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Characteristics of IEF Patterns and SDS-PAGE Result of Indian EPO Biosimilars

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Abstract

The use of recombinant EPOs for doping is prohibited because of their performance enhancing effect. The similar biological medicinal products or “biosimilars” of EPO are available across the globe which may have isoform profiles, not exactly matching with already referenced (e.g., darbepoetin alfa, epoetin alfa, epoetin beta, epoetin, delta and/or omega, etc.) preparations. The aim of the present study was to investigate whether various biosimilar EPO preparations available in Indian market could be differentiated from the endogenous EPO by iso-electro-focusing (IEF) plus double blotting and SDS-PAGE for antidoping analysis. Eleven preparations of EPO manufactured in India were procured and their band pattern was studied. All Indian biosimilars showed discriminative isoelectric profiles from endogenous EPO proiles, but few showed different band patterns with reference rEPO. All the EPO preparations were detected in the basic region except Cresp (Dr. Reddy's Lab) which was detected in the acidic region. SDS-PAGE of all Indian biosimilar EPOs resulted in different molecular weight patterns which were distributed higher than endogenous EPO. The electropherograms of excretion study samples of Vintor showed that the pattern of bands arising from urinary rEPO was different from that of endogenous urinary EPO. Though, the bands of rEPO could be detected up to 95 hrs post administration but as per WADA criteria, the samples were positive only up to 28 hrs. As per SDS-PAGE, all the samples showed presence of rEPO upto 95 hours.

Introduction

The recombinant erythropoietin (rEPO) is often misused in sports due to its performance enhancing effect. Biosimilar version of epoetins have been available in developing countries for many years and are widely used for economic reasons. Few of them have shown differences compared to reference EPO preparations [(1)]. This is primarily due to the way in which biological EPOs and biosimilar EPOs are manufactured where slight differences in manufacturing conditions and processing can create small differences in the finished product. The existence of biosimilar versions of EPO is a major problem for drug testers. The aim of the present study was to investigate whether Indian version EPOs could be differentiated from the endogenous EPO by iso-electro-focusing (IEF) plus double blotting and SDS-PAGE for anti-doping analysis.

Experimental

1. Reference standards

Erythropoietin (EPO) reference standards (human urinary erythropoietin (NIBSC) and recombinant erythropoietin (BRP)) were procured from National Institute for biological standards and control (UK), and European Directorate for the Quality of Medicines (France). For darbepoetin alpha (NESP) and Methoxypolyethylene glycol epoietin beta (CERA), injectable preparations were used as reference material. The sources of all other drugs, reagents, and chemicals are as per the method of Reichel et al.[2]

Eleven preparations of EPO manufactured in India were procured to study the band pattern of Indian biosimilars, rhEPO and endogenous EPO for this study. viz. Ceriton (Ranbaxy), Eposis (Shantha Biotech), Wepox (Wockhardt), Erypro safe (Biocon), Shanpoietin (Shantha Biotech), Vintor (Gennova Biopharma), Epofit (Intas Pharma), Erykine (Intas Pharma), Epotrust (Intas Pharma), Eporise (Gennova Biopharma) and Cresp (Dr. Reddy`s Lab). All the preparations of EPO were diluted to the final concentration of 0.03 IU/mL or 0.2 ng/mL (approximately).

 ISBN 978-3-86884-038-4

2. Excretion study

Vintor was administered subcutaneously (4000 IU) to a healthy male volunteer and urine samples were collected for 95 hours. All the urine samples were analysed for EPO by IEF and SDS-PAGE [2,3]. The method for testing of EPO consisted of three major steps i.e. IEF separation, double blotting and chemiluminescence detection. The gel casting mould was manually prepared in NDTL [3]. The emitted light was captured with a CCD camera for image acquisition (Fuji Film, LAS 4000). SDS-PAGE method [2] involved additional immunoaffinity purification step as part of sample preparation process. The electrophoretic separation was used in combination with double blotting and chemiluminescence detection.

Results and Discussion

All the eleven Indian biosimilars showed discriminative isoelectric profiles from endogenous EPO profiles and were detected in the basic region except Cresp (Lane 16, Dr. Reddy's Lab) which was detected in the acidic region (Fig. 1).

On SDS-PAGE different molecular weight patterns were observed for all the EPO biosimilars, which led to mass distributions higher than endogenous EPO (Fig. 2). However, four EPO preparations namely Wepox (lane 5), Erypro (lane 6), Vintor (lane 9), and Eporise (lane 15), showed apparently different IEF patterns in comparison to the reference standard of rEPO (BRP) (Fig.1). The electropherograms of excretion study samples after subcutaneous injection of Vintor showed that IEF patterns from rEPO were different from that of endogenous EPO (NIBSC)(Fig. 3a and 3b).

