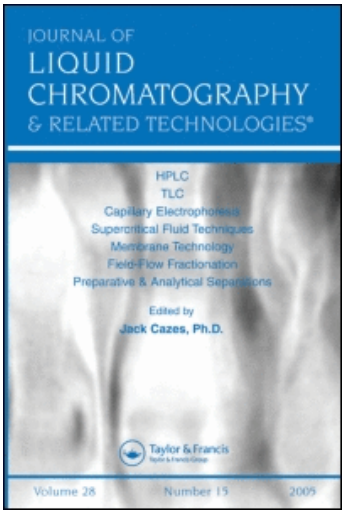

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Abstract: Gradient reversed-phase liquid chromatography (RP-LC) was validated for the analysis of rhG-CSF in pharmaceutical formulations. The LC method was carried out on a Jupiter C_4 column (250 mm × 4.6 mm I.D.), the mobile phase A consisted of water:acetonitrile (90:10, v/v) with 0.1% TFA and the mobile phase B was water:acetonitrile (20:80, v/v) with 0.1% TFA, run at a flow rate of 0.5 mL/min and detection at 280 nm. Validation parameters were evaluated and the method was linear in the range of 10–300 μg/mL. The dimers, high molecular mass forms, sulphoxides, and deamidates were analysed by the LC methods and then subjected to independent neutropenia mouse bioassay, giving overall biological activities within 13.47% and 15.63%. The pharmaceutical samples were analysed by the chromatographic methods and compared to the bioassay, showing mean difference between the estimated potency of 2.04% lower for the RP-LC, and 4.03% lower for the SE-LC, with significant correlation ($P > 0.05$). Due to the bioactivity of the rhG-CSF-related proteins, the SE-LC is

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proposed in combination with the RP-LC as an alternative to the bioassay for the potency assessment, improving the quality control of rhG-CSF in pharmaceutical dosage forms.

Keywords: Recombinant human granulocyte colony-stimulating factor (rhG-CSF), Neutropenia mouse bioassay, Size-exclusion chromatography, Reversed-phase chromatography, Validation

INTRODUCTION

The recombinant human granulocyte colony stimulating factor (rhG-CSF) is now currently available in two forms: non-glycosylated and glycosylated. The non-glycosylated form of the hG-CSF gene has been cloned and the recombinant protein produced in genetically engineered *Escherichia coli.* The hydrophobic protein is a 175 aminoacids chain containing an extra methionine at its N-terminus. Current clinical uses of rhG-CSF include promoting the recovery of neutrophil count in various conditions, such as neutropenia due to anticancer chemotherapy, neutropenia due to bone marrow transplantation, and neutropenia associated with the myelodysplatic syndrome or aplastic anemia.[1–4]

The biological activity of rhG-CSF can be assessed by the *in vivo* assays based on the evaluation of the neutrophils number of mice, leukocyte numbers after rhG-CSF treatment of cyclophosphamide treated mice, or 5-fluorouracil treated mice.[5–8] Growth factor dependent cell line assays using NFS-60 or G-NFS-60 cells, were used to determine the proliferative activity of the G-CSF molecule.[9–11]

The bioassays are useful to assess the quality, safety, and efficacy of those proteins, which could not be adequately characterized only by physicochemical tests. There have been decades of research on biologicals to reach a stage where the biological activity of proteins could be correlated with physicochemical analysis. The rhG-CSF is not described in any official literature and there is no correlation published between physicochemical methods and biological assays, that is highly recommended in the context of the reduction, refinement, and replacement of the bioassays.[12,13]

The chemical and physical instabilities of pharmaceutical proteins have been investigated and correlated to the biological properties of such forms. Chemical degradations include, at least, reduction, deamidation, and oxidation, that has been recognized to be an important cause of inactivation of therapeutic proteins and peptides, including rhG-CSF.[14,15] Protein aggregation may be induced by a variety of physical factors, and may result from chemical degradations or modifications as well, and may also have no or reduced activity, and altered immunogenicity.[16,17]

