

A.Chammakhi, S.Kraiem, H. Loueslati

Study of r-Met-hu-G-CSF (recombinant Granulocyte colony-stimulating factor) by isoelectric focusing and RP-HPLC

Laboratoire National de Contrôle des Médicaments, Laboratoire de dépistage du dopage, 11 bis Rue Jebel Lakhdar Bab Saâdoun 1006 Tunis, Tunisie

Introduction

Granulocyte colony-stimulating factor (G-CSF) is a 18 800 Dalton glycoprotein, it is a growth factor or cytokine produced by a number of different tissues: monocytes, fibroblasts and endothelial cells [1].

G-CSF then stimulates the bone marrow to release them into the blood. It induces proliferation, differentiation and activation of the neutrophil-granulocyte lineage and also enhances some mature neutrophil functions [2].

Recombinant human r-Met-hu-G-CSF is mainly applied to reduce febrile neutropenia in patients with non-myeloid malignancies that receive myelosuppressor. Drugs G-CSF is also used to increase the number of hematopoietic stem cells in the blood of the donor before collection by leukapheresis for use in hematopoeitic stem cell transplantation [3]. It may also be given to the receiver, to compensate for conditioning regimens.

Structure of r-Met-hu-G-CSF

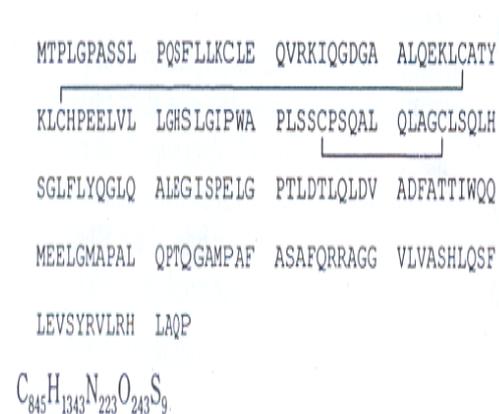


Figure 1. Sequence of amino acids of r-G-CSF

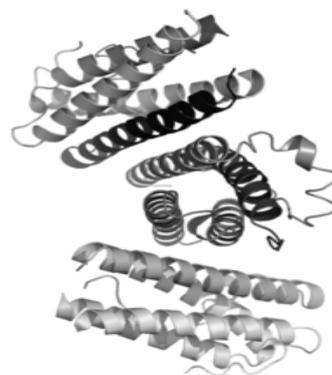


Figure 2. 3D structure of r-G-CSF

ISOELECTRIC FOCUSING

Experimental conditions

Gel characteristic Acrylamide T=5% -C=3%
 Ampholyte 5-8 (final dilution 1/6)
 12% sorbitol
 Thin layer 1mm

Sample preparation:

The sample used is a pharmaceutical product having as concentration 1 mg /ml. It was diluted with sodium acetate buffer to obtain a final concentration of 0.25 mg/ml.

Focusing condition

Cathode solution: sodium hydroxyde 1M
 Anode solution: glutamic acid 0.04M/ phosphoric acid (0.0025% V/V)
 First step: 250 V, 150 mA, 15 min
 Second step: 1500 V, 50 mA, 15 W, 1.5 h

Results

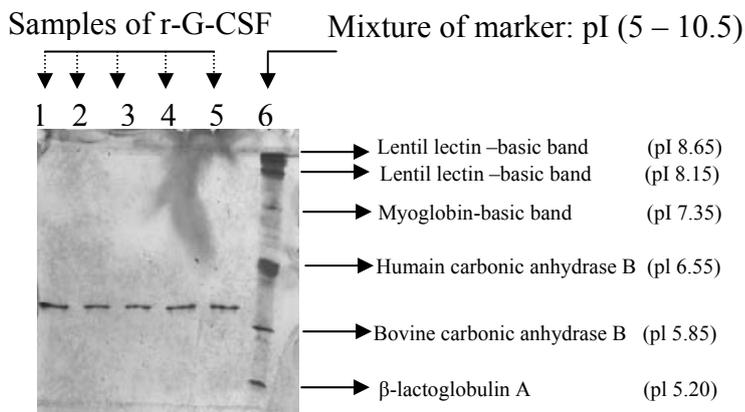


Figure 3. Focusing image of r-G-CSF

The figure 3 illustrates the focusing of r-G-CSF samples (lane1-5) on an IEF gel. On lane 6, the IEF markers with known pI's will be focused in their respective isoelectric points serving to determinate the pH gradient. The identity of the focused band of r-G-CSF samples can be determined by noting the positions of pI markers bands. The result of using pI calibration shows that the pI of r-G-CSF is 6.10 ± 0.15 .

Other studies of final pharmaceutical product of r-G-GSF confirm that the pI value should varied from 5.5 to 6.1 [4] according to the degree of glycosylation.

The researches concerning the focusing isoform G-CSF of treated neutropenia patient urine show that there is no band at pI 6.10. This fact may be explained by the low concentration of r-G-CSF in urine (about 0.025µg/mL) [5].

