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Characteristics of IEF Patterns, SDS-PAGE and SAR-PAGE: Result of Cuban rEPO Biosimilar

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Abstract

The aim of the present study was to investigate whether biosimilar alpha r-HuEPO – ior®-EPOCIM, produced in Cuba and also available in other countries, could be differentiated from endogenous urinary EPO, by isoelectric focusing (IEF) plus double blotting, SDS-PAGE and SAR-PAGE for antidoping analysis.
The band pattern of three lots of Cuban rEPO was studied (two Reference Materials and one Injectable Preparation).
All three lots were detected in the basic region of the IEF-gel and showed discriminative profiles from endogenous EPO. Additionally, slightly different band patterns compared to the rEPO reference (BRP-EPO) were observed. SDS-PAGE and SAR-PAGE of ior®-EPOCIM resulted in different molecular masses, which were higher than the mass of endogenous EPO. In conclusion, IEF with double blotting and SDS-PAGE/SAR-PAGE with immunoaffinity purification can be used to discriminate the tested Cuban rEPO biosimilar from endogenous EPO.

Introduction

EPO is a glycoprotein produced in the kidney, which stimulates the division and differentiation of stem cells in the bone marrow into red blood cells. Erythropoietin is available as a therapeutic agent produced by recombinant DNA technology in mammalian cell culture into which the human EPO gene has been transfected [1,2]. Biosimilar Epoetins are mostly erythropoietins of the Epoetin alfa, and beta type, which are being produced at much lower cost due to expired patents. Recombinant human erythropoietin (rh-EPO) contains the identical amino acid sequence of natural EPO (165 amino acids) and has a molecular weight of ca. 30,400 Da. Since glycosylation is not only dependent on the cell-line used for the expression of Epoetins but also on the entire biotechnological process the glycosylation patterns of biosimilars do not necessarily reflect the patterns of the originator compounds [2-4]. Today biosimilar Epoetins are manufactured and distributed worldwide and under many different names [2]. The use of recombinant EPOs for doping is prohibited because of its performance enhancing effect [5,6].
The current study investigates the electrophoretic behaviour of different batches of a Cuban rEPO biosimilar on IEF, SDS-PAGE, and SAR-PAGE.

Experimental

Materials

The rhEPO (EPO BRP) was purchased by Council of Europe European Pharmacopoeia, and NIBSC (endogenous urinary hEPO) was provided by National Institute for Biological Standards and Control. For Methoxypolyethyline glycol epoetin beta (CERA), Epoetin Delta (Dynepo) and Darbepoetin alpha (NESP), injectable preparations were used as reference material. The sources of all chemicals, reagents and other drugs are as per the the method of Reichel et al.[8,9]

The 3 batches of Cuban rEPO (ior®-EPOCIM-nominal concentration 2000 IU/mL (2 “Reference Materials”), and 10.000 IU/ 1,1 mL (1 “Injectable Preparation”)) (Table 1) was obtained from the Immunology Molecular Centre, Centre of Molecular Immunology, CIMAB, Atabey, Playa, Cuba, and supplied by the Laboratorio Antidoping, Instituto de Medicina Deportiva, Havana, Cuba.

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Stock Solution preparation
All the preparations of rEPO were diluted (in solution of 0,05% bovine serum albumin (BSA)/50 mM Tris-HCl pH 7.4) to the final concentration of, 0.03 IU/mL or 0.2 ng/mL (approximately).

IEF-PAGE, SDS-PAGE and SAR-PAGE
All the 3 batches of Cuban rEPO (ior®-EPOCIM) were analyzed along with EPO reference standards (BRP, NESP; NIBSC; CERA and Dynepo), by IEF, SDS-PAGE and SAR-PAGE. All the three methods were performed as described by Reichel et al., with minor modifications. Briefly, the methods for testing of EPO consisted of three major steps i.e. electrophoretic separation combined with double blotting and chemiluminescence detection.

EPO (Brand Name)	Manufacture´s Name	Marketed by	Type	Lot	Expire date	Unit (IU)
EPOCIM	CIMAB	CIMAB	Reference Material	030923	2/2011	2000
EPOCIM	CIMAB	CIMAB	Reference Material	031065	09/2012	2000
EPOCIM	CIMAB	CIMAB	Injectable Preparation	701410	5/2017	10.000

Table 1. Details of Cuban rEPO (ior®-EPOCIM)

Results and Discussion

The three batches of Cuban rEPO (ior®-EPOCIM) showed discriminative IEF-profiles from endogenous urinary EPO and slightly different band patterns compared to BRP rEPO. Two batches ("Reference Material") had bands 4 and 5 as most intense bands, while for the other one ("Injectable Preparation") bands 2 and 3 were the most intense (same as for BRP-EPO) (Fig.1). This results could be because the two rEPOs were already expired at the time of analysis (02/2011 and 09/2012) and it is known that a loss of sialic acids due to degradation leads to a shift in the IEF-profile towards the basic region.
Since the EPOCIM is a epoetin-α, the profile is as expected, similar to BRP (although BRP is a equimolar mix of epoetin-α and epoetin-ß), and not to the other reference rEPO (Dynepo) and analogues (NESP, CERA) used in this study.

