Artigo / Article

Biological potency evaluation and characterization of rhG-CSF in pharmaceutical products

Avaliação biológica da potência e caracterização de rhG-CSF em produtos farmacêuticos

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*The identification of rhG-CSF was carried out in pharmaceutical preparations by non-reducing polyacrylamide gel electrophoresis and western blotting with specific antibodies, showing a single band in the 19 kDa region. The potency was assessed by the neutropenia mouse bioassay giving values between 88.4 and 122.4% of the stated potency. The precision index expressed by weight was between 141 and 432 for the independent assays. Batch-to-batch, the samples met the requirements for the safety test and bacterial endotoxins test. The biological and immunological results showed the quality of the products in clinical use and the specifications established contribute to assuring the safety and efficacy of biological medicines. Rev. bras. hematol. hemoter. 2004;**26**(2):104-108.*

Key words: *Filgrastim; recombinant human granulocyte colony-stimulating factor; neutropenia; bioassay.*

Introduction

Granulocyte colony-stimulating factor (G-CSF) is a member of the haematopoietic colony stimulating factors family that is primarily produced by monocyte-macrophages, fibroblasts, bone marrow stromal cells and endothelial cells.[1]

The advances in recombinant DNA technology have facilitated the production of biological medicines and rhG-CSF is now available for clinical use in two forms: non-glycosylated and glycosylated. The non-glycosylated form of the hydrophobic protein, known as filgrastim, contains an extra methionine at its N-terminus, consisting of 175 aminoacids, derived from expression in *E. Coli.*[2,3] The glycosylated G-CSF, known as lenograstim, produced in Chinese hamster ovary (CHO) cells possesses an O-linked carbohydrate chain attached to threonine-133 of the molecule. Glycosylation has been reported to confer many advantages over non-glycosylation in terms of *in vitro* stability, although it does not appear to be essential for the biological activity of the G-CSF molecule.[4]

rhG-CSF stimulates the survival and proliferation of myeloid progenitor cells, as well as their differentiation towards neutrophilic granulocytes. In addition, G-CSF stimulates the release of mature neutrophils from bone marrow and brings about their activation. Clinical evaluation of rhG-CSF has demonstrated that it is useful in treating patients suffering from neutropenia during or after chemotherapy and in mobilising peripheral blood progenitor cells for harvesting and transplantation.[5-7]

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The activity of rhG-CSF can be assessed using biological assay models based on the evaluation of the leukocyte numbers after rhG-CSF treatment of cyclophosphamide-treated mice[8] or 5-fluorouracil-treated mice.[9] Growth factor-dependent cell line assays, using NFS-60 and GNFS-60 cells, can determine the proliferative activity of the G-CSF molecule.[10,11] The myeloid bone marrow colony assay evaluates both the proliferative and differentiation-inducing properties of the molecule.[5,12]

The present report describes the identification and biological potency evaluation of the recombinant non-glycosylated human G-CSF demonstrating the quality of pharmaceutical preparations in clinical use and suggesting specifications that will contribute to the safety and efficacy of these biological medicines.

Materials and Methods

Reagents and pharmaceutical products

The filgrastim reference substance was generously supplied as an NIBSC (National Institute for Biological Standards and Control) in-house reference substance, together with the sheep polyclonal anti-human G-CSF antibody (NIBSC, UK). Anti-goat IgG rabbit monoclonal antibody (whole molecule-alkaline phosphatase conjugate) was from Sigma-Aldrich, USA. Electrophoresis reagents were from Pharmacia Biotech, Sweden. Filgrastim pharmaceutical products containing 300 g of active substance per millilitre were identified by Arabic numerals from 1 to 5 and 6 to 10, respectively from manufacturers A and B; all preparations were within their shelf-life. Cyclophosphamide was from Asta Medica, Brazil, and May-Grünwald and Giemsa stains were from Inlab, Brazil.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Non-reducing SDS-PAGE was carried out on a vertical slab-gel electrophoresis apparatus using 1.5-mm thick polyacrylamide gels (17x13 cm). SDS was added to the samples, with final concentrations generally of 1% (w/v). Samples 1 to 10 (1.71 g) were left to migrate for about 12 h under 340 V, 20 mA and 50 W at 4 ºC. Resolved proteins were stained with silver nitrate.

