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Characteristics of IEF Patterns and SDS-PAGE Result of Indian Darbepoeitin (CRESP) and its detection window following single subcutaneous injections.

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Abstract

Various recombinant erythropoietin (EPO) biosimilars are easily available the world over out of which few have different isoform profiles compared to standard reference preparations. Indian darbepoetin alfa (CRESP), manufactured by Dr Reddy`s Lab, Hyderabad, India, is the first generic biosimilar preparation of Darbepoetin alfa (NESP) having been launched in 2011. The present study aims to investigate the IEF band pattern of different batches of CRESP and its detection window following single subcutaneous injection. Six different batches of CRESP were used for the study in which urine samples till 15 days and blood samples on day 0, 4, 7 and 9 were collected after single subcutaneous injection CRESP® (4000 IU) to a volunteer. Isoelectric focusing (IEF) and SDS-PAGE followed by immunoblotting of urinary proteins were performed to determine the detection window of Darbepoietin-α (CRESP) as per the positivity criteria of World Anti-Doping Agency (WADA). Significant inter batch variation of the IEF pattern was observed amongst the CRESP batches. As per WADA recent positivity criteria (TD2013EPO), the IEF and SDS-PAGE based urinary EPO test determined the detection window of CRESP close to 12 days after single injection of CRESP which was much longer than for NESP.

Introduction

NESP, darbepotin alfa marketed by Amgen with trade name Aranesp having been approved in September 2001 by the Food and Drug Administration (FDA, USA) is produced by recombinant DNA technology in genetically modified CHO (*Chinese hamster ovary*) cell line. It is a synthetic EPO and differs from other recombinant erythropoietin (rEPO) by having two more N-linked oligosaccharide chains. NESP (Novel Erythropoietin Stimulating Protein) has a longer half life as compared to other rEPO preparations [1]. The use of NESP is prohibited in sports on account of its performance enhancing effect and was abused by athletes in the Winter Olympics in Salt City [2].
Despite the number of studies conducted on rEPO biosimilars, a few of them have shown different isoform profiles compared to already referenced preparations [3,4]. Similar studies conducted with Indian biosimilars have shown parallel results in 2012 [4]. It was observed that CRESP (Dr Reddy`s Lab, Hyderabad, India) on IEF did not meet the positivity criteria as per WADA technical document (TD2009EPO)[5]. The present study aims to investigate the IEF band pattern of different batches of CRESP and its detection window following single subcutaneous injection as per WADA technical document both old (TD2009EPO)[5] and new (TD2013EPO)[6].

Experimental

Reference Standards

Erythropoietin (EPO) reference standards (human urinary erythropoietin (NIBSC) & recombinant erythropoietin (Biological Reference Preparation, BRP)) were procured from the National Institute for Biological standards and Control (UK), and the European Directorate for the Quality for Medicines (France). For Darbepoetin-α (NESP, Amgen AG, Zug, Switzerland) and Methoxypolyethyline glycol epoetin beta (Continuous Erythropoietin Receptor Activator, CERA, Roche Diagnostics GmbH, Mannheim, Germany), injectable preparations were used as reference material.

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Experimental design:
Six different batches of CRESP (Lot No. - DAAS00810, DABS00312, DAAS00912, DAAS00712, DABS01512, & DABS01612) were procured for the study. They were characterized by IEF and SDS-PAGE followed by immunoblotting after making final concentrations of 0.03 IU/mL (or 0.2 ng). For the excretion study of CRESP, two volunteers, one 25 year old male and one 30 year old female participated in the study. The study design was approved by ethical committee of National Dope Testing Laboratory (NDTL). Both the participants gave their consent before administration of single injection of 40 µg of CRESP® (Lot No. - DABS00312 and Lot No. - DABS01612) on the upper arm. Urine samples were collected before injection (blank urine) and further urine samples were collected for 15 days after administration of CRESP and stored at - 80°C after aliquoting. For safety reasons, blood tests were carried out on 0, 4, 7 and 9th day to check the hemoglobin concentration and other blood parameters on automated anaylser (SYSMEX XT 2000i). The first urine samples (morning) of all the days were analysed for EPO by IEF and SDS-PAGE method [4].