Today, many analytical techniques are available to monitor the purity, the chemical stability, and the potency of pharmaceutical proteins obtained through recombinant technology, but no single technique can satisfactorily provide sufficient information about a protein. Thus, a combination of

physicochemical, immunological, and biological method is recommended for the characterization and to monitor protein instability.[3,17] The size exclusion liquid chromatography (SE-LC) method was validated for the analysis of rhG-CSF in pharmaceutical formulations, demonstrating it to be able to detect and separate the dimers, related substances of higher molecular mass (HMM), and the intact protein.[18] The aggregation of rhG-CSF was investigated under physiological and native-like conditions.[16,19] Furthermore, the RP-LC has been widely used to separate and quantitate oxidized and deamidated proteins, and most likely influence the pharmacological activity of the compound by changing its polarity and/or lipophilicity. One drawback of this technique is that proteins are usually denatured or dissociated as they are adsorbed to the column matrix, and, thus, was found not to be an appropriate technique for establishing the potency of preparations that contain biologically inactive, non-covalent oligomers and polymers. The oxidation of rhG-CSF has been investigated, demonstrating that the RP-LC is also stability indicating.[20–22]

The aim of this paper, was to develop and validate a sensitive and specific RP-LC method that could be used in combination with the SE-LC for the analysis of the non-glycosylated rhG-CSF in pharmaceutical formulations, evaluating the correlation between the physicochemical methods and the biological assay, seeking for alternatives that can contribute to replace the bioassay, and to improve the quality control of this biological medicine.

EXPERIMENTAL

Chemicals and Reagents

The rhG-CSF reference substance was an in-house reference preparation generously supplied by the National Institute for Biological Standard and Control (NIBSC, UK). A total of eleven batches of rhG-CSF (filgrastim) sample preparations containing 300 μg of active substance per millilitre were obtained from commercial sources and used within their shelf life period, and were identified by Arabic numbers from 1 to 11. Hydrogen peroxyde 30% in aqueous solution was from Merck (Darmstadt, Germany). HPLC grade acetonitrile, sodium hydroxide, potassium phosphate monobasic, sodium phosphate dibasic, sodium chloride, trifluoroacetic acid, and phosphoric acid were purchased from Merck (Darmstadt, Germany). Ifosfamide was from Asta Medica, Brazil, and May-Grünwald and Giemsa stains were from Inlab, Brazil. For all analyses, ultrapure water (Labconco, Kansas City, USA) filtered through a 0.22 μm membrane filter was used.

Laboratory Animals

Male 7-8-week-old BALB/c mice were housed in air conditioned controlled conditions (room temperature $22 \pm 2^{\circ}C$ and relative humidity of 65%;

artificial illumination, 12 h per day), and they were used weighting between 19 and 24 g. They were given food and water ad libitum.

Biological Assay

The animals were allocated to sample, standard, and control groups in a fully randomised order and identified by color code for the assay, usually with 6 mice per treatment group. Standard and test samples were diluted to the concentrations with 4, 12, and 36 μg per mL with phosphate buffered saline containing 0.1% bovine serum albumin. A single dose of 200 mg of ifosfamide/0.5 mL per mouse was injected intraperitoneally into the respective animal on day 0. Multiple injections of 0.5 mL rhG-CSF per mouse were injected, to the ifosfamide treated mice, from day 1 to day 4. 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, and the white cells counted and expressed as percentage of the total number of neutrophils.

Statistical Analysis

Statistical analyses of the bioassay data were carried out according to Finney[23] by parallel line methods (3 × 3), using a PLA 1.2 Program (Stegmann Systemberatung, Rodgau, Germany). 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 potency. 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 ($P > 0.05$).[24]

Apparatus and Chromatographic Conditions

A Shimadzu LC system (Shimadzu, Kyoto, Japan) was used equipped with an SCL-10A_{VP} system controller, LC-10 AD_{VP} pump, DGU-14A degasser, CTO-10A_{vp} column oven, SIL-10AD_{VP} autosampler, and an SPD-M10A_{VP} photodiode array (PDA) detector. The detector was set at 214 nm for size exclusion and 280 nm for reversed phase, and peak areas were integrated automatically by computer using a Shimadzu Class VP® V 6.12 software program.