On the basis of IEF findings, the bands of rEPO could be detected up to 95 hrs post administration, but did not pass the criteria of WADA for the reporting of adverse analytical finding (AAF). These samples passed the WADA criteria of AAF only up to 28 hrs. However, by application of SDS-PAGE which is recommended in WADA technical document in case the profile is not consistent with a typical endogenous /exogenous profile, all the samples showed presence of rEPO upto 95 hours (Fig.4). Similar findings were also observed by Kang et al.[1] in EPO preparations available in Korea. Further work is in progress with a higher number of volunteers and rest of the EPO preparations which showed apparently different band pattern in comparison to the reference standard.

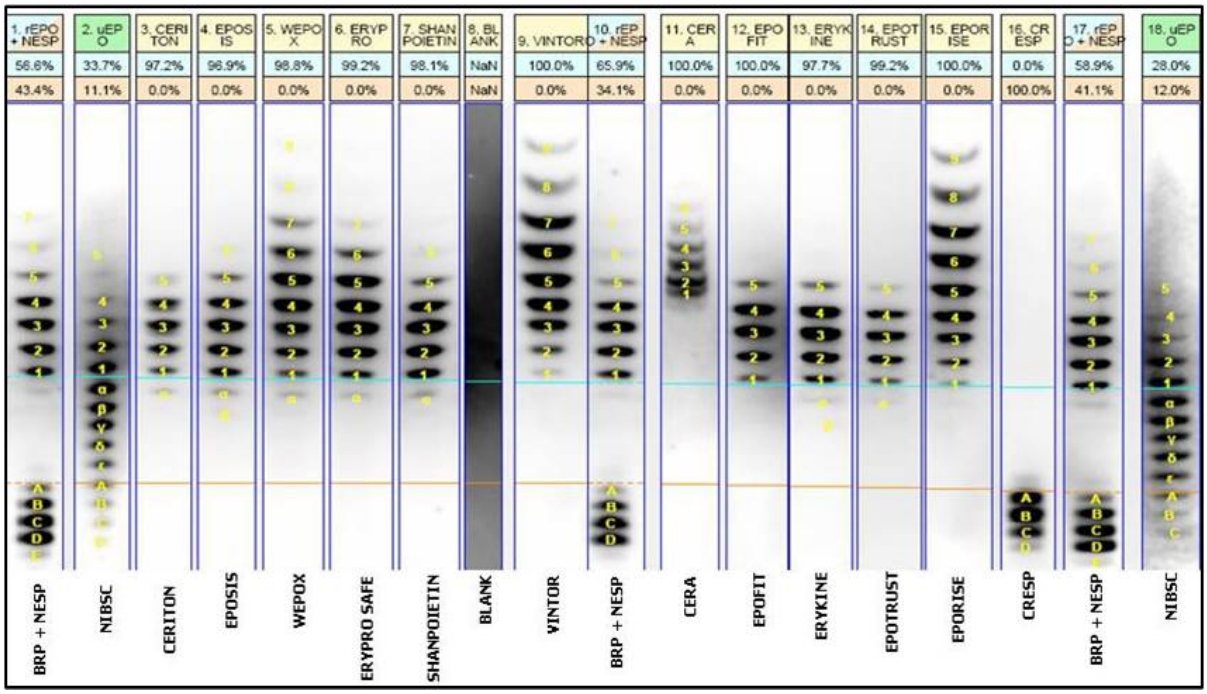
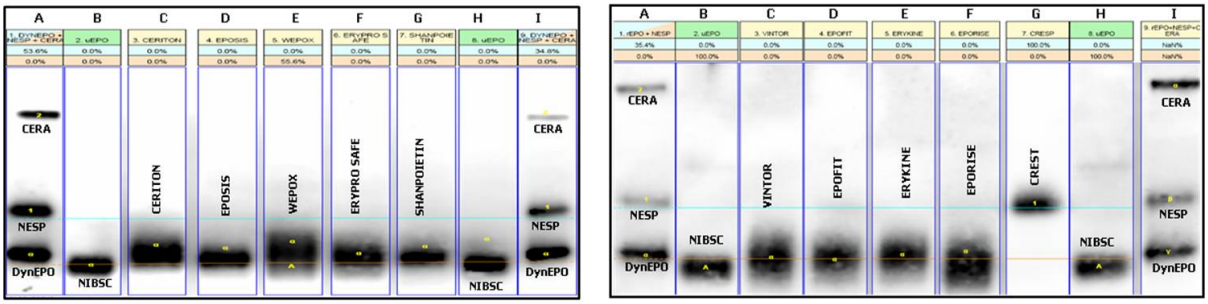