Sample preparation and analysis:
The method for testing of EPO consisted of three major steps i.e. IEF separation, Western double blotting and chemiluminescence detection [7]. The gel casting mould was manually prepared in NDTL. Briefly, 20 mL of urine was subject to ultrafiltration and total EPO content in the retentates were determined using Enzyme Linked Immuno- sorbent Assay (ELISA, Stem Cell Technologies, Vancouver, Canada) intended both for endogenous and recombinant EPO isoforms, including darbepoetin alpha. Then, the retentate was focused on an IEF gel (pH 2–6). Proteins were double-blotted (Western). The emitted light was captured with a CCD camera for image acquisition (Fuji Film, LAS 4000). SDS-PAGE involved additional immunoaffinity purification step as a part of the sample preparation process. The electrophoretic separation was used in combination with double blotting and chemiluminescence detection. Isoelectric profile analysis was performed using "GASepo" v2.1 software from Austrian Research Centers GmbH- ARC.

WADA Positivity criteria
To determine if a sample was considered as positive or negative, WADA criteria of positivity for NESP by both old and new technical document on EPO as detailed below were applied in order to find the detection window of CRESP.

Old Criteria (TD2009EPO)
i) In the acidic area, there must be at least 3 acceptable, consecutive bands assigned as "B", "C" and "D" in the corresponding reference preparation.
ii) The most intense band measured by densitometry must be "C" or "D";
iii) Both bands "C" or "D" must be more intense than band "B".

New Criteria (TD2013EPO)
i) In the acidic area, there must be at least 3 acceptable, consecutive bands assigned as "A", "B", "C" or "D" as defined in the corresponding NESP preparation used as reference preparation.
ii) The two most intense bands measured by densitometry shall be in the acidic area.

Results and Discussion

Analysis of different batches of CRESP
The band pattern of reference standards of human erythropoietin (NIBSC), recombinant EPO (BRP) and darbepoetin (NESP) and CRESP on IEF is depicted in Figure 1. In case of CRESP, band- A was more intense in comparison to band- D in NESP (Figure 1c & 1a). The overall isoform distribution pattern of CRESP was distinctly different from that of NESP.

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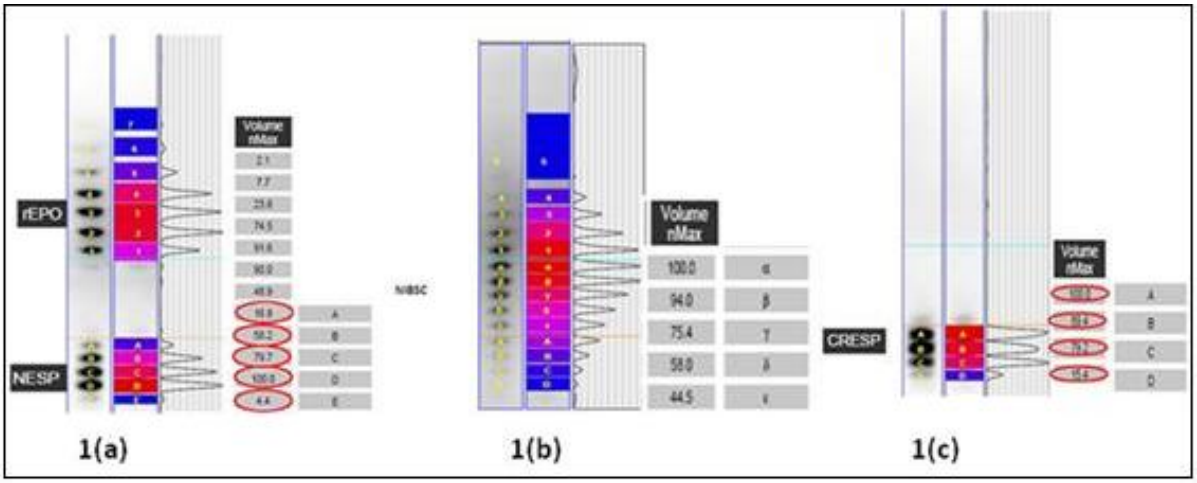

Fig.1: Comparison of band pattern of reference standards a) BRP (Biological Reference Preparation) standard & NESP (Aranesp®/Recormon®) standards, b) NIBSC (National Institute for Biological Standards and Control) standard and c) Biosimilar preparation of CRESP on IEF Gel.