Western blotting

After SDS-PAGE, the separated components were transferred to a nitrocellulose membrane (Bio-Rad, USA) by electrophoresis at a constant voltage of 24 V in 192 mmol/L glycine + 25 mmol/L Tris, containing methanol (1:4), for 1 h using a Trans-blot apparatus (Bio-Rad, USA). The membrane was washed for 1.5 h at room temperature in 50 mmol/L sodium phosphate + 150 mmol/L sodium chloride pH 7, containing 5% (w/v) dried skimmed milk powder. This was then reacted for 14 h at room temperature with anti-goat IgG rabbit monoclonal antibody (1:1000), in milk-containing phosphate buffered saline as above. Membrane-bound antibody was detected by reaction with an anti-goat IgG whole molecule-alkaline phosphatase conjugate at 1:5000, using a phosphatase substrate solution, containing (1:2:18) 5 mg/mL of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in DMF, 1 mg/mL of nitro-blue tetrazolium (NAT) in water, and alkaline phosphatase buffer (0.1M Tris + 0.1M NaCl + 5 mmol $MgCl_2$, pH 9.5), to develop the colour.

Laboratory animals

Male 8-week-old CF1 mice bred in specific pathogen-free conditions and weighing between 29 and 34 g were housed under controlled conditions (room temperature 22 ± 2 ºC; artificial illumination, 12 hours per day). Guinea pigs weighing between 300 and 350 g were also used.

Biological assay

The animals were allocated in a fully randomised manner into sample and standard groups and identified by colour coding for the assay with 6 mice per treatment group. Standard and samples were diluted to concentrations of 30, 60 and 120 g per mL with phosphate buffered saline containing 0.1% bovine serum albumin. Mice were injected intraperitoneally with 220 mg per mouse per 0.5 mL of cyclophosphamide on day 0. Multiple injections of 0.5 mL rhG-CSF per mouse were given to the cyclophosphamide-treated mice, from day 1 to day 4. Control mice were injected with the same volume of phosphate buffered saline. Six hours after the last rhG-CSF injection, peripheral blood was collected from the orbital venous sinus. Smears were prepared on glass slides and stained by the May-Grünwald-Giemsa method.[13] Three fields were selected and 25, 50 and 25 white cells were counted under a microscope (magnification x 40). The neutrophil numbers were expressed as a percentage of the total number of white cells and used for the statistical analysis.

Statistical analysis

Statistical analyses of the bioassay data were carried out according to Finney,[14] by parallel line methods (3 x 3), using SAS 6.1 for Windows (SAS Institute Inc., USA). Analysis of variance was performed for each assay and the assumption of linearity and parallelism of the log dose-log response lines was tested ($P<0.05$). Statistical weights were computed as the reciprocal of the variance of the log potencies. Estimates of log potency were examined for heterogeneity using a χ^2 test ($P=0.05$) and were combined as weighted geometric means of homogeneous estimates ($P>0.05$).[14,15] The specification suggested for the potency was between 90 and 110% of the stated potency with confidence limits of between 80 and 125%.

Safety test

The safety test was performed by intraperitoneal injection of the neat sample at 300 μg per mL in a volume of 0.5 mL in five mice and 5 mL in two guinea pigs. The animals were observed for 7 days,[16] and evaluated for any apparent toxic effect, loss of weight and mortality.

Bacterial endotoxins test

The test was carried out as previously described.[17] The endotoxins limit was calculated as 667 EU per mg and the maximum valid dilution established as 2 EU per mL for the samples with 300 μg per mL, using a *Limulus* amoebocyte lysate with sensitivity of 0.06 EU per mL.

