CHARACTERISTICS OF IEF, SDS AND SAR-PAGE OF CUBA EPO BIOSIMILAR

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INTRODUCTION

EPO is a glycoprotein produced in the kidney, which stimulates the division and differentiation of red cells in the bone marrow. 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, beta or omega 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, with a molecular weight of 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[2,3,4]. 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 aim of the present study was to investigated whether biosimilar alpha r-HuEPO – ior®-EPOCIM, produced in Cuba and also available in other countries in all continents[7], could be differentiated from endogenous one by iso-electro-focusing plus double blotting, SDS-PAGE and SAR-PAGE for antidoping analysis.

MATERIAL AND METHODS

Materials

The rhEPO (EPO BRP) was purchased by Council of Europe European Pharmacopoeia, and NIBSC (endogenous 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 EPO (ior®-EPOCIM-nominal concentration 2000 IU/mL (2), and 10.000 IU/1,1 mL (1)) (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, Habana, Cuba.

Stock Solution preparation

All the preparations of EPO 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 EPO (ior®-EPOCIM) were analyzed along with reference standards of EPO (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[8,9] 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.

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

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	Preparation	701410	5/2017	10.000

RESULTS & DISCUSSION

All three batches of Cuban EPO (ior®-EPOCIM) were detected in the basic region, showed discriminative isoelectric profiles from endogenous EPO profiles, but showed slight difference band patterns with the reference rEPO (BRP).The two Cuban EPO (ior®-EPOCIM) Reference Material, showed the two most intense bands (4 and 5) in the basic region, and the preparation, showed the same two most intense bands (2 and 3) like the reference rEPO (BRP) (Fig.1).

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.

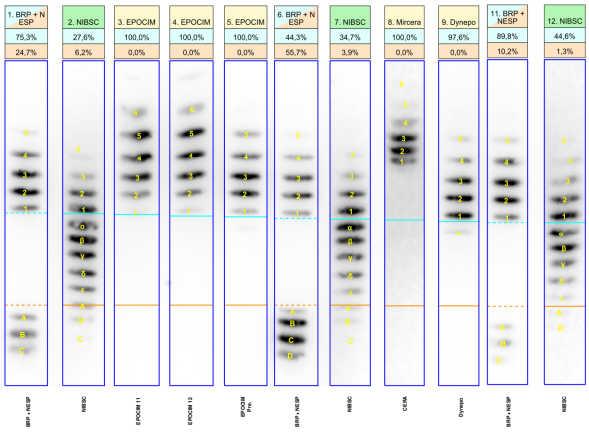

Fig.1. IEF Patterns of 3 batches of Cuban EPO (ior®-EPOCIM). Lane 1, 6 & 11:BRP + NESP Standard; Lane 3: EPOCIM Ref. Mat. batch 030923; Lane 4: EPOCIM Ref. Mat. batch 031065; Lane 5: EPOCIM Preparation batch 701410; Lane 2, 7 & 12: NIBSC; Lane 8: CERA; Lane 9: Dynepo.

On SDS-PAGE and SAR-PAGE, ior®-EPOCIM resulted in different molecular weight patterns and band shape, which led to mass distributions higher than endogenous EPO. 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 & 3).

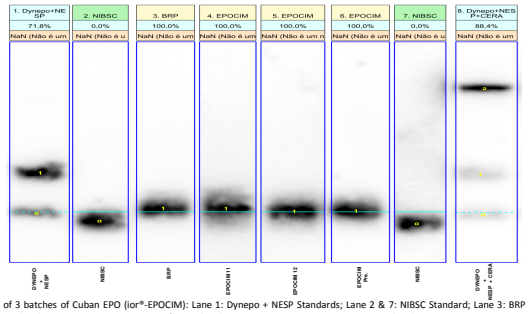

Fig.2. SDS-PAGE of 3 batches of Cuban EPO (ior®-EPOCIM): Lane 1: Dynepo + NESP Standards; Lane 2 & 7: NIBSC Standard; Lane 3: BRP Standard; Lane 4:EPOCIM Ref. Mat. batch 030923; Lane 5: EPOCIM Ref. Mat. batch 031065; Lane 6: EPOCIM Preparation batch 701410; Lane 8: Dynepo + NESP + CERA Standards.

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

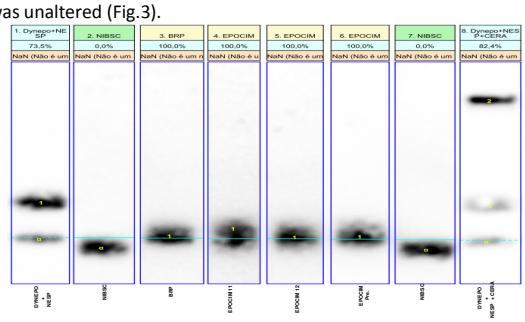

Fig.3. SAR-PAGE of 3 batches of Cuban EPO (ior®-EPOCIM): Lane 1: Dynepo + NESP Standards; Lane 2 & 7: NIBSC Standard; Lane 3: BRP Standard; Lane 4:EPOCIM Ref. Mat. batch 030923; Lane 5: EPOCIM Ref. Mat. batch 031065; Lane 6: EPOCIM Preparation batch 701410; Lane 8: Dynepo + NESP + CERA Standards.

CONCLUSIONS

The results of the present study indicate distinguishable profiles of Cuban EPO (ior®-EPOCIM) from that of endogenous erythropoietin.

Between the two material reference, 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 EPOs from endogenous EPO.

Further work will be done, to establish the pattern of bands arising from urinary EPO (excretion study) with the Cuban EPO.

REFERENCES

1.Pucaj et al.; *Journal of Pharmaceutical Sciences* **2014**,103:3432-3441.
2.Reichel C, Gmeiner G.; Erythropoietin and Analogs (Chap.) in *Doping in sports, Handbook of Experimental Pharmacology;* Thieme and P. Hemmersbach (eds.)., **2009.**
3.Wolfgang J.; *British Journal of Haematology* **2008**, 141, 287-297.
4.Reichel C, Thevis M.; *Bioanalysis* **2013**, 5 (5), 587-602.
5.Jain et al.; 30th Manfred Donike Workshop on "Dope Analysis", Cologne, Germany, February, **2012**.
6.Kang et al.; *Korean Chem. Soc.* **2010**, Vol.31,No 9, 2493.
7.Díaz J.; *Revista Habanera de Ciencias Médicas* **2013**, 12(3): 464-471.
8.Reichel C. et al.; *Drug Test. Analysis* **2009**, 1, 43-50.
9.Reichel C. et al.; *Drug Test. Analysis* **2009**, 1, 494-504.
10.WADA-TD2014EPOv1 Harmonization of Analysis and Reporting of ESAs by Electrophoretic Techniques