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EPO immunoaffinity columns – a powerful tool for purifying erythropoietin and its recombinant analogues

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Abstract

In this study, disposable immunoaffinity columns targeting both endogenous and recombinant EPO molecules were evaluated and validated in two WADA accredited anti-doping laboratories. The use of the columns improved the resolution of the IEF profiles considerably when compared with the classical ultrafiltration method, and the columns' ability to ensure the isoform integrity of the endogenous and exogenous EPO molecules was confirmed. Immunoaffinity columns will significantly improve the sensitivity and specificity of the actual urinary EPO test.

Introduction

The sample preparation method preceding the urinary EPO doping test [1] is based on several concentration and ultrafiltration steps, yielding a retentate enriched in EPO as well as in many unwanted urinary proteins. When present in high amounts, these other urinary proteins can potentially interfere with the isoelectric focusing (IEF) of EPO, causing smear and bleeding between lanes, as well as interact non-specifically with the antibodies used in the following double-blotting procedure. The use of two distinct anti-EPO antibodies for purification and detection of EPO reinforces the specificity of the test, making the likelihood of cross-reactivity phenomena extremely low [2-4]. SDS-PAGE is a complementary method to IEF recently added to the EPO doping test repertoire, which can discriminate between endogenous

and recombinant EPO due to differences in the apparent molecular weight [5-7]. The purified eluate from the EPO affinity columns is also suitable for SDS-PAGE.

Materials and Methods

Prior to IEF, urine samples were concentrated and purified using an anti-EPO column (EPO Purification Kit, MAIIA Diagnostics, Uppsala, Sweden). Briefly, 20 mL of urine passed through a 6 µL monolith (Ø 7 mm, height 0.15 mm) containing about 40 µg immobilized monoclonal anti-EPO antibody 3F6 which specifically captures both endogenous and recombinant human EPOs (rEPOs). The procedure followed the instructions of the kit. Ultrafiltration (UF) and IEF were performed as described by Lasne et al [1]. Bands' intensities were calculated using "GASepo" v1.2 software from Smart Systems [8]. The validation samples consisted of eight negative urines, buffer (50 mM Tris pH 7.4, 50 mM NaCl, 0.05% BSA, 0.02% NaN_3) spiked with low and high concentration of the standard for urinary EPO; NIBSC (National Institute for Biological Standards and Control), and a mixture of the standard for rEPO; BRP (Biological Reference Preparation; an equimolar mix of epoetin-α and -β), and NESP (Novel Erythropoiesis Stimulating Protein), as well as two positive urines (EPO-β and NESP [9]). In addition, buffer spiked with one of several EPO-analogues, and a blank sample containing buffer only, were also prepared. The validation procedure took place in two different WADA-accredited laboratories in the same time period to assess the inter-laboratory reproducibility. All samples were concentrated with both IAC and UF on the same day and in triplicate. 20 µl of the obtained eluates (IAC) and retentates (UF) were deposited on IEF gels. Single eluates were prepared from the samples spiked with EPO analogues and deposited on an IEF gel next to direct deposits of standard. The entire procedure was repeated on the negative urine samples in one lab on another day by a different technician. The obtained IEF-profiles were analyzed with the GASepo software and the results interpreted according to WADAs technical document TD2009EPO [10].

Results and Discussion

The quality of the IEF profiles was greatly improved after affinity purification (Figures 1, 2). The signal-to-noise ratio was higher than with UF, and smear and bleeding phenomena were clearly reduced. Applying the WADA criteria [10], there were no false-positive or false-negative results using IAC, and the result for each sample was the same independent of preparation method.

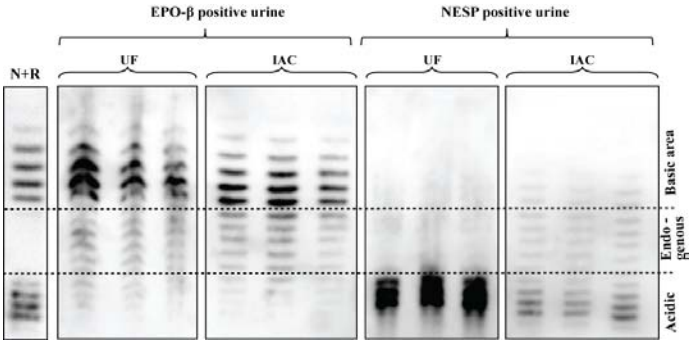

Figure 1. IEF gel representing the eluates and retentates prepared from EPO-β and NESP excretion samples by ultrafiltration (UF) and immunopurification (IAC), with positive controls (N; NESP, R; Recormon). Please note that the poor resolution of the UF-lanes shown here for the NESP positive urine is not a representative result; this sample is unique in that the NESP-concentration is very high, causing an overload in the acidic area after UF. This is not seen after IAC due to the lower recovery of NESP.