Size-Exclusion Chromatography (SE-LC)

The experiments were carried out on a size exclusion Phenomenex (Torrance, USA) BioSep-SEC-S 2000 column (300 mm × 7.8 mm I.D.).

A GCF-2000 Kit Security Guard Cartridge (Phenomenex) was used to protect the analytical column. The LC system was operated isocratically at controlled ambient temperature (25°C), using a mobile phase of phosphoric acid 0.1 M, pH 2.5 adjusted with sodium hydroxide 3 M. This was filtered through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA), and run at a flow rate of 1.0 mL/min. At the beginning of each experiment, 50 μL bovine albumin (1 mg/mL) was injected onto the column to reduce non-specific adsorption. The injection volume was 50 μL for both standard and samples.[18]

Reversed-Phase Chromatography (RP-LC)

The experiments were carried out 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) and a C_4 Kit Security Guard Cartridge was used to protect the analytical column. The LC system was operated at controlled ambient temperature (25°C). The elution was performed by a fast gradient at a constant flow rate of 0.5 mL/min. Mobile phase A consisted of water:acetonitrile (90:10, v/v) containing 0.1% trifluoroacetic acid (TFA), and mobile phase B consisted of water:acetonitrile (20:80, v/v) containing 0.1% TFA. The applied gradient was as follows: 0.1 min 66% of B, from 0.1–35 min linear to 85% of B, from 35.1–38 min linear back to 66% and 38.1–48 min 66% of B. The mobile phases were filtered through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA). The injection volume was 50 μL for both standard and samples.

Procedure

Samples and Standard Solutions

Working standard and sample solutions of rhG-CSF were prepared daily by diluting the reference standard and the sample of pharmaceutical formulation in water to a final concentration of 200 μg/mL for the LC methods, and with phosphate buffered saline containing 0.1% bovine serum albumin, to appropriate concentrations for the bioassay.

Dimers and HMM Solutions

The dimers and HMM were artificially produced subjecting the sample of pharmaceutical formulations at a concentration of 300 μg/mL, under strong mixing during 5 min, and maintaining the sample in water bath at 70°C during 1 h, respectively. The samples were analysed by SE-LC and used for the biological activity assessment.

Sulphoxide and Deamidate Solutions

The sulphoxide and deamidate related proteins were produced by adding 15 μL of hydrogen peroxyde 30% during 8 min, to the sample of pharmaceutical formulations, at a concentration of 300 μg/mL. The degraded samples were analysed by RP-LC and used for the biological activity evaluation.

Validation of the RP-LC Method

Once the chromatographic and the experimental conditions were established, the method was validated by the determination of the following parameters: specificity, linearity, range, precision, accuracy, robustness, limit of detection (LOD), limit of quantitation (LOQ), and system suitability tests, following the ICH guidelines.[25]

Specificity

Specificity of the method towards the drug was established through the determination of the peak purity of samples of pharmaceutical formulation of rhG-CSF, subjected to degradation by oxidative conditions and analysed by the RP-LC method using a PDA detector.

Linearity

The range of linearity was determined by constructing three calibration curves. For the construction of each calibration curve, eight standard concentrations of rhG-CSF in the range of 10–300 μg/mL were prepared. Before injection of the solutions, the column was equilibrated for at least 45 min with the mobile phase flowing through the system. Each measurement was carried out in three replicates of 50 μL injection for the standard solution to verify the repeatability of the detector response at each concentration. The peak areas of the chromatograms were plotted against the concentrations of rhG-CSF to obtain the calibration curve. The eight concentrations of the standard solution were subjected to regression analysis to calculate calibration equation and correlation coefficients.

Precision

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was determined by performing six repeated analysis of the same samples of rhG-CSF, on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on three different days (inter-day) and, also, by another analyst performing the analysis in the same laboratory (between-analysts).

