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Chromatographic and electrophoretic assessment of Filgrastim biosimilars in pharmaceutical formulations

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ABSTRACT

An orthogonal testing protocol was developed and validated to assess the quality of Filgrastim biosimilars. Results were compared to those obtained from the innovator product. Initial screening was carried out using reducing and non-reducing gel electrophoresis. RP-LC was employed for the determination of Filgrastim in the presence of its oxidative degradation products. SEC and CIEF were used under non-denaturing conditions to reveal high molecular weight and charged impurities, respectively. RP-LC assay was found accurate (99.78 ± 0.89) and precise over a linear concentration range of 9.38–300.00 μg/ml with a LOD of 8.26 μg/ml (0.44 mM). SEC was carried out over a molecular weight range of 5.0–150.0 kDa. CIEF was optimized using neutrally coated capillaries over a wide-range pH gradient (pH 3.0–10.0). Differences between the studied products were revealed using all these techniques. Impurities above the acceptable limits were detected in both biosimilar products. CIEF revealed heterogeneity in the active ingredient that has not been investigated by the manufacturers. Correlation of the obtained results indicated the presence of not only product-related impurities, but also process-related impurities. Results confirmed the need for in-house validated orthogonal testing protocols to be developed by local regulatory authorities. This should prevent access of substandard biosimilars to price-sensitive markets.

1. Introduction

Human proteins expressed in heterologous systems are of an unpredictable nature and assessment of their activity and stability is of primary importance. Some variability is not only anticipated but is also acceptable as long as it is well understood by the manufacturer [1]. Biosimilars, “follow-on biopharmaceuticals” are products that are biologically similar but not identical to an authorized innovator product. Unlike generic pharmaceuticals, there is a strong correlation between the manufacturing processes of biopharmaceuticals and the characteristics of the final product. Small changes in the manufacturing process can affect both efficacy and safety. Comparison of a biosimilar product to its lead (innovator) product is essential in order to evaluate “biosimilarity” [2,3]. A well-defined analysis strategy that addresses the key features of a recombinant product has to be developed and validated [1,4,5].

Being more economic, biosimilar products continue to spread especially in price-sensitive markets. This has been accompanied by the emergence of dozens of biotech companies all over the world. Production systems in such newly established companies might not be equally characterized as those employed by the innovator manufacture. Unfortunately, this might result in products with a different purity and safety profiles from the innovator and even biosimilars produced by well-recognized companies. The European Medicine Agency (EMA) and the US Food and Drug Administration (FDA) regularly issues and updates guidelines for manufacture and approval of biosimilars [6–9]. Elsewhere, the cost is a major factor in production of biosimilars in emerging markets. In such cases, there is a more complex negotiation between safety and access to the market and a lower bar is set in terms of assessment and clinical-trial requirements. In-house, reliable testing protocols should be developed and implemented by local regulatory authorities in order to ensure safety and efficacy of new products.

Filgrastim is a recombinant human granulocyte colony-stimulating factor (rhuG-CSF) produced in *Escherichia coli* and is used for treatment of neutropenia. It is a hematopoietic cytokine that stimulates and regulates the proliferation and differentiation

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of neutrophils [10,11]. Filgrastim is a 175-amino acid protein with a molecular weight of 18.8 kDa and isoelectric point p*I* ~ 6.0 [12,13]. Few comparability studies have been published on Filgrastim biosimilars [14,15]. Hydrophobicity, related proteins and oxidized impurities were inspected using RP-HPLC [14,15]. SDS-PAGE and SEC were used to detect impurities with different molecular masses [12,14,15]. Charged impurities were investigated using ion chromatography [14,15] and gel IEF [12,14]. Several spectroscopic techniques were employed to evaluate high order protein structure such as advanced mass spectrometry, circular dichroism and NMR [14,15]. Biological characterization has also been reported using cell proliferation assays [12,14,15].

The aim of this work was to establish a reliable protocol for the assessment of Filgrastim in biosimilar pharmaceutical formulations. An in-house purity-indicating testing protocol is described and validated. RP-LC, SEC and CIEF along with conventional gel electrophoresis tools are implemented. A comparability study between two Filgrastim biosimilars and the innovator product is presented with respect to content and impurity profile. Correlation of the analysis results is carried out in order to better understand the nature of impurities.

2. Experimental

2.1. Chemicals and samples

Filgrastim working standard (300.00 μg/ml) was kindly supplied by F. Hoffmann-La Roche Ltd. (Switzerland). Standard solution stability was assessed at 2–8 °C and 15 °C according to ICH guidelines [16] for HPLC and CIEF assays, respectively. Samples of two different batches of Filgrastim Innovator (Neupogen, F. Hoffmann-La Roche Ltd., Switzerland), Biosimilar-1 and Biosimilar-2 were obtained from the Egyptian market. Commercial names and batch numbers of biosimilar products were not disclosed for confidential purposes. NaOH (1.0 M), H_3PO_4 (1.0 M), carrier ampholytes (CA) covering a pH range of 3.0–10.0, CIEF gel, and protein standards (4.1, 5.5, 7.0, 9.5 and 10.0) were obtained as part of the Beckman CIEF kit (Beckman Coulter, USA). All other chemicals were of HPLC grade or higher and were purchased from Sigma (USA).