When six different batches of CRESP preparations were run on IEF gel (Figure 2a), different patterns of isoform distribution were observed. The most intense bands were either **BA** or **BC** instead of **CD** as found in NESP (Figure 2b).

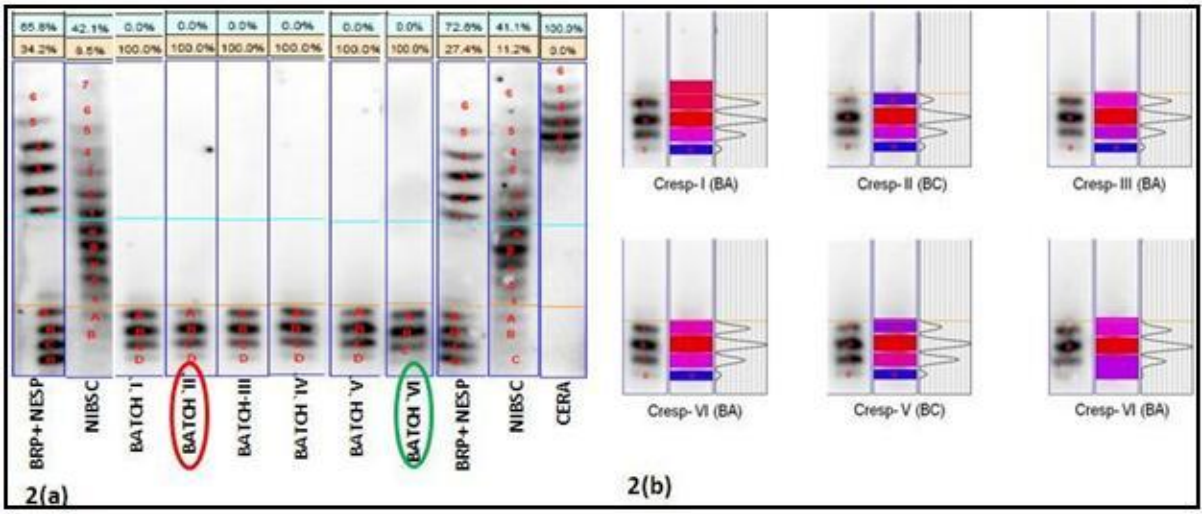

Fig. 2: IEF gel showing 2(a) isoform pattern of reference standards of BRP, NESP, NIBSC and CERA, and six different batches of CRESP in the acidic region, 2(b) different isoform distribution pattern of six batches of CRESP after GASEPO evaluation.

As per positivity criteria of WADA technical document (TD2009EPO), the electropherogram of all six different batches of CRESP showed negative findings on IEF. However, the band of all six batches of CRESP was in the same region as NESP on SDS-PAGE indicating the presence of darbepoetin in urine (Figure 3).

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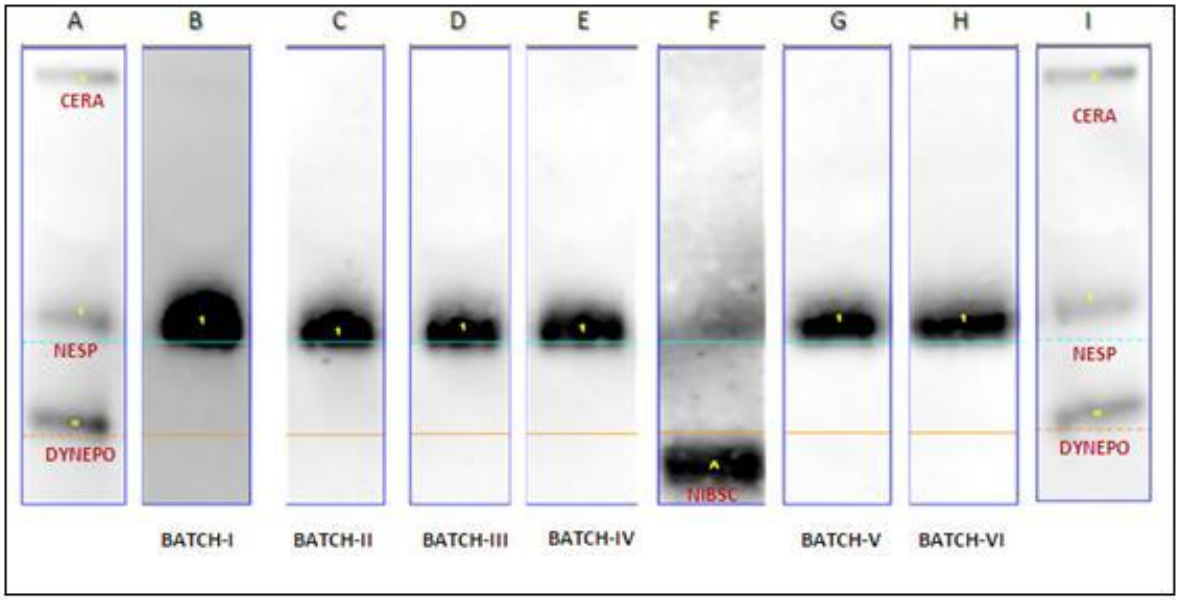