Accuracy

The accuracy was evaluated applying the proposed method to the analysis of the in-house mixture of the formulation excipients with known amounts of the reference drug, corresponding to the concentrations of 80, 100, and 120%. The accuracy was calculated as the percentage of the drug recovered from the formulation matrix.

Robustness

The robustness was assessed by altering the following experimental conditions such as: changing the flow rate from 0.3 to 1.0 mL/min, the injection volume from 30 to 60 μL, the wavelength in the range of 210 to 320 nm, the temperature of the column between 25 to 40°C, and stability of the analytical solution in the autosampler at controlled ambient temperature (25°C).

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

For the calculation of the LOD and the LOQ, a calibration equation was generated by using the mean values of the three independent calibration curves. The LOD and the LOQ were obtained by using the mean of the slope and the standard deviation of the intercept of the independent curves, determined by a linear regression line as defined by ICH.[25]

System Suitability

To ensure the validity of the analytical procedure, data from five injections of 50 μL of the working standard solution containing 200 μg/mL were used for the evaluation of the system suitability parameters like asymmetry, number of theoretical plates, and retention time and area, through the CLASS-VP® V 6.12 software.

RESULTS AND DISCUSSION

Validation of the RP-LC Method

A typical chromatogram was obtained by the proposed RP-LC method, demonstrating the resolution of the symmetrical peak corresponding to rhG-CSF with the retention time of 31.9 min, and no interfering peaks due to the excipients is shown in Figure 1. The specificity of the analytical method was indicated, by the oxidative conditions that generated additional peaks

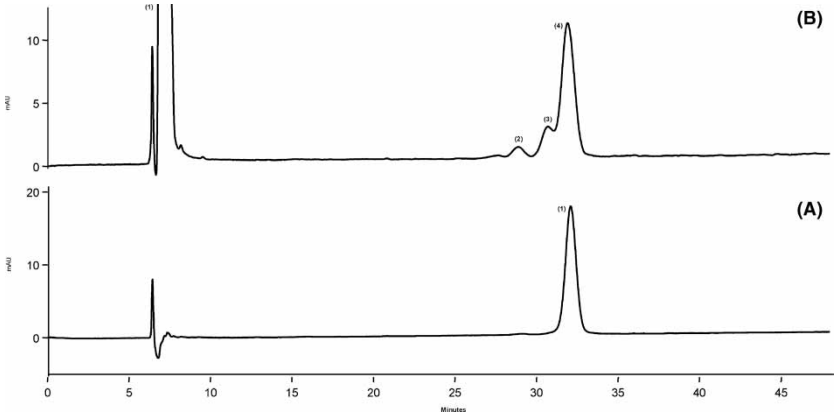

Figure 1. RP-LC chromatograms of rhG-CSF. (A) Reference substance (200 μg/mL): peak 1–main peak. (B) degraded pharmaceutical sample: peak 1–hydrogen peroxide, peak 2–deamidates, peak 3–sulphoxides, and peak 4–main peak.

with retention times of 28.9 and 30.7 min, of deamidates and sulphoxides. The studies with the PDA detector showed that the rhG-CSF peak was free from any coeluting peak, thus demonstrating that the proposed method is specific for the analysis of rhG-CSF.

The calibration curves for rhG-CSF were constructed by plotting the area of the peaks versus concentration. Linearity was observed in a concentration range from 10 to 300 μg/mL. The value of the determination coefficient ($r^2 = 0.9999$, $y = 3773.4x - 16617.9$) indicated significant linearity of the calibration curve for the method.

The precision of an analytical method was studied as the repeatability, by calculating the relative standard deviation (RSD) for six determinations of the concentration of 200 μg/mL. The mean of the determinations of rhG-CSF in the samples of pharmaceutical formulation was 98.79%, with the RSD calculated as 0.36%.

