), B.T. Rosamed Co. (Tehran, Iran). The products containing 300 μg of filgrastim in a fill volume of 1 ml were from Poyesh Darou Co. (Tehran, Iran), Zahravi Co. (Tabriz, Iran) and Actover Co. (Teharn, Iran).

The original brand, Neupogen was obtained from the Iranian market. Other products were among the samples submitted to the laboratories of quality control of the Iranian Ministry of Health for the evaluation of their quality. We received the permission from AryaTinaGene Co. to reveal the name of Tinagrast. The commercial names of other therapeutics were not disclosed for confidential purposes. A code name of A–F was given to these products.

2.2. Reagents

Filgrastim control reference standard (CRS, 1.95 mg/ml) was purchased from EDQM (Strasbourg, France). The filgrastim internal reference standard (IRS, 1000 μg/ml) prepared using filgrastim CRS, was provided by AryaTinaGene Co. (Gorgan, Iran). Filgrastim working standards were prepared from the IRS in blank formulation (pH 4) of 10 mM acetate, 50 mg/ml sorbitol and 40 μg/ml polysorbate 80. HPLC grade methanol, acetonitrile and analytical grade sodium dihydrogen phosphate monohydrate, sodium hydroxide, sodium acetate, 85% phosphoric acid, acetic acid, dichloromethane, ammonium hydrogen carbonate and polysorbate 80 were obtained from E. Merck (Darmstadt, Germany). Water for injection (Shiraz Serum Co., Shiraz, Iran) was used throughout the study. Sorbitol, fructose and glucose were purchased from Samchun Pure Chemical Co. (Gyeonggi-do, Korea). RPMI-1640 media (with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate), FBS (fetal bovine serum), tripan blue stain 0.4%, phosphate buffered saline (PBS), all purchased from Gibco, Invitrogen, USA. 2-Mercaptoethanol was purchased from Sigma–Aldrich (Steinheim, Germany). CellTiter 96® aqueous one solution reagent (MTS) was purchased from Promega (USA). NFS-60 cells were obtained from Pasteur Institute of Iran (Karaj, Iran).

2.3. Instrumentation

For SE-HPLC, the chromatographic system equipped with a Smartline 1000 solvent delivery pump, Smartline 2500 ultraviolet detector, Jet stream column heater/cooler, ChromGate HPLC software (Knauer, Berlin, Germany), Rheodyne 7725i loop injector (USA), and a TSK-Gel G3000SWXL column (7.8 mm × 300.0 mm, 5 μ, Tosoh, Japan). For determination of polysorbate 80, the same system but with a Chromolith RP-18e column (100 × 4.6 mm) with a RP-18e guard column (5 × 4.6 mm), both from Merck (Darmstadt, Germany) were used for the chromatographic separation. For determination of sorbitol, the chromatographic system consisted of a LKB Bromma 2150 pump (LKB, Sweden), Jet stream column heater/cooler (Knauer, Berlin, Germany), Rheodyne 7725i loop injector (California, USA), RID-6A refractive index detector (Shimadzu, Kyoto, Japan), Clarity Lite HPLC software (Data Apex, Petrzilkova, Czech Republic), and a Nucleodur NH2-RP column (250 × 4.6 mm, 5 μ particle size and 100 Å pore size) with a guard column (5 × 4.6 mm), both from Macherey Nagel (Duren, Germany).

2.4. Determination of filgrastim monomer and aggregates

SE-HPLC was performed according to the European Pharmacopoeia (EP) monograph for filgrastim [22]. To determine the monomer, dimer, oligomers and high order aggregates, 30 μl of filgrastim sample was mixed with 15 μl of acetate buffer and 40 μl was injected. The mobile phase comprised of 50 mM ammonium hydrogen carbonate, adjusted to pH 7 with concentrated phosphoric acid. The mobile phase was kept on ice–water bath at below 10 °C. Analyses were run at a flow rate of 0.5 ml/min on TSK-Gel column at 20 °C and UV detection was carried out at 215 nm. A resolution solution for filgrastim dimer and aggregates was prepared by mixing 30 μl of filgrastim IRS with 15 μl of acetate buffer and vortex-mixing for 2 min. A volume of 40 μl was injected after centrifugation for 2 min at 12,000 × *g*. The concentration of filgrastim monomer was determined using calibration curves prepared in the concentration range of 10–1000 μg/ml. Calibration curves were constructed by plotting peak area (*y*) of filgrastim monomer versus its concentration (*x*). A linear regression was used for quantitation.

2.5. In vitro biological assay

The stimulatory effect of filgrastim on proliferation of the G-CSF adapted NFS-60 cells was used to assess the biological activity. Filgrastim samples were tested in triplicate at the initial concentration of about 200 IU/ml followed by 7 twofold dilutions to obtain a standard curve. Each plate also had a blank (A1–H1, medium only), negative (A11–H11, cells only) and positive (A12–H12, cells plus filgrastim) controls. The quantity of produced formazan was estimated at 490 nm. The potency of the samples was calculated in a parallel line assay according to the EP method [22]. The specificity of bioassay test was investigated by applying the formulation buffer or erythropoietin instead of filgrastim.