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Assessing analytical comparability of biosimilars: GCSF as a case study

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ABSTRACT

The biosimilar industry is witnessing an unprecedented growth with the newer therapeutics increasing in complexity over time. A key step towards development of a biosimilar is to establish analytical comparability with the innovator product, which would otherwise affect the safety/efficacy profile of the product. Choosing appropriate analytical tools that can fulfil this objective by qualitatively and/or quantitatively assessing the critical quality attributes (CQAs) of the product is highly critical for establishing equivalence. These CQAs cover the primary and higher order structures of the product, product related variants and impurities, as well as process related impurities, and host cell related impurities. In the present work, we use such an analytical platform for assessing comparability of five approved Granulocyte Colony Stimulating Factor (GCSF) biosimilars (Emgrast, Lupifil, Colstim, Neukine and Grafeel) to the innovator product, Neupogen®. The comparability studies involve assessing structural homogeneity, identity, secondary structure, and product related modifications. Physicochemical analytical tools include peptide mapping with mass determination, circular dichroism (CD) spectroscopy, reverse phase chromatography (RPC) and size exclusion chromatography (SEC) have been used in this exercise. Bioactivity assessment include comparison of relative potency through in vitro cell proliferation assays. The results from extensive analytical examination offer robust evidence of structural and biological similarity of the products under consideration with the pertinent innovator product. For the most part, the biosimilar drugs were found to be comparable to the innovator drug anomaly that was identified was that three of the biosimilars had a typical variant which was reported as an oxidized species in the literature. But, upon further investigation using RPC-FLD and ESI-MS we found that this is likely a conformational variant of the biotherapeutic been studied.

1. Introduction

Biosimilars are subsequent versions of a commercial biotherapeutic (referred to as innovator drug) that is manufactured by a different sponsor following imminent patent and exclusivity expiry [1]. The past decade has led to a significant expansion in the biosimilar space, fuelled in part by the improved affordability when compared to novel biotherapeutics [2]. EU has been a leader in setting up the regulatory pathway to enable 21 biosimilars approved to date. Of other developed countries, Japan has 7 biosimilars approved so far and the US just approved their first biosimilar, Zarzio® (from Sandoz) in 2015 [3]. The Indian regulators have also been quite supportive of biosimilars and have approved 25 biosimilars to date, a fact that highlights the significant potential of Indian biopharmaceutical companies in development and commercialization of biosimilars. With respect to establishing analytical comparability, the Indian regulators as well as the manufacturers are aligned to the international practices [4].

The inherent complexity that is associated with biotherapeutics when compared to chemical entities manifests itself in the form of critical quality attributes (CQAs) [5,6]. While

Abbreviations: CQA, critical quality attributes; GCSF, granulocyte colony stimulating factor; CD, circular dichroism spectroscopy; RPC, reverse phase chromatography; SEC, size exclusion chromatography; FLD, fluorescence detector; mAbs, monoclonal antibodies; PTM, post translational modification; ICH, International Conference on Harmonisation; CID, collision induced dissociation; ETD, electron transfer dissociation; HSQC NMR, heteronuclear single quantum coherence nuclear magnetic resonance spectroscopy; CDSCO, Central Drugs Standard Control Organization; OPA, ortho-phosphoric acid; TFA, trifluoroacetic acid; APS, ammonium persulfate; SDS, sodium dodecylsulfate; EDTA, ethylenediaminetetraacetic acid; TEMED, N,N,N′,N′-tetramethylethylenediamine; GMCSF, granulocyte macrophage colony stimulating factor; ESI-TOF-MS, electrospray ionization-time of flight-mass spectrometer; HPLC, high performance liquid chromatography; Gn-HCl, guanidine hydrochloride; Tris-HCl, Tris hydrochloride; DTT, dithiothreitol; IAM, iodoacetamide; pMod, peak modeling; MFE, molecular feature extraction; MRE, mean residue ellipticities; PAGE, polyacrylamide gel electrophoresis; UPLC, ultra performance liquid chromatography; DAD, diode array detector; IU, international unit; TIC, total ion chromatogram; TCC, total compound chromatogram; PK, pharmacokinetic; PD, pharmacodynamic; UV/VIS, ultraviolet/visible λ_{max} maximum wavelength mAU milli absorbance unit.

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pharmaceutical products may have 1 to 5 CQAs, biological products are likely to have anywhere from 10 to 30 CQAs, ranging from simpler biological products such as the granulocyte colony stimulating factor (GCSF) to more complex monoclonal antibodies (mAbs) [1]. These CQA may be related to degradation, post translational modification (PTM) or other biochemical changes to the product. As a result, there is a critical requirement for demonstration of a high degree of similarity between the biosimilar which may have been produced by different manufacturing processes, equipment and/or sites and the pertinent innovator drug such that no clinically relevant quality, safety and efficacy concerns are observed [7]. The typical strategy that manufacturers employ to establish biosimilarity involves a thorough analytical characterization of the products under consideration. This is achieved by using a wide array of orthogonal, high resolution, analytical tools to characterize biosimilar(s) together with the innovator product [8,9]. Any concerns that arise from this exercise have to be allayed by performing suitably designed non-clinical, preclinical, and clinical studies. An undesirable outcome of these could be the conclusion that the product in question is not a biosimilar and has to be considered as a novel drug. Considering the critical role that analytical comparability plays in the regulatory approval process, there is an immense need of establishing analytical platforms that are capable of fingerprinting the concerned biotherapeutic and also to update the platform in view of recent developments in analytical methodologies [10].