The intermediate precision was assessed by analyzing two samples of the pharmaceutical formulation on three different days (inter-day, Table 1); the RSD values obtained were 1.12 and 0.48%, respectively. Between analysts, precision was determined by calculating the RSD for the analysis of three samples of the pharmaceutical formulation by two analysts; the values were found to be 1.40, 1.18, and 0.69%, respectively (Table 2).

The accuracy was assessed from three replicate determinations of three different solutions containing 160, 200, and 240 μg/mL. The absolute means obtained were 99.42, 100.37, and 99.87% respectively, with a mean value of 99.90% and RSD of 0.47% as shown in Table 3. It is evident that the method is accurate within the desired range.

Table 1. Inter-day precision data of RP-LC for rhG-CSF in samples of pharmaceutical formulation

Sample	Day	Recovery[a] (%)	Mean[b] (%)	RSD[c] (%)
1	1	101.20	100.40	1.12
	2	99.09		
	3	100.84		
2	1	100.35	99.80	0.48
	2	99.74		
	3	99.39		

[a]Mean of three replicates.
[b]Mean of three days.
[c]RSD = Relative standard deviation.

The robustness was determined by analyzing the same pharmaceutical samples under the variety of conditions described. The results and the experimental range of the selected variables are given in Table 4, together with the optimized values. There were no significant changes in the chromatographic pattern when the above modifications were made in the experimental conditions, thus showing that the method is robust.

The LOD and the LOQ calculated were 0.66 and 2.20 $\mu g/mL$, respectively. The LOQ evaluated in an experimental assay, with the precision lower than 5% and accuracy within $\pm 5\%$, was found to be 10 $\mu g/mL$.

The system suitability tests were also carried out to evaluate the resolution and reproducibility of the system for the analysis to be performed. Results of system suitability tests are given in Table 5, showing that the parameters are within the suitable range.

Table 2. Between-analyst precision data of RP-LC for rhG-CSF in samples of pharmaceutical formulation

Sample	Analyst	Recovery[a] (%)	Mean[b] (%)	RSD[c] (%)
1	A	98.71	99.70	1.40
	B	100.68		
2	A	100.29	101.13	1.18
	B	101.97		
3	A	100.37	99.88	0.69
	B	99.39		

[a]Mean of three replicates.
[b]Mean of two analysts.
[c]RSD = Relative standard deviation.

Table 3. Accuracy of RP-LC for rhG-CSF in samples of pharmaceutical formulation

Nominal concentration (μg/mL)	Mean concentration found[a] (μg/mL)	RSD[b] (%)	Accuracy (%)
160	159.07		
200	200.75	0.47	99.90
240	239.70		

[a]Mean of three replicates.
[b]RSD = Relative standard deviation.

Analysis of Pharmaceutical Dosage Forms by Physicochemical and Biological Methods

The analysis of eleven commercial batches of pharmaceutical dosage forms, carried out in parallel by the chromatographic and biological methods, resulted in a mean difference between estimated potency of 2.04% ± 2.67 lower for the RP-LC compared to the bioassay, except for the sample 10, that due to the altered forms, showed 5.34% higher value than the biological potency, according to the Table 6. The mean difference of the estimated potency was also 4.03% ± 1.32 lower for the SE-LC compared to the bioassay, and the highest difference was 5.50%, with significant correlation as calculated by the Student's *t*-test ($P > 0.05$). As shown by the samples

Table 4. Chromatographic conditions and range investigated during robustness testing

Variable	Range investigated	rhG-CSF[a] (%)	Optimized value
Flow rate (mL/min)	0.3	101.98	
	0.5	100.01	0.5
	0.8	101.25	
	1.0	99.96	
Column temperature (°C)	25	100.53	25
	30	101.20	
	35	101.77	
	40	102.10	
Injection volume (μL)	30	100.42	
	50	100.05	50
	60	101.10	
Solution stability	Autosampler 24 h	99.20	—
Wavelength (nm)	210–320	—	280

[a]Mean of three replicates.

