Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 1028–1032

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Erythropoietin (EPO) immunoaffinity columns—A powerful tool for purifying EPO and its recombinant analogues

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ARTICLE INFO

Article history:
Received 15 March 2010
Received in revised form 17 June 2010
Accepted 18 June 2010
Available online 26 June 2010

Keywords:
Erythropoietin
Affinity purification
Isoelectric focusing
Validation
Doping control

ABSTRACT

The sample preparation method preceding the urinary erythropoietin (EPO) doping test is based on several concentration and ultrafiltration steps. In order to improve the quality of isoelectric focusing (IEF) gel results and therefore, the sensitivity of the EPO test, new sample preparation methods relying on affinity purification were recently proposed. This article focuses on the evaluation and validation of disposable immunoaffinity columns targeting both endogenous and recombinant EPO molecules in two World Anti-Doping Agency (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 constitute therefore a potent and reliable tool for the preparation of urine samples and their use will significantly improve the sensitivity and specificity of the actual urinary EPO test.

1. Introduction

The main physiological role of the renal hormone EPO is the stimulation of red blood cell production. Recombinant forms of EPO (rEPO) have been produced since the late eighties for the treatment of anemia, and due to its effect on the oxygen transport to tissues, rEPO rapidly became one of the most frequently used doping agents in endurance sport. In 2000, an assay based on IEF followed by a double-blotting process allowing the identification of rEPO intake in athletes' urines was introduced [1–3]. This method is currently the only official method accredited by the WADA and is used on a routine-basis in the anti-doping laboratories around the world. In 2003, the WADA requested the publication of an external evaluation report on the urine EPO test [4]. This report notably suggested some improvements of the classical sample preparation method, which is based on a series of concentration and ultrafiltration (UF) steps. Progress in this matter has been hampered by difficulties in producing antibodies not discriminating EPO isoforms and by cumbersome immunopurification procedures, though several laboratories have started to develop immunoaffinity-based preparative methods [5,6]. Immunoaffinity purification is a valuable and particularly powerful tool to improve the signal-to-noise ratio of immuno-based methods [7]. It presents the advantage to specifically purify urine samples by isolating the various isoforms of EPO thanks to a specific antibody targeting both endogenous and exogenous forms of the hormone. Such purification prevents smears and "smiling" phenomena as well as eventual non-specific interactions of the second antibody with diverse urinary proteins. Altogether, this results in a lower background noise, a better resolution of the different isoforms and, consequently, a better quality of IEF gels. In addition, 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. Cross-reactivity has indeed been suggested by several authors [8–10], and even if it has been demonstrated that the anti-EPO antibody used for IEF detection (the AE7A5 clone from R&D Systems) recognizes a second urinary protein, it was concluded that this cross-reactivity in no case interfered with the interpretation of IEF gel results [11].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is another method which can discriminate between endogenous and recombinant EPO due to differences in the apparent molecular weight [12–15], and the method was recently added to the EPO doping test repertoire to meet the challenges posed by new EPO-analogues and biosimilars entering the marked. The purified eluate from the EPO affinity columns is also suitable for SDS-PAGE analysis, which otherwise requires affinity purification of the urine ultrafiltrate, most commonly performed with EPO enzyme-linked immunosorbent assay (ELISA) [14,15].

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doi:10.1016/j.jpba.2010.06.017

Our article focuses on the evaluation and validation of a new sample preparation method based on immunoaffinity columns (IACs) produced by a Swedish company in two WADA accredited anti-doping laboratories. Though immunoaffinity purification per se is well known, the herein validated columns represent the first commercially available affinity tool suitable for EPO purification from urine samples.

2. Materials and methods

2.1. Immunoaffinity purification and IEF

All urine samples were collected, aliquoted and stocked following the standard procedures of an anti-doping laboratory. 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 (Ø7 mm, height 0.15 mm) containing about 40 μg immobilized monoclonal anti-EPO antibody 3F6 which specifically captures both endogenous and recombinant human EPOs. 55 μL of eluate was recovered after desorption by micro-centrifugation. The procedure followed the instructions of the kit, which includes a precipitate dissolution buffer and buffers for washing, desorption and pH adjustment. The flow rate was standardized for all extractions and fixed at 1 mL/min. UF and IEF were performed as previously described by Lasne et al. [2]. The IEF-bands' intensities were calculated using "GASepo" v1.2 software from Smart Systems [16]. The percentage of basic isoforms (PBIs), respectively the percentage of acidic isoforms (PAIs) for NESP-containing samples, were determined on the basis of the bands' intensities and positions compared to those of standard reference preparations, as described in the 2009 WADA technical document [17]. The actual WADA positivity criteria were applied to the obtained profiles [17]. With regards to the 3rd positivity criterion, the "ratio" was defined as the quotient of the intensity of the second most intense basic isoform by the intensity of the most intense endogenous isoform.

