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Granulocyte-macrophage colony stimulating factor: Evaluation of biopharmaceutical formulations by stability-indicating RP-LC method and bioassay

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ABSTRACT

The granulocyte-macrophage colony stimulating factor (GM-CSF) is a cytokine that regulates the proliferation and differentiation of hematopoietic cells and activates granulocytes and macrophages. A reversed-phase liquid chromatography (RP-LC) method was validated for the assessing of the stability of non-glycosylated recombinant rhGM-CSF (Molgramostim) in biopharmaceutical formulations. The RP-LC method was carried out on a Jupiter C_4 column (250 mm × 4.6 mm i.d.), maintained at 45 °C. The mobile phase A consisted of 0.1% TFA and the mobile phase B was acetonitrile with 0.1% TFA in acetonitrile, run at a flow rate of 1 mL/min, and using photodiode array (PDA) detection at 214 nm. Chromatographic separation was obtained with a retention time of 29.2 min, and was linear over the concentration range of 2–300 μg/mL ($r^2 = 0.9992$). Specificity was established in degradation studies. Moreover, the *in vitro* cytotoxicity test of the degraded products showed significant differences ($p < 0.05$). The method was applied to the assessment of rhGM-CSF and related proteins in biopharmaceutical dosage forms, and the results were correlated to those of a bioassay. It is concluded that the employment of RP-LC in conjunction with current methods allows a great improvement in monitoring stability, quality control and thereby assures the therapeutic efficacy.

1. Introduction

The granulocyte-macrophage colony stimulating factor is a cytokine that regulates the proliferation and differentiation of hematopoietic progenitor cells and activates mature granulocytes and macrophages. Human granulocyte-macrophage colony stimulating factor, produced by recombinant DNA technology (rhGM-CSF), expressed in *Escherichia coli*, Molgramostim, is now marketed world-wide for clinical use in enhancing hematopoietic recovery after cancer chemotherapy and bone marrow transplantation [1–3].

The rhGM-CSF molecule is a non-glycosylated polypeptide chain consisting of 127 amino acids, with four cysteine residues which form two disulphide bonds, between Cys^{54} and Cys^{96} and Cys^{88} and Cys^{121}. The molecular mass is approximately 14.5 kDa [4–6].

The biological assay based on the proliferation of the factor-dependent cell line TF-1 (ATCC CRL-2003), has been used for the potency assessment of rhGM-CSF. The growth-promoting activity has been evaluated by incubation with tetrazolium bromide (MTT) or alamarBlue and measuring absorbance [7–10].

Three enzyme-linked immunosorbent assay (ELISA) have been described and compared to the cell proliferation bioassay for their ability to quantify the non-glycosylated rhGM-CSF present in mixtures with variable protein content. This proved to be useful during the production process [8]. Also, the ELISA was applied in pharmacokinetic studies [11,12] and validated for the quantitation of adducts of rhGM-CSF and human serum albumin in stressed solution mixtures [13].

Currently, *in vivo* or *in vitro* biological assays are required for the potency assessment of biotechnology-derived medicines. Physico-chemical techniques cannot yet predict their biological activity, but can produce information about structure and composition and are used to monitor content, purity, and chemical stability of the product. No single technique can satisfactorily provide sufficient information about the protein. Thus, a combination of physico-chemical, immunological, and biological methods is recommended [9,14,15]. Liquid chromatography (LC) has been successfully applied

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in correlation studies between physicochemical and biological assays, in attempts to find methods for characterization and for monitoring the stability of different biotherapeutics [16–20]. However, the development of analytical methods for the analysis of products obtained through recombinant technology presents some difficulties, due to the low dose of the microheterogeneous protein present relative to the large amounts of human serum albumin. This is added to prevent adsorption of the protein to the vial walls and to increase stability during storage [13,21,22]. Due to health concerns related to transmissible spongiform encephalopathies, the use of human serum albumin is now decreasing.

Reversed-phase liquid chromatography (RP-LC) exploits the hydrophobic properties of biomolecules in the separation process and offers a high level of accuracy and sensitivity for the analysis of closely related protein variants or degradation products which may have reduced activity and altered immunogenicity [14,21]. The rhGM-CSF expressed in *E. coli* was characterized by fast atom bombardment mass spectrometry combined with RP-LC. The fractions of the enzymatic digests were separated by RP-LC using a TSK-ODS column and detection at 230 nm, demonstrating that the sulfoxidation of methionine residues occurs during renaturation and/or purification steps [23]. Also, samples of rhGM-CSF were analyzed by gradient RP-LC using a C_4 column with UV detection at 214 nm, and compared to the capillary electrophoresis method [24]. The monopegylated rhGM-CSF was characterized by SDS-PAGE, size-exclusion (SE-LC) and RP-LC, capillary electrophoresis and mass spectrometry, demonstrating the applicability of these analytical techniques [9]. However, validation of the method is essential to show that the procedure is suitable for its intended purpose [25].

