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Competition testing of Erythropoietin (EPO) during Commonwealth Games – 2010

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Introduction

Erythropoietin (EPO) is a glycoprotein hormone that is produced naturally by the human body. EPO is released from the kidneys and acts on the bone marrow to stimulate red blood cell production. An increment in red blood cells increases the amount of oxygen that the blood can carry to the body's muscles. Recombinant human EPO (rhEPO) is illicitly used to improve performance in endurance sport and its use is banned in sports since 1990. The method for the testing of EPO as per double blotting technique described by F. Lasne et. al. [1] and has been adopted by the World Anti-Doping Agency (WADA) to screen rhEPO in urine samples [2, 3, 4, 5]. The well established SDS-PAGE procedure can be used complementarily to the IEF method for confirmation of the exogenous or endogenous origin of the finding, especially when the profile is not consistent with a typical endogenous profile and does not fulfill the strict criteria defined in WADA technical document TD2009EPO. However, this atypical profile may be due to other biosimilar rEPOs or a combination of substances which show a shift of bands towards the basic area. The objective of the present paper is to evaluate the findings of EPO testing during Commonwealth Games (CWG) 2010.

Materials and Methods

Erythropoietin (EPO) reference standards (human urinary erythropoietin & recombinant erythropoietin) were procured from National Institute for biological standards and control (NIBSC), and European directorate for the quality of medicines. The sources of all other drugs, reagents, and chemicals are as per the method of Christian etal (6).

The Doping Commission of Commonwealth games (Oct.3 -14. 2010) collected 121 urine samples for EPO analysis. The method for testing of EPO consisted of four major steps i.e. urine concentration, IEF separation, double blotting and chemiluminescence detection [4]. The gel casting mould was manually prepared in NDTL[7]. Urine samples were centrifuged, micro- and ultra filtrated as described elsewhere(5).

IEF – PAGE and Western double blot

20 mL of urine was subjected to ultrafiltration, and the retentate containing EPO was focused in an IEF gel (pH 2–6). The isoforms were double-blotted and visualized by chemiluminescence substrate (Thermo Scientific, West Pico) (7). The emitted light was captured with a CCD camera for image acquisition (Fuji Film, LAS 4000).

Active urine test (stability test) and SDS - PAGE

To avoid false positive results and for confirmation of suspicious samples, Active Urine test (AUT) or Stability test was performed as described in TD2009EPO [4]. Urine samples with atypical profile were confirmed by SDS-PAGE according to the method described by Christain etal (6). For this method, an immunoaffinity purification step was added as necessary part of the sample preparation process. The electrophoretic separation was used in combination with double blotting and chemiluminescence detection.

Protein Analysis: Strip method (Combistix, M/s Bayer, India) was used to test the presence of protein in urine samples those who showed atypical profile on IEF.

Results and Discussion

All the samples tested for EPO were found negative. However, out of 121 samples, there was mild to moderate shifting of bands of uhEPO to the basic region in 70 samples though it did not fulfill the WADA evaluation criteria for rhEPO (Fig.1 & 2). On the basis of isoform intensity, 10 samples were found suspicious for rhEPO (Biosimilar EPOs) and were further processed for the confirmation and stability test along with double immunoblotting after SDS-PAGE. The samples were found stable (fig. 2) and showed only uhEPO on SDS PAGE (Fig. 3 & 4). Out of 70 samples tested for protein by strip method, 63 samples showed elevated protein concentration (traces to ++++) indicating that proteinuria after competition may be responsible for the shifting of bands (Fig. 4 & Table-1). Our results are in conformity to the findings of Beullens et.al [2]. who also reported shifting of bands towards basic region in post exercise protein-rich urine, probably because the adopted monoclonal anti—EPO antibodies are not monospecific. The present method of testing of EPO from urine samples can be refined with the use of specific high-affinity antibodies coupled with immuno purified assay in the beginning[8].

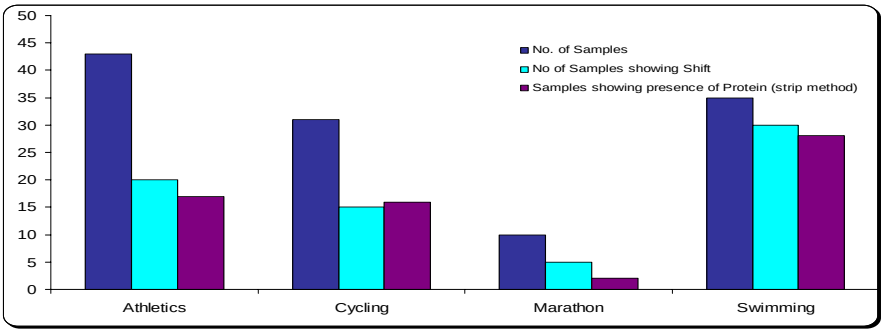

Fig 1: Discipline wise number of urine samples received, band shift observed & elevated Protein detected