Results

Polyacrylamide gel electrophoresis and immunoblotting

SDS-PAGE of the ten pharmaceutical samples revealed the same general pattern for each, as shown in Figure 1, with a single diffuse band in the 19 kDa molecular weight range, as indicated by the molecular weight markers and similar to the reference substance for G-CSF applied to the same gel. No dimeric or oligomeric bands were apparent within the detection limits of the system, indicating the absence of covalent (disulphide-linked) associations.

The same general pattern was revealed when the samples shown in figure 1 were subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane and immunodetection with a G-CSF-specific antiserum (Figure 2).

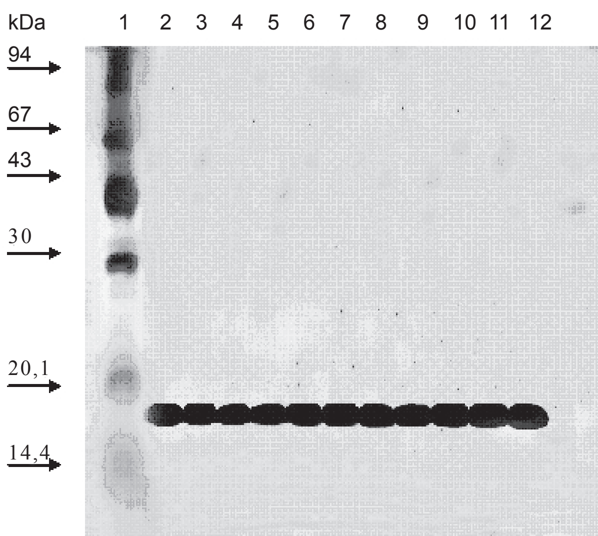

***Fig. 1** – Non-reducing SDS-PAGE showing a single band for rhG-CSF pharmaceutical preparations after detection with silver staining. Lane 1: Low molecular weight markers; Lane 2: Reference substance for G-CSF (1.71 g); Lanes 3-12: pharmaceutical products, respectively, 1 to 10 (1.71 g)*

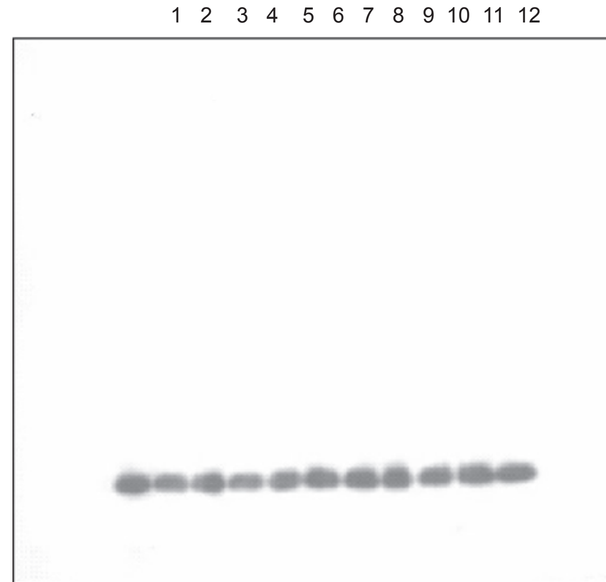

***Fig. 2** – Western blot showing a single band for rhG-CSF on the nitrocellulose membrane after immunodetection with a specific polyclonal antiserum. Lane 1: molecular weight markers; Lane 2: Reference substance for G-CSF (1.71 g); Lanes 3 to 12: pharmaceutical products, respectively, 1 to 10 (1.71 g).*

Biological potency evaluation by the neutropenia mouse assay

The neutrophil counts were analysed statistically by the parallel line assay (3 x 3), and the results of the independent or combined assays are presented in Table 1.