RP-HPLC

Experimental conditions

Sample preparation

The pharmaceutical sample having as initial concentration 1mg/mL was diluted with sodium acetate buffer pH 4 to obtain 0.3 mg/ml

RP-HPLC parameters

Mobile phase: 95% A (99.90 %H₂O/ 0.1% TFA)

5% B (99.90 % ACN/ 0.1% TFA)

Stationary phase: column C4 (250mm x 4.6 mm, 5 microns of particle size)

Temperature of column compartment: 25°C

Flow rate: 0.8 ml/min isocratic mode

Detection: UV detection at 214 nm

Injection volume: 50 µl

Results

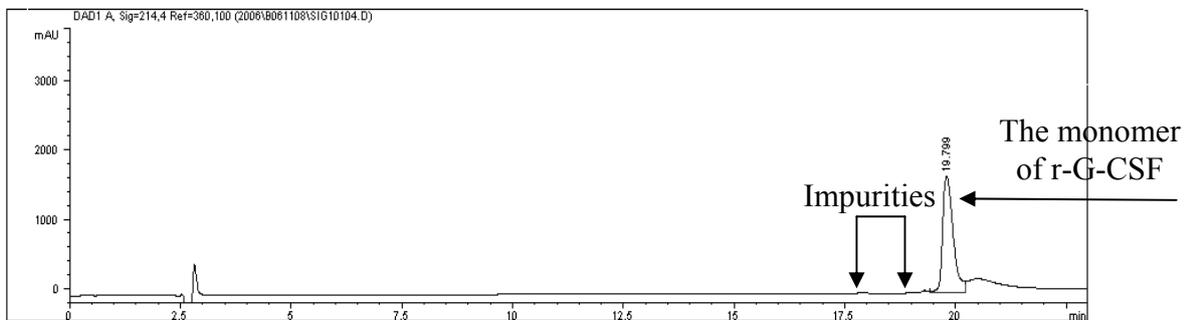


Figure 4. Typical chromatogram of r -G-CSF

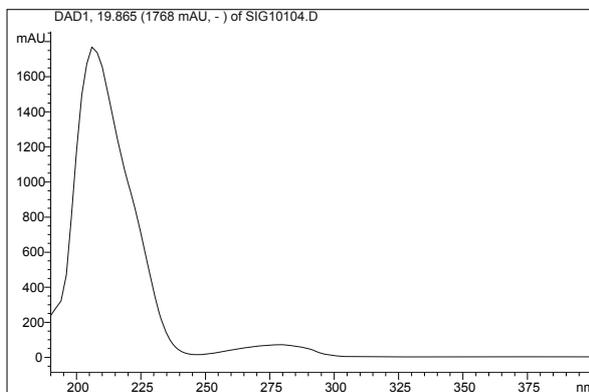


Figure 5. UV spectra of r-Met-hu-G-CSF

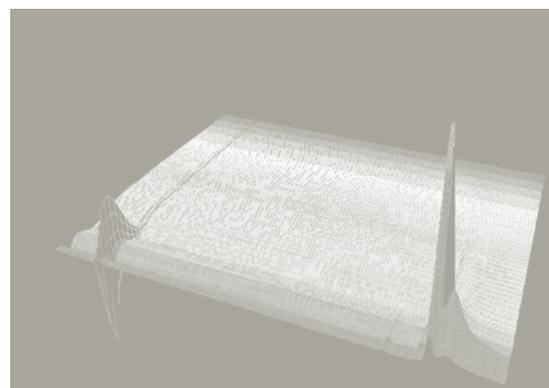


Figure 6. 3D chromatogram of r-Met-hu-G-CSF

The Typical chromatogram (figure 4) proves that the main pic presenting r-G-CSF is eluted at about 20 min while the retentions time of impurities pics are inferior to 20 min. The study of UV spectra (figure 5) shows that the ideal wave length for the detection of r-G-CSF is 214 nm. The 3D chromatogram (figure 6) shows that there is a good separation between the monomer form and the impurities (oxides forms of r-G-CSF). In fact the impurities may be eluted at the same time of the main pic of r-G-CSF.

The limit of HPLC method is in the range of 30 µg/ml but the concentration of r-G-CSF in the urine is in the range of monogram according to the results of ELISA dosage [5].

Conclusion and perspectives

The study of Recombinant Granulocyte Factor r-Met-hu-G-CSF can give some information about chemical and biological behavior of the human G-CSF due to the similarity of the two hormones.

In order to optimize the IEF result, the sample preparation will be performed to obtain a higher concentration.

The use of immuno-detection can improve the quality of revelation and decrease the limit of detection.

Liquid Chromatography coupled at mass spectrometry could be the method of choice to differentiate the two forms: endogenous and exogenous

References

- [1] Lieschke GJ, Burgess AW. (1992) Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (1). *N Engl J Med.* **327**, 28–35.
- [2] Thomas J, Liu F, Link DC. (2002) Mechanisms of mobilization of hematopoietic progenitors with granulocyte colony-stimulating factor. *Curr Opin Hematol.* **9**, 183-189.
- [3] Hirbe AC, Uluçkan O, Morgan EA, Eagleton MC, Prior JL, Piwnica-Worms D, Trinkaus K, Apicelli A, Weilbaeher K . (2007) Granulocyte colony-stimulating factor enhances bone tumor growth in mice in an osteoclast-dependent manner. *Blood.* **109**, 3424–31.
- [4] European Directorate for the Quality of Medicine (edqm), (2007) Filgrastim concentrated solution. PHARMEUROPA 19.1, 104-108.
- [5] Darlene A. Calhoun, Mathilde Lunøe, Yan Du, Alan D. Hutson, Mark Veerman, and Robert D. Christensen. (2000) Granulocyte colony-stimulating factor serum and urine concentrations in neutropenic neonates before and after intravenous administration of recombinant granulocyte colony-stimulating factor. *Pediatrics.* **105**, 392-397