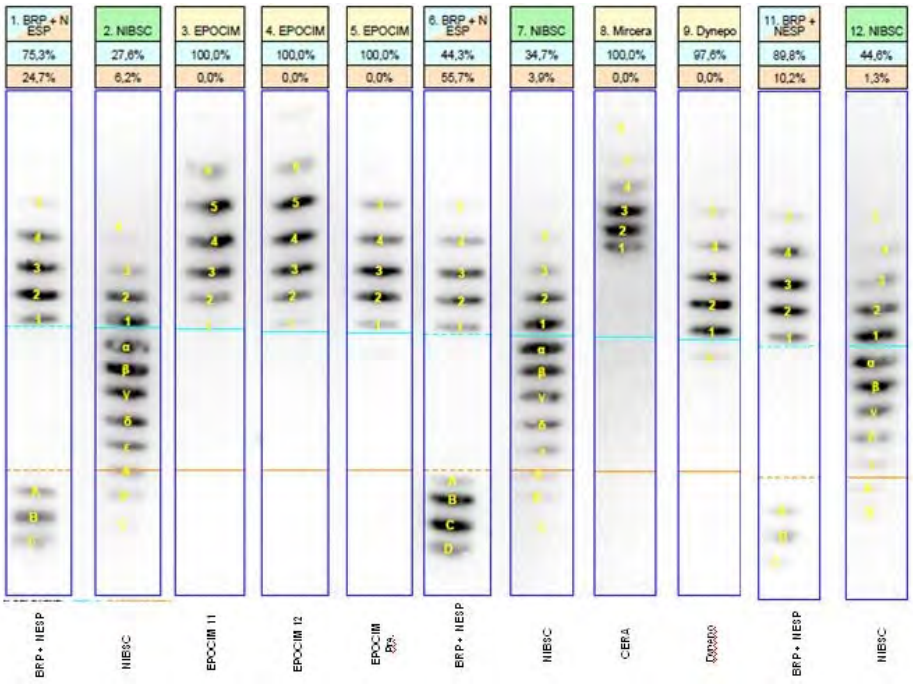

Fig.1. IEF Patterns of 3 batches of Cuban EPO (ior®-EPOCIM). Lane 1,6 & 11: BRP + NESP Standard; Lane 3: EPOCIM Ref. Mat. batch 030923; Lane 4: EPOCIM Ref. Mat. batch 031065; Lane 5: EPOCIM Inject. Preparation batch 701410; Lane 2,7 & 12: NIBSC; Lane 8: CERA; Lane 9: Dynepo.

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On SDS-PAGE and SAR-PAGE, ior®-EPOCIM resulted in a higher molecular mass and different band shape than endogenous urinary EPO (NIBSC). All 3 batches showed a migration behaviour (band) and shape (“broad band”) characteristic of epoetin-α, according the WADA Technical Document – TD2014EPO [10] (Fig. 2 and 3).

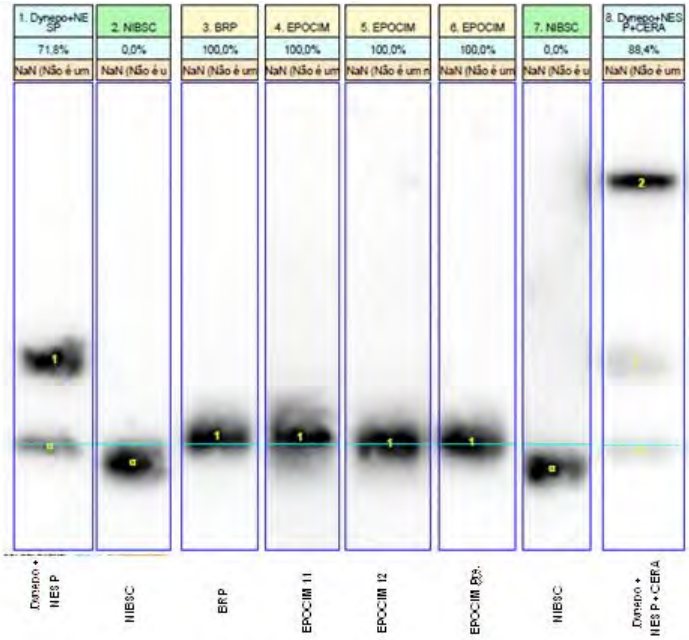

Fig.2. SDS-PAGE of 3 batches of Cuban EPO (ior®-EPOCIM).: Lane 1: Dynepo + NESP Standards; Lane 2 & 7: NIBSC Standard; Lane 3: BRP Standard; Lane 4:EPOCIM Ref. Mat. batch 030923; Lane 5: EPOCIM Ref. Mat. batch 031065; Lane 6: EPOCIM Inject. Preparation batch 701410; Lane 8: Dynepo + NESP + CERA Standards.