2.2. Samples collection

2.2.1. Negative samples

Four "normal" (N) and four "concentrated" (C) negative urine samples were selected with regards to their respective specific gravity (SG) (mean $SG_{N\ urines} = 1.009$, mean $SG_{C\ urines} = 1.019$) and total protein concentration (TPC) (mean $TPC_{N\ urines} = 19$ mg/L, mean $TPC_{C\ urines} = 136$ mg/L). TPC in urine was estimated using a Dimension XPand system (Siemens Healthcare Diagnostics, Deerfield, USA).

2.2.2. Spiked buffers

A buffer containing 50 mM Tris pH 7.4, 50 mM NaCl, 0.05% BSA, 0.02% NaN_3 was spiked with the standard for urinary EPO, NIBSC (National Institute for Biological Standards and Control), and with a mixture of the standard for rEPO, BRP (Biological Reference Preparation, an equimolar mixture of epoetin-α and epoetin-β, the European Directorate for the Quality of Medicines, Strasbourg, France) and Aranesp® (NESP, Novel Erythropoiesis Stimulating Protein, Amgen AG, Zug, Switzerland) in two different concentrations defined as "low" and "optimal". The traditional amounts of standards deposited on IEF gels are 0.2 ng for NESP, 0.3 ng for BRP and 0.325 ng for NIBSC. "Low" and "optimal" concentrations corresponded to one third and to 1.5 times of these amounts, respectively. In addition, buffer was spiked with one of several EPO-analogues (0.75 ng of analogue was added to 20 mL buffer, final volume of eluates after IAC was 55 μL) (Erythrostim, Epocrine (both from Russia), Epomax (epoetin omega from Slovenia), Ning Hong Xin (China), Repotin (South-Africa) and ESPO (Japan)). A blank sample containing buffer only was also prepared.

2.2.3. Positive samples

Two positive urine samples (one NESP-positive sample, one EPO-β positive sample) coming from excretion studies performed in the past [18] were selected for analysis.

2.3. Validation process

The validation was performed following the EN ISO/IEC 17025 (2005) standard [19]. In order to assess the repeatability of each up-concentrating method, each extraction using UF or IAC was performed in triplicate for each sample of negative and positive urine, and spiked buffer. The samples spiked with EPO-analogues were only extracted once. On the same day, each sample was extracted using both UF and IAC. The obtained extracts were defined as retentates and eluates, respectively. For calculation of recovery, EPO concentration was estimated in the extracts using