Fig. 3: SDS PAGE image of different batches of CRESP (Direct). Lane A & I: CERA (Continuous Erythropoietin Receptor Activator), NESP standard, DYNEPO (epoietin delta) standards, Lane F: NIBSC standard, Lane B to E: CRESP- Batch I to IV & Lane G & H: CRESP Batch V & VI showing the band of all the six batches of CRESP in the same region as NESP.

Excretion study

EPO concentrations were measured in tested urine samples. EPO concentration varied greatly for each volunteer depicting a significant total EPO concentration increase following CRESP injection. However, no correlation could be observed between the EPO concentration and the positivity of the sample (Figure 4). Similar findings have also been reported by Laman et al.[2]

Urine samples of both volunteers collected as first urine sample on all days were analysed for EPO by IEF and SDS PAGE. The IEF patterns showed discriminative isoelectric profiles from endogenous and four bands (A, B, C & D) were detected in the acidic region (Figure 4).

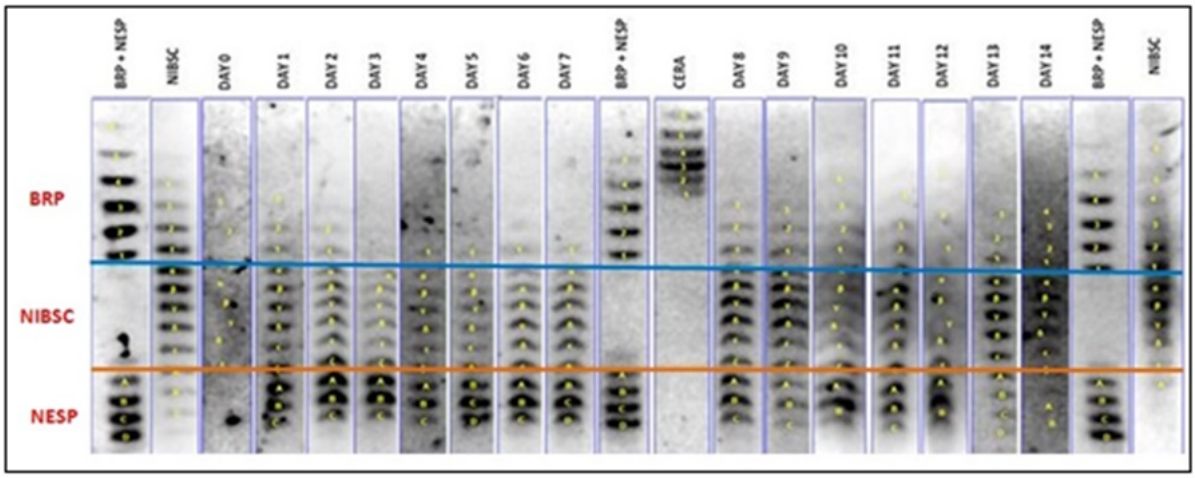

Fig. 4: IEF Gel of Day 0 to Day 14 samples (volunteer 1) with reference standards BRP (Biological Reference Preparation) standard), NESP (Aranesp®/Recormon®) standards), NIBSC (National Institute for Biological Standards and Control) standard) and CERA (Continuous Erythropoietin Receptor Activator), Mircera, standard). Four bands (A, B, C &D) of NESP standard were defined in the acidic region. The urine samples from Day 1 to Day 12 showed shifting of bands towards endogenous area (NIBSC).