Since Sarcosyl-PAGE only improves the electrophoretic performance of PEGylated EPO [4], performance characteristics of the three batches of Cuban EPO tested, was unaltered (Fig.3).

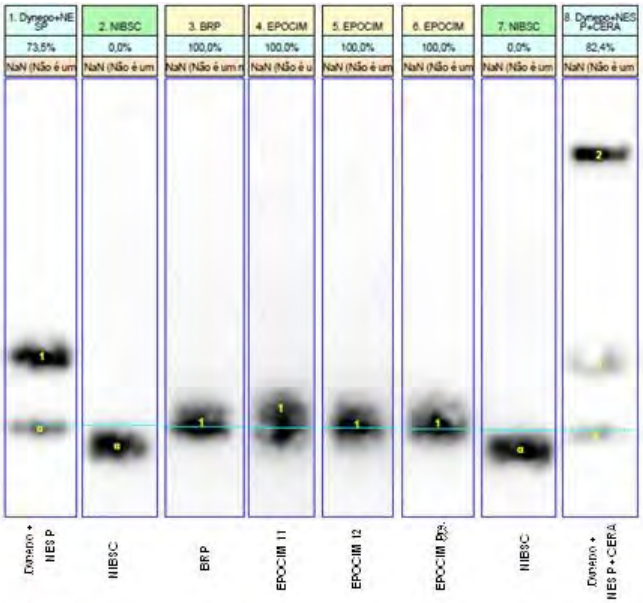

Fig.3. SAR-PAGE of 3 batches of Cuban EPO (ior®-EPOCIM): Lane 1: Dynepo + NESP Standards; Lane 2 & 7: NIBSC Standard; Lane 3: BRP Standard; Lane 4:EPOCIM Ref. Mat. batch 030923; Lane 5: EPOCIM Ref. Mat. batch 031065; Lane 6: EPOCIM Preparation batch 701410; Lane 8: Dynepo + NESP + CERA Standards.

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Conclusions

The results of the present study indicate distinguishable profiles of Cuban rEPO (ior®-EPOCIM) from that of endogenous erythropoietin. Between the two reference materials, no batch-to-batch variation was observed. The IEF-PAGE and SDS-PAGE/ SAR-PAGE in combination with immunological methods (western blotting), allow fast and sensitivity comparisons of pharmaceutical products on the molecular level and can be used to discriminate biosimilar rEPOs from endogenous EPO. Further work will be in progress, to establish the pattern of bands arising from urinary EPO (excretion study) with the Cuban EPO.

References

1. Pucaj et al. (2014**)** Safety and Biosimilarity of ior®EPOCIM Compared with Eprex® Based on Toxicologic, Pharmacodynamic, and Pharmacokinrtic Studies in the Sprague-Dawley Rat.*Journal of Pharmaceutical Sciences* **103**, 3432-3441.
2. Reichel C, Gmeiner G**.** (2009**)** Erythropoietin and Analogs. In: Thieme and P. Hemmersbach (eds.) *Doping in sports, Handbook of Experimental Pharmacology*, pp 251-286.
3. Wolfgang J.(2008**)** *British Journal of Haematology* **141**, 287-297.
4. Reichel C, Thevis M.(2013**)** Gel electrophoretic methods for the analysis of biosimilar pharmaceuticals using the example of recombinant erythropoietin. *Bioanalysis* **5 (5)**, 587-602.
5. Jain et al., (2012**)** Characteristics of IEF Patterns and SDS-PAGE Result of Indian EPO Biosimilars. In: Schänzer W, Geyer H, Gotzman A, Mareck U. (eds.) *Recent Advances in Doping Analysis* (20), Cologne, pp 259-262.
6. Kang et al. (2010) Characteristics of IEF Patterns and SDS-PAGE Results of Korean EPO Biosimilars. *Korean Chem. Soc*. **9**, Vol.31, 2493.
7. Díaz J.(2013**)** 15 años de Eritropoyetina Recombinante Humana cubana. Beneficios y retos. Revista Habanera de Ciencias Médicas **12(3),** 464-471**.**
8. Reichel C. et al. (2009) SDS-PAGE of recombinant and endogenous erythropoietins:benefits and limitations of the method for application in doping control. *Drug Test. Analysis* **1**, 43-50.
9. Reichel C. et al. (2009) Sarcosyl-PAGE: a new method for the detection of Mircera and EPO-doping in blood. *Drug Test. Analysis* **1**, 494-504.
10. World Anti-Doping Agency. Harmonization of analysis and reporting of erythropoiesis stimulating agents (ESAs) by electrophoretic techniques (2014) https://wada-main-prod.s3.amazonaws.com/resources/files/WADA-TD2014EPO-v1-Harmonization-of-Analysis-and-Reporting-of-ESAs-by-Electrophoretic-Techniques-EN.pdf [access date 9.02.2015].

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