Table 5. Results of the system suitability test

Parameter	Minimum	Maximum	RSD (%)	Status
Asymmetry	1.04	1.05	0.42	Passed
Theoretical plates	13075	13626	1.55	Passed
Retention time	31.81	31.90	0.10	Passed
Area	878897	896292	0.85	Passed

10 and 11, these values can change due to the content of the rhG-CSF related contaminants of each product, indicating the importance of the identity and purity evaluation of the samples before the quantitative control of the potency by SE-LC.

The results of the content/potency were calculated against the reference substance and expressed as percentage of the main peak, for the RP-LC, and the monomer for the SE-LC, also illustrated with the mean values for each method (Table 6). The results of the analysis of the aggregates and

Table 6. Determination of the rhG-CSF potency in pharmaceutical dosage forms against the reference standard

			Bioassay[a]	
Sample	SE-LC[a,b] (%)	RP-LC[a,b] (%)	Potency (%)	Confidence limits (P = 0.95)
1	97.20	98.30	102.70***	90–117
2	95.81	96.53	100.20**	82–122
3	94.50	97.11	98.40*	71–136
4	93.02	94.25	98.00*	71–134
5	99.22	101.60	103.10*	79–134
6	110.00	111.50	113.50*	87–150
7	94.76	96.20	99.20**	77–127
8	95.92	98.19	101.10*	83–123
9	121.56	124.59	126.10*	103–156
10	74.80	83.54	78.20*	64–95
11	86.90	83.80	87.50*	72–106
Mean	96.70	98.69	100.73	—
SD[c]	11.84	11.52	12.31	—

*Number of independent assays.
[a]Non-significant difference ($P > 0.05$).
[b]Mean of three replicates.
[c]SD = Standard deviation.

Table 7. Analysis of the rhG-CSF-related proteins in pharmaceutical dosage forms

	SE-LC[a]			RP-LC[a]	
Sample	Monomer (%)	Dimer (%)	HMM (%)	Main peak (%)	Deamidates/ sulphoxides (%)
1	99.26	0.18	0.56	99.88	0.12
2	99.84	0.08	0.08	99.93	0.07
3	99.78	0.22	0.00	99.90	0.10
4	99.53	0.00	0.47	99.49	0.51
5	99.34	0.00	0.66	100.00	0.00
6	99.93	0.00	0.07	99.95	0.05
7	99.34	0.00	0.66	99.58	0.42
8	99.51	0.41	0.08	99.61	0.39
9	99.78	0.00	0.22	99.81	0.19
10	83.76	11.80	4.44	99.89	0.11
11	99.81	0.00	0.19	95.68	4.32
Mean	98.17	1.15	0.68	99.43	0.57
SD[b]	4.79	3.53	1.27	1.25	1.25

[a]Mean of three replicates.
[b]SD = Standard deviation.

degraded forms were expressed as percentage of the total area obtained in the respective chromatographic procedure, as shown in Table 7.

Besides, some pharmaceutical samples were artificially degraded and analysed by the RP-LC validated method, and subjected to the neutropenia mouse bioassay, resulting, for the sulphoxides and deamidates forms, in a mean biological activity of $15.63 \pm 5.73\%$ ($n = 3$). Moreover, the dimers and HMM were also analysed by SE-LC and subjected to the bioassay, resulting in mean biological activities of $14.60 \pm 3.81\%$ ($n = 3$) and $13.47 \pm 5.22\%$ ($n = 3$), respectively. Therefore, the RP-LC method is recommended for the analysis of the related proteins, and the SE-LC for the evaluation of dimers and HMM, and for the potency assessment of rhG-CSF in pharmaceutical formulations.

A current concern in the administration of recombinant derived proteins, is that the presence of rhG-CSF related contaminants can have undesirable side effects and usually may have no or reduced activity, as demonstrated in the present article. So, proper quality controls must be taken to ensure that the levels of such forms are accurately determined, and the limits in the pharmaceutical products must be well established. The combination of the validated physicochemical techniques employed, offered a high degree of resolving power and selectivity and is suggested as an alternative in the context of the replacement of the bioassay for the quality control of rhG-CSF in pharmaceutical dosage forms.