Filgrastim or recombinant methionyl-granulocyte colony stimulating factor (rGCSF) is a glycoprotein that is known to significantly impact proliferation and differentiation of cells of hematopoietic lineage. It is licensed for reducing the incidence and duration of post-chemotherapy neutropenia in patients with non-myeloid malignancies and for the mobilization of hematopoietic progenitor cells in transplantation patients [11]. It is an 18.8 KDa protein and consists of 175 amino acids with one free cysteinyl residue (C17) and two disulphide bonds (C36-C42 and C64-C74) [12]. Filgrastim is manufactured in *E. coli* and has been reported to have same potency as naturally occurring GCSF, which is O-glycosylated at Thr-133 demonstrating that glycosylation is not essential for bioactivity of rGCSF. However, studies on Lenograstim, a glycosylated form produced in Chinese Hamster Ovary (CHO) has been revealed to exhibit 25% greater in-vitro bioactivity [13,14]. In a major advancement, Zarzio® has gradually overtaken Neupogen® in Europe in terms of prescriptions and illustrates the growing acceptance of biosimilars worldwide, the faith in analytical comparability programs and in the strength and effectiveness of pharmacovigilance programmes [15].

Extensive literature exists on the topic of establishing physicochemical and functional comparability of filgrastim with respect to the innovator product. Zarzio® (EP2006) has been a subject of several analytical and clinical comparability exercises and has succeeded in receiving regulatory approval both in the EU and the US [16,17]. The extensive characterization study on different batches of filgrastim from Hospira (Nivestim) has been shown to have similar physicochemical properties, molecular characteristics, and biological activity as Neupogen® using state of art analytical methods in accordance with International Conference on Harmonisation (ICH) [11]. A filgrastim biosimilar (BK0023) has been demonstrated to be comparable to Neupogen® in terms of not only the analytical but also in-vitro biological and in-vivo PK and PD studies [11]. Another comparability exercise involving use of more than ten analytical methods on a laboratory purified GCSF has also showcased the effectiveness of such analytical platforms [18]. High performance liquid chromatography-mass spectrometry (HPLC–MS) followed by top down fragmentation (collision induced dissociation/electron transfer dissociation (CID/ETD)) and 2D heteronuclear single quantum coherence nuclear magnetic resonance spectroscopy (HSQC-NMR) have been exploited recently as orthogonal approaches for characterizing physicochemical properties such that comparability assessment could be carried out with greater conviction [19].

The Central Drugs Standard Control Organization (CDSCO) under the Ministry of Health and Family Welfare evaluates safety, efficacy and quality of drugs in India. The draft guidelines on 'Similar Biologics' were released in 2012, where the demonstration of similarity involves detailed product characterization, preclinical and clinical studies carried out in accordance to the reference product. Numerous filgrastim biosimilars (about 8) have received marketing authorization in India [20]. The present study demonstrate results from an evaluation of physicochemical and functional comparability of five such approved filgrastim biosimilars against Neupogen® using an array of advanced analytical methods for detection of changes in protein structure, identity, purity and bioactivity.

2. Materials and methods

2.1. Materials

Filgrastim reference standard (Neupogen®from Amgen; 300 μg/0.5 ml) and biosimilars manufactured by five Indian companies (Emgrast from Gennova Biopharmaceuticals Ltd.; 300 μg/0.5 ml, Lupifil from Lupin Pharmaceuticals; 300 μg/0.5 ml, Colstim from Zydus Cadila; 300 μg/ml, Neukine from Intas Biopharmaceuticals Ltd.; 300 μg/ml, Grafeel from Dr. Reddy's Laboratories Ltd.; 300 μg/ml)(all single use, pre-filled syringes) were purchased from two distributors (Farma Glow, Gurgaon and Vardhman Health Specialities, Bangalore) and stored at 4 °C as per manufacturer's instructions. All the product consisted of similar formulation buffer i.e. sodium acetate, acetic acid, sodium hydroxide, sorbitol and polysorbate 80. Samples of 0.3 μg/0.5 ml were diluted to 0.3 μg/ml with the formulation buffer prior to analysis.

Glacial acetic acid, sodium acetate (anhydrous), sodium hydroxide, bromophenol blue, methanol, silver nitrate, ammonium hydrogen carbonate, *ortho*-phosphoric acid (OPA), sodium carbonate, acetonitrile (HPLC grade), and trifluoroacetic acid (TFA) were purchased from Merck Chemicals, India. Acrylamide, ammonium persulfate (APS), bisacrylamide, beta-mercaptoethanol, glycine, sodium dodecylsulfate (SDS), potassium salt of ethylenediaminetetraacetic acid (EDTA), sodium thiosulphate, and N,N,N′,N′-tetramethylethylenediamine (TEMED), Sterile RPMI-1640 media, and granulocyte macrophage colony stimulating factor (GMCSF) were purchased from Sigma Aldrich Co., India.

2.2. Methods

Physicochemical and functional characterization was successfully performed using a wide range of state-of-the-art analytical methods adapted from the European [21] and Indian Pharmacopeia [4]. The methodology was designed such that it could determine any disparity in protein structure, mass, size, hydrophobicity, and bioactivity.

2.2.1. Reverse-phase liquid chromatography-electrospray ionization-mass spectrometry (RPC-ESI-MS) for intact mass and peptide mapping (primary structure)

Intact mass of the biosimilars was performed to determine the molecular mass of GCSF. RPC was conducted using a Phenomenex Jupiter C4 (4.6 × 250 mm) column and an Agilent 1260 Infinity Bioinert Quaternary Liquid Chromatography (LC) system coupled to an Agilent 6230 electrospray ionization-time of flight-mass spectrometer (ESI-TOF-MS) instrument. Gradient elution was carried out with 0.1% (v/v) TFA in 10% acetonitrile (A) and 0.1% (v/v) TFA in 80% acetonitrile (B) at 0.6 ml/min and 60 °C from 66 to 73% B in