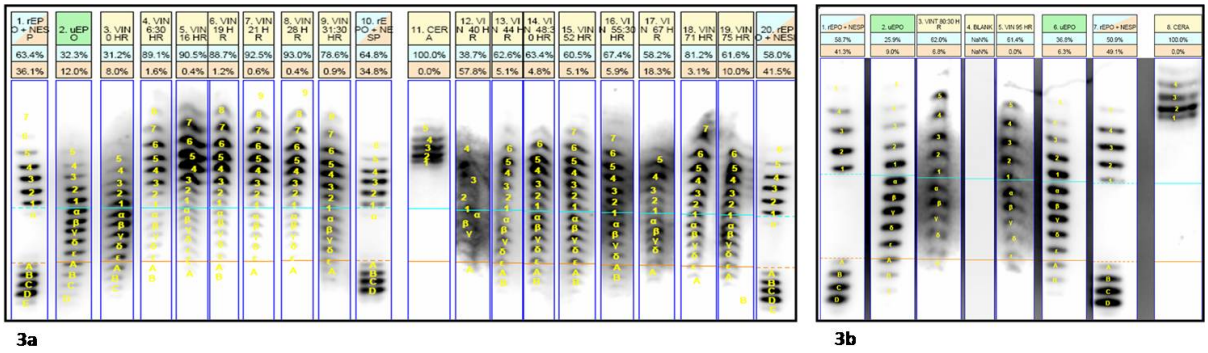
Figure 1. IEF Pattern of various EPO biosimilars (Indian Manufactured). Lane 1,10 & 17: BRP + NESP standards, Lane 2 & 18: NIBSC standard, Lane 11: CERA (Roche manufactured), Lane 3: CERITON (Ranbaxy), Lane 4: EPOSIS (Shantha Biotech), Lane 5: WEPOX (Wockhardt), Lane 6: ERYPROSAFE (Biocon), Lane 7: SHANPOIETIN (Shantha Biotech), Lane 8: Blank, Lane 9: VINTOR (Gennova Biopharma), Lane 12: EPOFIT (Intas Pharma), Lane 13: ERYKINE (Intas Pharma), Lane 14: EPOTRUST (Intas Pharma), Lane 15: EPORISE (Gennova Biopharma), Lane 16; CRESP (Dr Reddy's Lab).

ISBN 978-3-86884-038-4



2a 2b

Figure 2a. SDS-PAGE of various Indian manufactured EPO biosimilars. Lane A & I: DYNEPO + NESP + CERA standard, Lane B & H: NIBSC standard, Lane C: CERITON, Lane D: EPOSIS, Lane E: WEPOX, Lane F: ERYPROSAFE, Lane G: SHANPOIETIN. Figure 2b. SDS-PAGE of various Indian Manufactured EPO biosimilars. Lane A & I: DYNEPO + NESP + CERA standard, Lane B& H: NIBSC standard, Lane C: VINTOR, Lane D: EPOFIT, Lane E: ERYKINE, Lane F: EPORISE, Lane G: CRESP.



3a 3b

Figure 3a. Excretion study of Vintor (4000 IU) on IEF Gel. Lane 1, 10 & 20: BRP + NESP standards, Lane 2: NIBSC standard, Lane 11: CERA standard, Lane 3: 0 hrs, Lane 4: 6:30 hrs, Lane 5: 16 hrs, Lane 6: 19 hrs, Lane 7: 21 hrs, Lane 8: 28 hrs, Lane 9: 31:30 hrs, Lane 12: 40 hrs, Lane 13: 44 hrs, Lane 14: 48:30 hrs, Lane 15: 52 hrs, Lane 16: 55:30 hrs, Lane 17: 67 hrs, Lane 18: 71 hrs, Lane 19: 75 hrs. Figure 3b. Excretion study of Vintor (4000 IU) on IEF Gel. Lane 1 & 7: BRP + NESP standards, Lane 2: NIBSC standard, Lane 8: CERA standard, Lane 3: 80:30 hrs, Lane 4: Blank, Lane 5: 95 hrs

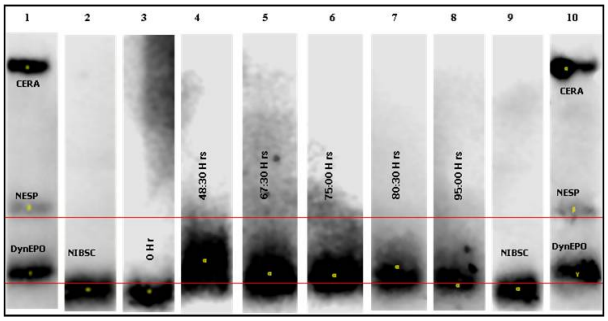


Figure 4. Excretion study of Vintor (65 IU/kg) on SDS-PAGE Gel. Lane 1 & 10: DYNEPO + NESP + CERA standards, Lane 2 & 9: NIBSC standard, Lane 3: 0 hr, Lane 4: 48:30 hrs, Lane 5: 67:30 hrs, Lane 6: 75:00 hrs, Lane 7: 80:30 hrs, Lane 8: 95:00 hrs.

Conclusions

The results of the present study reveals different band patterns of EPO isoforms in few of Indian EPO biosimilars. The excretion study results with one of the preparation (Vintor) did not fulfill the WADA criteria of AAF reporting on the basis of IEF only. However, application of electrophoretic SDS-PAGE along with the IEF method facilitated confirming exogenous origin of EPO. The window for detection of rEPO was superior in SDS-PAGE as compared to IEF-PAGE. Further work is in progress with rest of the EPO preparations which showed apparently different band patterns in comparison to the reference standard.

References

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