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In all the samples, there was a slight shifting of band towards the endogenous region (Figure 5). All samples failed to pass the 2nd and 3rd positivity criteria of WADA technical document (TDEPO2009) as the most intense band must be either “C” or “D” and both bands “C” or “D” must be more intense than band “B”. When new positivity criteria (TDEPO2013) was applied, all the samples of volunteer -1 were positive until the 12th day except two samples on 8th and 9th day which were negative as they failed to pass the 2nd positivity criteria inspite of having four bands in acidic region. In these two samples, the most intense bands were located in the endogenous area. During the positivity period, bands in acidic region were more intense than any endogenous EPO band. In volunteer-2, urine samples till 9th day passed the new positivity criteria. Thereafter, most intense bands were found in the endogenous area though four bands were present in the acidic area. Moreover, endogenous bands became less intense following CRESP injection which could be explained by a possible feedback regulation of endogenous EPO production by this subject 2. The intensity of endogenous bands increased on 5th day onwards. Similarly, when urine samples collected in the evening (8th day to 14th day), were analysed by IEF, they did not pass the positivity criteria of WADA due o presence of most intense band in the endogenous area inspite of presence of bands in acidic region.

Similar findings have been also reported by Lamon et al [2]. Physiologically, this could eventually be explained by a sudden increase of endogenous EPO production. However it is known that among each person, endogenous EPO levels can vary in natural way [8]. When this specific criterion is applied, athletes with a natural elevated endogenous EPO production and abusing of NESP or CRESP will be more often considered as negative [2]. In case of NESP or CRESP abuse, the main considered information should be the position and the specific distribution of the bands in the most acidic area of the gel instead of band intensity ratio as suggested by Lamon et al.[2] The risk of declaring a NESP or CRESP false positive is extremely improbable as none of the studies have shown any changes in the acidic area of gel following strenuous physical activity. 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 in the region of NESP (CRESP) upto 12th day in both the volunteers except one sample of volunteer-1 on 8th day (Figure 5).

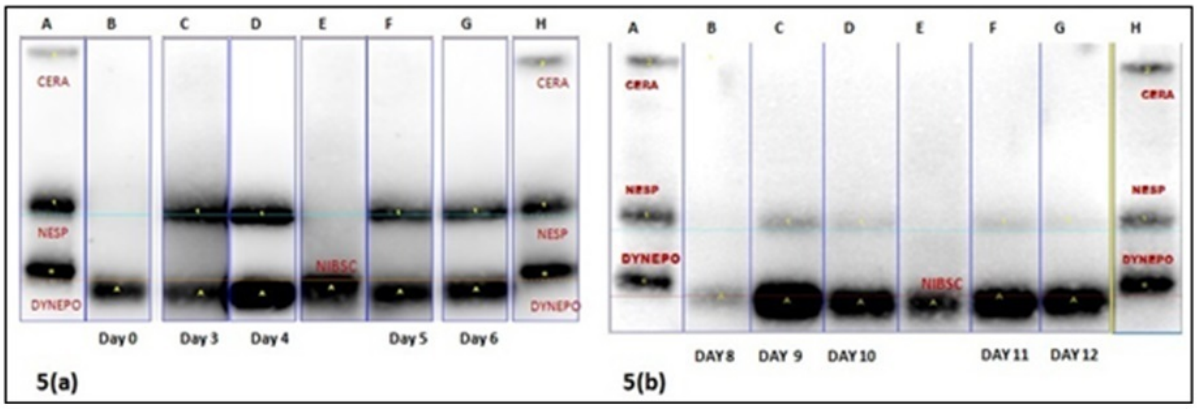

Fig. 5: SDS-PAGE images showing Excretion study samples of Volunteer-I along with reference standards; (a) Lane A & H: CERA), NESP, DYNEPO, Lane E:, Lane B, C, D, F & G: excretion sample of different days, b) Lane A & H: CERA, NESP, DYNEPO, Lane E: NIBSC, Lane B,C, D, F & G: excretion sample of different days. All the samples showed presence of band in the region of NESP upto 12th day in both the volunteers except one sample of volunteer-1 on 8th day indicating abuse of darbepoetin alfa (CRESP).