CONCLUSION

The results of the validation show that the proposed RP-LC method is specific, accurate, precise, and possesses excellent linearity characteristics, and has been used for the analysis of the pharmaceutical formulations. Moreover, based on the reduced biological activity of the rhG-CSF related forms, and the correlation between the SE-LC and the neutropenia mouse bioassay, the SE-LC method is proposed in combination with the RP-LC, as an alternative to the bioassay for the potency evaluation of rhG-CSF, that can be applied to the purification process and to the quality assessment of the pharmaceutical product.

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REFERENCES

1. Zsebo, K.M.; Cohen, A.M.; Murdock, D.C.; Boone, T.C.; Inoue, H.; Chazin, V.R.; Hines, D.; Souza, L.M. Recombinant human granulocyte colony stimulating factor: Molecular and biological characterization. Immunobiol. **1986**, *172*, 175–184.
2. Lu, H.S.; Clogston, C.L.; Narhi, L.O.; Merewether, L.A.; Pearl, W.R.; Boone, T.C. Folding and oxidation of recombinant human granulocyte colony stimulating factor produced in *Escherichia coli*. J. Biol. Chem. **1992**, *267* (13), 8770–8777.
3. Herman, A.C.; Boone, T.C.; Lu, H.S. Characterization, formulation, and stability of Neupogen® (Filgrastim), a recombinant human granulocyte colony stimulating factor. In *Formulation, Characterization, and Stability of Protein Drugs*; Pearlman, R., Wang, Y.J., Eds.; Plenum Press: New York, 1996, 303–326.
4. Rubenstein, E.B. Colony stimulating factor in patients with fever and neutropenia. Intl. J. Antimicrob. Agents **2000**, *16*, 117–121.
5. Bowen, S.; Tare, N.; Inoue, T.; Yamasaki, M.; Okabe, M.; Horii, I.; Eliason, J.F. Relationship between molecular mass and duration of activity of polyethylene glycol conjugated granulocyte colony stimulating factor mutein. Exp. Hematol. **1999**, *27*, 425–432.
6. Matsuzaki, G.; Li, X.Y.; Ohyama, Y.; Nomoto, K. Kinetics of serum granulocyte colony stimulating factor (G-CSF) concentration and G-CSF receptor expression during G-CSF treatment of cyclophosphamide treated mice. Intl. J. Immunoph. **1996**, *18*, 363–369.
7. Kaneko, M.; Kawakita, T.; Kumazawa, Y.; Takimoto, H.; Nomoto, K.; Yoshikawa, T. Accelerated recovery from cyclophosphamide induced leukopenia in mice administered a japanese ethical herbal drug, *Hochu-ekki-to*. Immunopharmacol. **1999**, *44*, 223–231.