Table 1
Potency, confidence limits (P = 0.95) and precision of the estimates of the rhG-CSF in pharmaceutical products, by the neutropenic mouse bioassay

Sample	Potency			Confidence limits	Weight
	Stated	Found			
	μg/ml	μg/ml	%	(P = 0.95)	
1***	300	300.6	100.2	81 - 124	469
2*	300	367.2	122.4	98 - 155	420
3**	300	315.0	105.0	84 - 122	582
4***	300	307.2	102.4	92 - 124	805
5*	300	330.9	110.3	82 - 152	232
6*	300	265.2	88.4	66 - 117	269
7**	300	312.6	104.2	87 - 124	680
8**	300	284.7	94.9	80 - 112	756
9**	300	325.8	108.6	84 - 121	610
10**	300	324.9	108.3	83 - 123	599

** = number of independent assays*

Safety test

The pharmaceutical samples met the requirements of the test as none of the mice or guinea pigs lost weight, showed signs of ill health or died in the time interval of seven days.

Bacterial endotoxins test

All the samples gave semi-quantitative results between 0.06 and 0.12 EU per mL and met the requirements of the test.

pH

The samples gave pH values between 3.85 and 4.12 and met the specifications of pH between 3.7 and 4.3.

Discussion

The identification and purity evaluation carried out by non-reducing SDS-PAGE (Figure 1) showed a single band in the 19 kDa region, and the western blotting with specific antibodies confirmed the typical profile with a single band (Figure 2), according to the literature (5,11,18). Polyacrylamide gel electrophoresis (PAGE) and detection with specific antibodies are important for the identification of the recombinant proteins.

The dose-response curve was carried out with the reference substance for G-CSF and the doses for the biological assay were selected from the linear region of the curve. The bioassay was standardised on an animal model of neutropenia induced by anticancer drugs[8,9] and used for the potency evaluation of the biological medicines. The results obtained were between 88.4% and 122.4% of the stated potency. Table 1 shows that some samples are not approved for clinical use and also, the necessity of combination of independent assays in order to achieve the recommended precision. It is important to highlight that filgrastim is not described in any Pharmacopoeia, and in this paper values for the potency of between 90 and 110% are suggested, with confidence limits between 80 and 125%, following the procedures recommended for biological medicines.[19] The statistical requirements for assay validity, that is, significant regression and non significant deviation from linearity or parallelism of the log dose-log response lines were fully met.[14] The precision index calculated for the independent assays gave weight values between 141 and 432 (data not shown), considered to be a parameter of this biological assay.[15] The evaluation of the toxicity and bacterial endotoxins test, recommended to be carried out on a batch-to-batch basis, gave results according to the specifications for recombinant hormones.[16,19]

Extensive characterisation and quality control of rhG-CSF therapeutic products can be achieved using a combination of physicochemical, immunological and biological methods. However, the biological and immunological results presented here demonstrated the quality of the granulocyte colony-stimulating factor pharmaceutical preparations in clinical use and the specifications suggested will contribute to assuring the efficacy and safety of the products.

Resumo

*Realizou-se a identificação do fator estimulador da colônia de granulócitos humanos recombinante em produtos farmacêuticos por eletroforese em gel de poliacrilamida não redutora e imunodetecção com anticorpos específicos, que apresentaram banda única na região de, aproximadamente, 19 kDa. A avaliação de potência baseada na contagem do número de neutrófilos em camundongos com neutropenia forneceu valores entre 88,4 – 122,4 % em relação à potência declarada. A precisão expressa pela ponderação, calculada nos ensaios independentes, forneceu valores entre 141 e 432. As amostras lote-a-lote cumpriram os requisitos dos testes de toxicidade e endotoxinas bacterianas. Os resultados dos ensaios biológicos e imunológicos demonstram a qualidade dos produtos farmacêuticos em uso clínico, e as especificações sugeridas contribuem para assegurar a inocuidade e eficácia terapêutica dos produtos biológicos. Rev. bras. hematol. hemoter. 2004;**26**(2):104-108.*

Palavras-chave: *Filgrastima; fator estimulador da colônia de granulócitos humanos recombinante; neutropenia; bioensaio.*

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