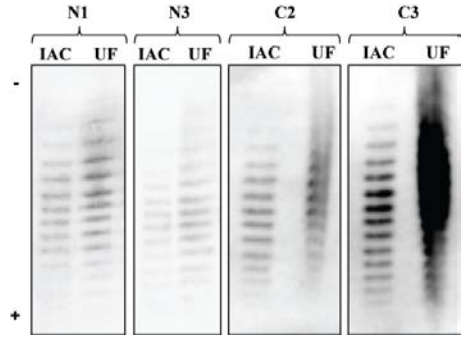

Figure 2. IEF gel with representative samples of eluates and retentates prepared from normal (N) and concentrated (C) negative urine samples by immunopurification (IAC) and ultrafiltration (UF).

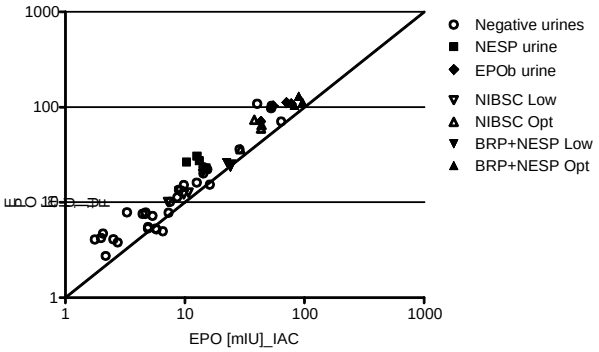

Figure 3. Two-by-two representation of the EPO amounts [mIU] measured in the retentates (UF) and eluates (IAC) prepared from the negative urines, excretion urines (NESP and EPO-β) and spiked buffer validation samples.

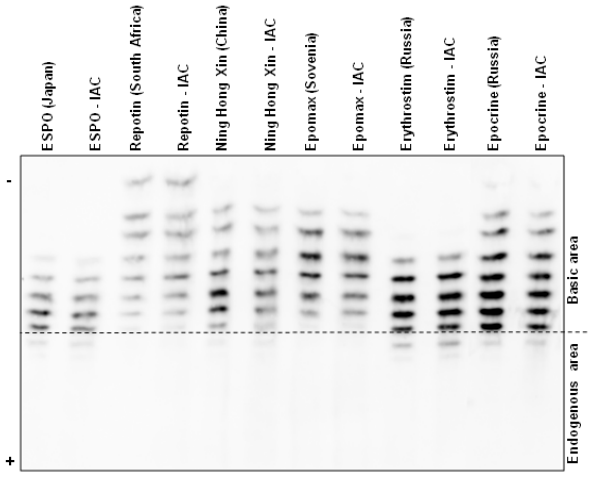

Figure 4. IEF gel representing eluates of buffers spiked with various rEPOs (IAC), and standards loaded directly on the gel. The standards were loaded in the following amounts: 0.3 ng ESPO; 0.2 ng Repotin; 0.3 ng Ning Hong Xin; 0.3 ng Epomax; 0.25 ng Erythrostim and 0.25 ng Epocrine. The amount of standards in the eluates was estimated to be 0.24 ng, assuming a recovery of 0.7 after IAC.

Repeatability and reproducibility of the EPO concentration procedure using IAC was both satisfactory and improved when compared to UF (Table 1, Figure 1). No isoform discrimination of the columns was observed comparing the IEF profiles of various EPO-analogues after IAC with the profiles of directly deposited material (Figure 4). Both labs experienced a slight shift from the basic to the endogenous are using IAC, which could be explained by the reduced background often seen in the basic area using UF. The columns affinity for NESP was lower compared to other epoetins. In both labs, the EPO amounts

measured in the eluates were lower than those measured in the retentates (Figure 3). The ratio $EPO_{eluates}$ / $EPO_{retentates}$ obtained in the two laboratories was 0.7 (SD = 0.2) and 0.8 (SD = 0.2), respectively. The EPO immunoaffinity columns fulfilled all criteria required to fit for the purposes of anti-doping analyses. They allowed a significant improvement of the quality of IEF gels by augmenting the signal to noise ratio. The slightly reduced recovery of the columns was almost fully counterbalanced by the high resolution of the IEF profiles following IAC. It can therefore be concluded that IAC constitute a specific and powerful tool to prepare urinary EPO samples prior to IEF analysis[11].

Table 1 **Summary analytical variance**

		IAC	UF
Negative urines, PBI and PAI*	Intra-assay CV	17 %	25 %
	Inter-assay CV	20 %	25 %
	Inter-laboratory CV	19 %	31 %
Negative urines, Ratio	Intra-assay CV	27 %	29 %
	Inter-assay CV	21 %	25 %
	Inter-laboratory CV	26 %	37 %
Spiked buffers, PBI and PAI*	Intra-assay CV	16 %	19 %
	Inter-laboratory CV	35 %	17 %
Positive urines, PBI and PAI*	Intra-assay CV	6 %	2 %
	Inter-laboratory CV	3 %	6 %

* PAI calculated for NESP-containing samples only

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