The aim of this article was to validate a specific, sensitive and stability-indicating gradient RP-LC method to determine the content of rhGM-CSF in biopharmaceutical formulations; to correlate the results to the bioassay, and to evaluate the bioactivity and the cytotoxicity of the related proteins, thus contributing to the development of methods to monitor stability, improve quality control, and thereby assure therapeutic efficacy of the biological medicine.

2. Materials and methods

2.1. Chemicals and reagents

European Pharmacopoeia Certificated Reference Standard for rhGM-CSF (*CRS rhGM-CSF*) was obtained from the EDQM (Strasbourg, France) and the 1st International Standard for rhGM-CSF, WHO 88/646, was obtained from the National Institute for Biological Standards and Control-NIBSC (Herts, UK). A total of eight batches of Leucocitin (Blausiegel, São Paulo, Brazil), containing 300 μg/vial of rhGM-CSF were identified by numbers from 1 to 8. The samples were obtained from commercial sources within their shelf life period. Hydrogen peroxide 30% in aqueous solution, potassium phosphate monobasic, polysorbate 80, acetonitrile and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). All chemicals used were of pharmaceutical or special analytical grade. For all the analyses, ultrapure water was obtained using an Elix 3 coupled to a Milli-Q Gradient A10 system Millipore (Bedford, MA, USA).

2.2. Apparatus and analytical conditions

The LC method was carried out on a Shimadzu LC system (Kyoto, Japan) equipped with a SCL-10A_{VP} system controller, an LC-10 AD_{VP} pump, a DGU-14A degasser, a SIL-10AD_{VP} auto sampler, and a SPD-M10A_{VP} PDA detector. The peak areas were integrated automatically by computer using a Shimadzu Class-VP® V 6.14 software program. The experiments were performed on a reversed-phase Phenomenex (Torrance, USA) Jupiter C_4 column (250 mm × 4.6 mm i.d., with a pore size of 300 Å), maintained at 45 °C. A security guard holder was used to protect the analytical column. The elution was performed using a linear gradient at a constant flow rate of 1 mL/min. Mobile phase A consisted of 0.1% trifluoroacetic acid (TFA) and mobile phase B was acetonitrile with 0.1% TFA, run as follows: time 0–0.1 min 37% of B; from 0.1 to 34 min linear up to 50% of B; from 34.01 to 35 min linear down to 37% of B, maintained up to 40 min. The mobile phases were filtered through a 0.22 μm membrane filter Millipore (Bedford, MA, USA). The injection volume was 50 μL for both the *CRS rhGM-CSF* and the samples.

2.3. Procedure

2.3.1. In vitro bioassay

The bioassay was performed as described elsewhere [10,26], and the growth-promoting activity of rhGM-CSF assessed on TF-1 cell line (ATCC number CRL-2003). The cells were maintained in culture medium RPMI 1640 containing 10% (v/v) fetal bovine serum Sigma-Aldrich (St. Louis, MO, USA), adding concentrations of rhGM-CSF (1–20 ng/mL) for cell proliferation in 75 cm^2 flasks for 24 h, seeding at approximately 2.0–6.0 × 10^5 cells/mL. The assay was performed in triplicate, the cells were seeded in 96-well cell culture plates BD Biosciences (San Jose, CA, USA) at a density 4 × 10^5 cells/mL (2 × 10^4 cells/well) and dosed on seeding with two fold dilution series, range starting with 65 IU/mL (6.5 ng/mL) of rhGM-CSF. The WHO (88/646) rhGM-CSF was used as standard and the negative control was RPMI 1640 culture medium. Briefly, the plates were incubated at 37 °C, 5% CO_2 for a minimum of 24 h. Then 25 μL/well of MTT solution (5 mg/mL) was added and the plates were incubated for a further 5 h. Following the addition of 100 μL/well of sodium dodecyl sulfate (240 mg/mL) overnight, the absorbance was assessed at 595 nm, using microplate reader Thermo Scientific Multiskan FC (Vantaa, Finland).

2.3.2. In vitro cytotoxicity test

The *in vitro* cytotoxicity method was performed as described elsewhere [27], based on the neutral red uptake (NRU) assay, with the exposure of NCTC clone 929 cell line (mammalian fibroblasts, ATCC number CCL-1) to the degraded samples of rhGM-CSF. The pH of the samples was adjusted to 7.0, and positive and diluents controls, together with the *CRS rhGM-CSF* solution, were included in the assay. The NRU assay was performed on 96-well microplates, maintained at 37 °C in a CO_2 incubator for 24 h, with a cell suspension density of approximately 2 × 10^5 cells/mL. Neutral red released was evaluated by the addition of extractant solution, and the absorbance measured at 540 nm.

2.3.3. Samples and standard solutions

Working standard and sample solutions were prepared daily by diluting the *CRS rhGM-CSF* and the samples of pharmaceutical formulations in phosphate buffer 0.05 M pH 7.4 containing 0.5 mg/mL of polysorbate 80, to a final concentration of 100 μg/mL.

2.4. Validation of the method

The method was validated using samples of biopharmaceutical formulations of rhGM-CSF with a label claim of 300 μg/vial by determination of the following parameters: specificity, linearity, range, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), robustness, and system suitability test, following the ICH guidelines [25].