8. Shimamura, M.; Kobayashi, Y.; Yuo, A.; Urabe, A.; Okabe, T.; Komatsu, Y.; Itoh, S.; Takaku, F. Effect of human recombinant granulocyte colony-stimulating factor on hematopoietic injury in mice induced by 5-fluorouracil. Blood. **1987**, *69*, 353–355.
9. Shirafuji, N.; Asano, S.; Matsuda, S.; Watari, K.; Takaku, F.; Nagata, S. A new bioassay for human granulocyte colony-stimulating factor (hG-CSF) using murine myeloblastic NFS-60 cells as targets and estimulation of its levels in sera from normal healthy persons and patients with infectionus and hematological disorders. Exp. Hematol. **1989**, *17*, 116–119.
10. Hammerling, U.; Kroon, R.; Sjödin, L. *In vitro* bioassay with enhanced sensitivity for human granulocyte colony-stimulating factor. J. Pharm. Biomed. Anal. **1995**, *13* (1), 9–20.
11. Oshima, Y.; Tojo, A.; Niho, Y.; Asano, S. Biological activity of human granulocyte colony stimulating factor with a modified c-terminus. Biochem. Biophys. Res. Commmun. **2000**, *267*, 924–927.
12. Mire-Sluis, A.R.; Gerrard, T.; Das, R.G.; Padilha, A.; Thorpe, R. Biological assays: Their role in the development and quality control of recombinant biological medicinal products. Biologicals **1996**, *24*, 351–362.
13. Bristow, A.F.; Jeffcoate, S.L. Analysis of therapeutic growth hormone preparations: Report of an interlaboratory collaborative study on growth hormone assay methodologies. Biologicals **1992**, *20*, 221–231.
14. Lu, H.S.; Fausset, P.R.; Narhi, L.O.; Horan, T.; Shinagawa, K.; Shimamoto, G.; Boone, T.C. Chemical modification and site-directed mutagenesis of methionine residues in recombinant human granulocyte colony-stimulating factor: Effect on stability and biological activity. Biochem. Biophys. **1999**, *362*, 1–11.
15. Yin, J.; Chu, J.W.; Ricci, M.S.; Brems, D.N.; Wang, D.I.C.; Trout, B.L. Effects of excipients on the hydrogen peroxide-induced oxidation of methionine residues in granulocyte colony-stimulating factor. Pharm. Res. **2005**, *22* (1), 141–147.
16. Raso, S.W.; Abel, J.; Barnes, J.M.; Maloney, K.M.; Pipes, G.; Treuheit, M.J.; King, J.; Brems, D.N. Aggregation of granulocyte-colony stimulating factor *in vitro* involves a conformationally altered monomeric state. Protein Sci. **2005**, *14*, 2246–2257.
17. Wang, W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Intl. J. Pharm. **1999**, *185*, 129–188.
18. Codevilla, C.F.; Brum, L., Jr.; Oliveira, P.R.; Dolman, C.; Rafferty, B.; Dalmora, S.L. Validation of an SE-HPLC method for the analysis of rhG-CSF in pharmaceutical formulations. J. Liq. Chromatogr. & Rel. Technol. **2004**, *27*, 2681–2690.
19. Krishnan, S.; Chi, E.Y.; Webb, J.N.; Chang, B.S.; Shan, D.; Goldenberg, M.; Manning, M.C.; Randolph, T.W.; Carpenter, J.F. Aggregation of granulocyte colony stimulating factor under physiological conditions: Characterization and thermodynamic inhibition. Biochemistry **2002**, *41*, 6422–6431.
20. Dalmora, S.L.; Oliveira, J.E.; Affonso, R.; Gimbo, E.; Ribela, M.T.C.P.; Bartolini, P. Analysis of recombinant human growth hormone directly in osmotic shock fluids. J. Chromatogr. A. **1997**, *782*, 199–210.
21. Leon, J.; Reubsaet, E.; Beijnen, J.H.; Bult, A.; Hop, E.; Scholten, S.D.; Teeuwsen, J.; Underberg, W.J.M. Oxidation of recombinant methionyl human granulocyte colony stimulating factor. J. Pharm. Biomed. Anal. **1998**, *17*, 283–289.
22. Léon, J.; Reubsaet, E.; Beijnen, J.H.; Bennekom, W.P.V.; Bult, A.; Hoekstra, A.J.; Hop, E.; Os, P.J.H.J.V.; Teeuwsen, J.; Underberg, W.J.M. Reduction of Cys^{36}–Cy^{42} and Cys^{64}–Cy^{74} disulfide bonds in recombinant human granulocyte colony stimulating factor. J. Pharm. Biomed. Anal. **1999**, *19*, 837–845.

23. Finney, J.D. *Statistical Methods in Biological Assay*; Charles Griffin: London, 1978.
24. Gaines, D.R.E.; Tydeman, M.S. Iterative weighted regression analysis of logit responses. A computer program for the analysis of bioassays and immunoassays. Comput. Programs Biomed. **1980**, *15*, 13–22.
25. International Conference on Harmonisation (ICH) of Technical Requirements for the Registration of Pharmaceutical for Human Use, Validation of Analytical Procedures: Methodology (ICH-Q2B), November 1996; 1–8.

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