2.2. Instruments

All chromatographic separations were carried out using an Agilent 1200 HPLC system. Instrument control and data analysis were achieved using Chemstation software (Agilent Technologies, Germany). Capillary electrophoresis experiments were conducted using WinPrinCE system controlled by DAx data acquisition and analysis software (Emmen, Netherland). Both instruments were equipped with a temperature-controlled auto-sampler and column compartment. A Bio-Rad gel imaging system (USA) was used for documentation of stained gels.

2.3. Reversed phase liquid chromatography

2.3.1. Method optimization and system suitability

Optimization was carried out in the presence of oxidized impurities according to the European Pharmacopoeia [12]. An aliquot of 500.0 μl of Filgrastim working standard (300.0 μg/ml) was treated with 5.0 μl of 10% hydrogen peroxide. The treated Filgrastim solution was then incubated at 25 °C for 1.0 h. The method was optimized by changing the mobile phase composition and the flow rate. System suitability parameters were calculated [17] and compared to the reported method [12].

2.3.2. Optimized method

RP-HPLC analysis was performed using Vydac C4 column (4.6 mm × 250.0 mm, 5.0 μm) (Grace, USA). The temperature of the column compartment and sample tray was maintained at 60 °C and 4–8 °C, respectively. A gradient elution at a flow rate of 0.8 ml/min was employed and detection was carried out at 215.0 nm. The mobile phase composition was: (A) acetonitrile:water:trifluroacetic acid (30.0:70.0:0.1, v/v/v) and (B) acetonitrile:water:trifluroacetic acid (90.0:10.0:0.1, v/v/v), respectively. Run time was 20 min with linear gradient elution: 0.0–5.0 min (25–51% B), 5.0–15.0 min (51–58% B) and 15.0–20.0 min (58–25% B).

2.3.3. Calibration and validation

Calibration curve was constructed using a standard series covering a concentration range (9.38–300.00 μg/ml). The obtained regression equation was used to calculate the concentration of Filgrastim throughout the study. Validation was performed according to ICH guidelines and the following parameters were determined: specificity, linearity, range, accuracy, precision, LOD and LOQ. Specificity was assessed in the presence of oxidized impurities according to the European Pharmacopoeia. Results were compared to the reported Pharmacopoeia method [12].

2.4. Size exclusion chromatography

SEC was performed according to the European Pharmacopoeia for Filgrastim concentrated solution. Separation efficiency was demonstrated by using a sample prepared as previously described in the pharmacopoeia [12]. The degraded sample was prepared by vortexing an aliquot of Filgrastim working standard (300.0 μg/ml) for 1 min. A TSKgel G2000 SWXL column (7.8 mm × 300.0 mm, 5.0 μm and molecular weight range 5.0–150.0 kDa) was used for all determinations. The temperature of the column compartment and sample tray was maintained at 25 °C and 4–8 °C, respectively. A mobile phase of ammonium hydrogen carbonate pH 7.0 ± 0.05 was used at a flow rate 0.5 ml/min and detection was carried out at 215.0 nm.

2.5. Capillary isoelectric focusing

2.5.1. Method optimization

Polyacrylamide-coated neutral capillaries, 50.0 μm id, 325.0 μm od with a total/effective length of 67.0/47.0 cm were used in all investigations. On purchase, capillaries were tested for lack of electroosmotic flow (EOF) under the experimental conditions described by the manufacturer. Preconditioning of the capillary at the beginning of each day was carried out according to manufacturer specifications and previously optimized protocols [18]. Capillaries were flushed with MilliQ water for 2.0 min, CIEF gel for 5.0 min and finally with MilliQ water for 5.0 min.

Optimization of the focusing and mobilization conditions was carried out using a mixture of five reference standards (p*I* 4.1, 5.5, 7.0, 9.5 and 10.0). Suitable amounts of the reference standards were prepared in 2% carrier ampholytes/polymer solution as previously described [18]. The effect of addition of a cathodic stabilizer (500 mM L-arginine) was investigated. Different concentrations of the anolyte (phosphoric acid) and the catholyte (sodium hydroxide) were trialed. The effect of focusing time, applied voltages and mobilization pressure were studied and the current was monitored in each case.

2.5.2. Optimized method

Samples were prepared as described above but using p*I* markers 4.1 and 7.0 only. Capillary temperature was maintained at 25 °C and the sample tray at 15 °C. Capillaries were rinsed with