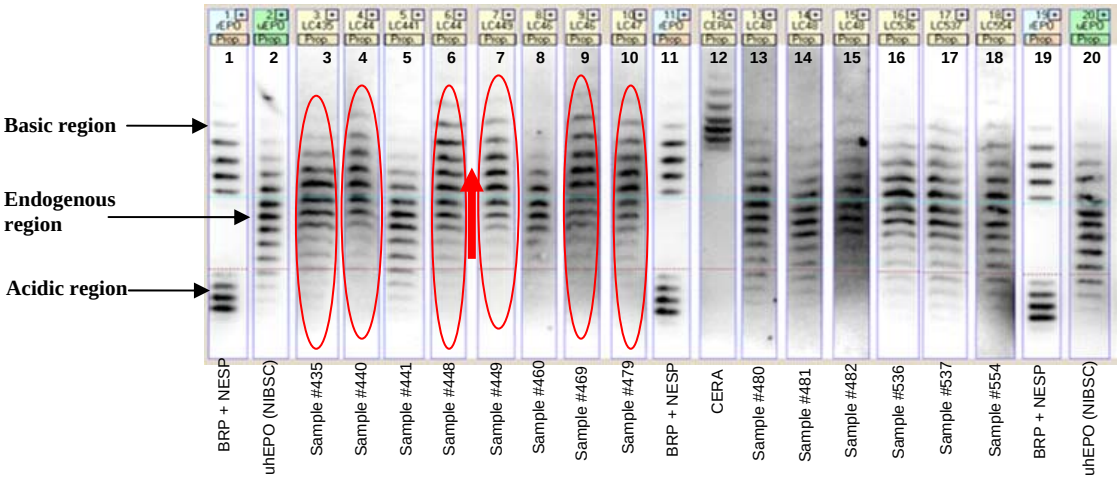

Fig 2: IEF gel showing recombinant human EPO STDs (BRP, NESP, CERA & urinary endogenous EPO in lane 1,12 &2. Lane # 3, 4, 6, 7, 9 & 10 showing mild to moderate shift towards basic region

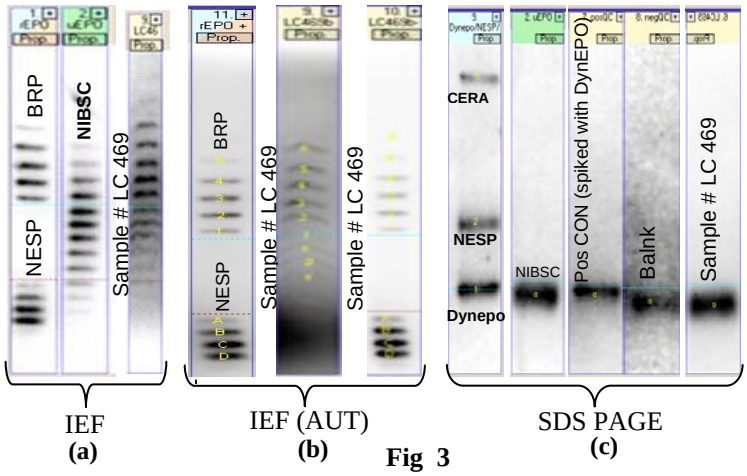

Fig 3

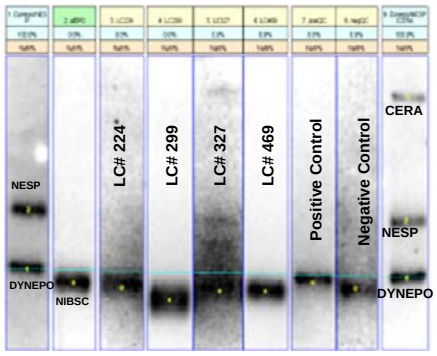

Fig 4

Fig 3: a) 1st IEF run of Sample LC # 469 showing shifting of bands towards basic region.
b) Confirmation of sample LC # 469 with stability test (Active urine test (AUT) showing stable urine and again shifting of bands towards basic region.
c) Confirmation of sample LC469 by SDS page showing endogenous EPO.

Fig 4: SDS-PAGE gel showing recombinant human EPO, STDS (DYNEPO, NESP, CERA & urinary endogenous (NIBSC)) and CWG Samples)

Table-1: Details of protein testing in urine samples (qualitative) by strip method

S.No	Discipline	No. of Samples	No of Samples showing Shift	Samples showing presence of Protein (strip method)	presence of proteins in the sample			
					Traces	+	++	+++
1.	Athletics	43	20	17	4	7	6	0
2.	Cycling	31	16	16	3	10	3	0
3.	Marathon	10	4	2	2	0	0	0
4.	Rugby	2	0	0	0	0	0	0
5.	Swimming	35	30	28	9	11	5	3
	Total	121	70	63				

Conclusion

The shift of bands to basic region leading to confirmation of samples may be due to proteinuria after competition. To avoid false positive detection of recombinant erythropoietin in post competition /post exercise urine, additional electrophoresis SDS-PAGE may be used complementarily to the IEF method to distinguish recombinant from endogenous EPO.

References

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