The findings of IEF and SDS-PAGE of both volunteers have been depicted in Table 1. No significant changes in blood parameters of both the volunteers were observed. As per WADA recent positivity criteria (TD2013EPO), the IEF and SDS-PAGE based urinary EPO test determined the detection window of CRESP close to 12 days after single injection of CRESP which was much longer than NESP.

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Time Window	As per WADA Technical Document			
	Volunteer -I		Volunteer – II	
	IEF GEL	SDS - PAGE	IEF GEL	SDS – PAGE
Day 0 (Control)	Negative	Negative	Negative	Negative
Day 1	Positive	Positive	Positive	Positive
Day 2	Positive	Not done	Positive	Positive
Day 3	Positive	Positive	Positive	Positive
Day 4	Positive	Positive	Positive	Positive
Day 5	Positive	Positive	Positive	Positive
Day 6	Positive	Positive	Positive	Positive
Day 7	Positive	Not done	Positive	Positive
Day 8	Negative	Positive	Positive	Positive
Day 9	Negative	Not done	Positive	Positive
Day 10	Positive	Positive	Negative	Positive
Day 11	Positive	Positive	Negative	Positive
Day 12	Positive	Positive	Negative	Positive
Day13	Negative	Not done	Negative	Not done
Day 14	Negative	Not done	Negative	Not done
Day 15	Negative	Not done	Negative	Not done

Fig. 6: The findings of IEF and SDS-PAGE of Volunteer I & II after administration of CRESP.

Conclusions

Significant inter batch variation was observed among all the batches of CRESP in terms of IEF pattern. All urine samples were negative as per old positivity criteria of WADA. However, as per new positivity criteria of WADA, the detection window of CRESP after administration to healthy volunteer was between 9-12 days which is longer than NESP. Further experimentation is in process with different batches of CRESP.

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References

[1]. Egrie JC, Dwyer E, Browne JK, Hitz A, Lykos MA (2003) Darbepoetin alfa has a longer circulating half-life and greater in vivo potency than recombinant human erythropoietin. *ExpHematol* **31**, 290–9.
[2]. Lamon S, Robinson N, Mangin P, Saugy M. (2007) Detection window of darbepoetin-α following one single subcutaneous injection.ClinChim Acta **379**, 145-9
[3]. Kang MJ, Shin SM, Yoo HH, Kwon OS, Jin C (2010) Characteristics of IEF Patterns and SDS-PAGE Results of Korean EPO Biosimilars.*Bulletin of the Korean Chemical Society* **31***,* 2493-2496.
[4]. Jain S., Lal R., Raj A., Tyagi D., Beotra A., Kaur T. (2012) Characteristics of IEF Patterns and SDS-PAGE of EPO Biosimilars available in Indian Market. In: Schanzer W, Geyer H, Gotzman A, Mareck U. (eds.) *Recent Advances in Doping Analysis* (20), Koln, pp 256-262.
[5]. World Anti-Doping Agency.Technical document on EPO, Montreal (2009) http://www.wada ama.org/ Documents/ World_Anti-Doping_Program/WADP-IS-Laboratories/ WADA_ TD2009EPO_EN.pdf (accesse date 06.01.2011).
[6]. World Anti-Doping Agency.Technical document on EPO, Montreal (2013) http://www.wada ama.org/ Documents/ World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_ TD2013EPO_EN.pdf. (access date 01.03.2013)
[7]. Reichel C. (2010) Practicing IEF-PAGE of EPO: The impact of detergents and sample application methods on analytical performance in doping control. *Drug Test Anal*. **2**, 603-19.
[8]. Klausen T, Dela F, Hippe E, Galbo H. (1993) Diurnal variations of serum erythropoietin in trained and untrained subjects. *Eur J ApplPhysiolOccupPhysiol* **67**, 545–8.

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