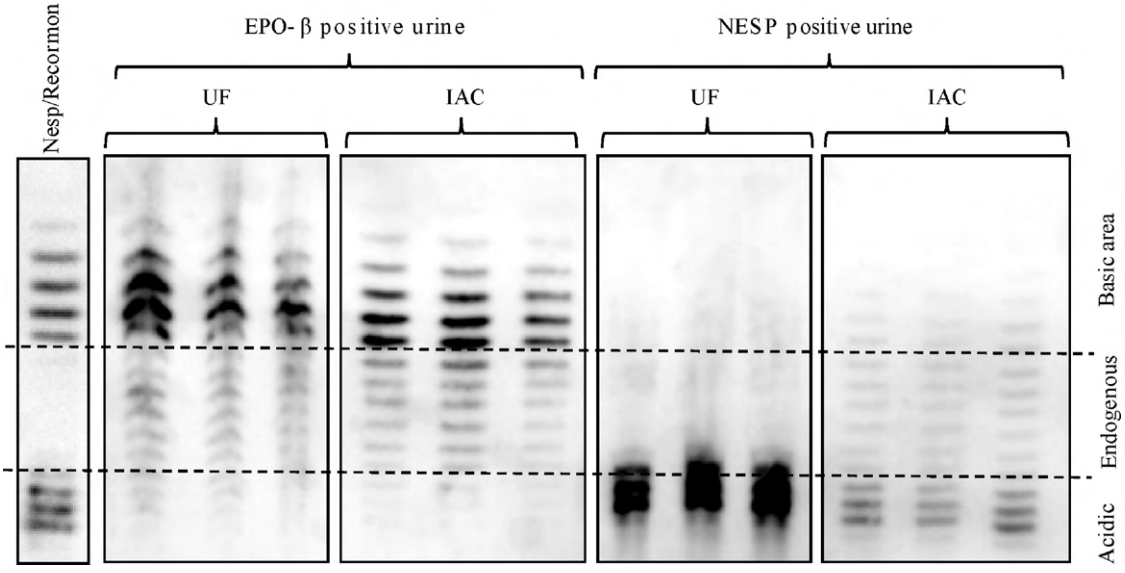

Fig. 1. IEF gel representing the retentates and eluates prepared from EPO-β and NESP-excretion samples by UF and IAC, with usual positive controls (Aranesp©/Recormon©). Please note that the poor resolution of the UF-lanes shown here for the NESP-positive urine is not a representative result; this sample 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.

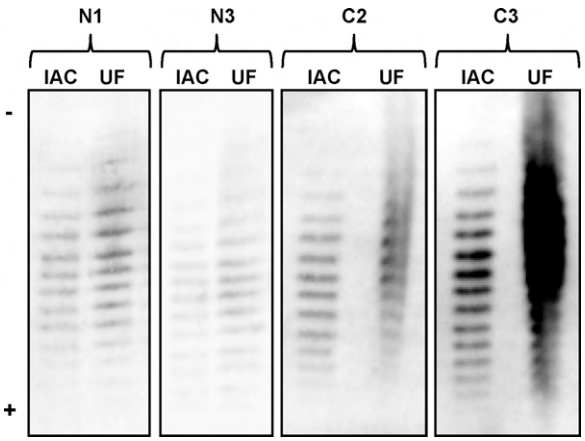

Fig. 2. IEF gel with representative samples of eluates and retentates prepared from normal (N) and concentrated (C) urine samples by IAC and UF.

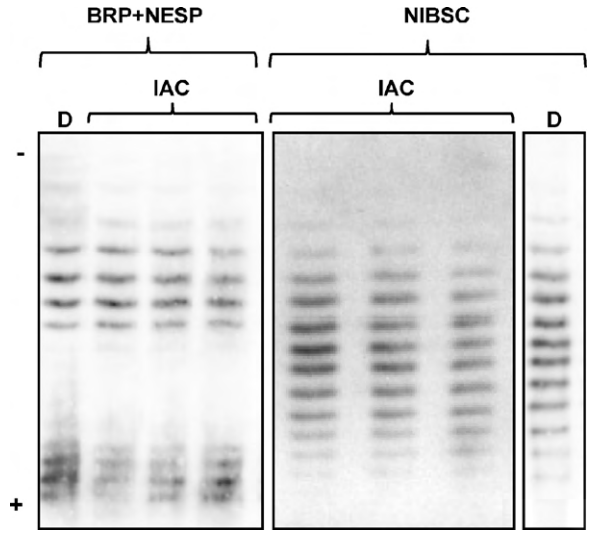

Fig. 3. IEF gel displaying eluates from buffer spiked with BRP and NESP, and NIBSC, after triplicate runs of IAC. Standards were directly deposited (D) on the gel for comparison of isoform profiles. No isoform discrimination by the columns was observed for any of the standards.

the IMMULITE© 2000 and 2500 immunoassay systems (Siemens Healthcare Diagnostics, Deerfield, USA). 20 μL of the obtained eluates and retentates were then 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 samples 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. In order to assess the inter-laboratory reproducibility, the process was carried out during the same period in two different WADA accredited laboratories.

3. Results and discussion

3.1. General quality of the gels

The general quality of IEF profiles was greatly improved following affinity purification (Figs. 1–3). The signal-to-noise ratio was evidently higher than with UF, while the smear and "bleeding" phenomena were clearly reduced. The detectable number of bands was systematically improved. Fig. 1 depicts a gel on which the eluates and retentates prepared from the EPO-β and NESP-excretion samples were deposited, Fig. 2 displays representative eluates and retentates from two normal and two concentrated urines, and in Fig. 3 a triplicate set of IAC eluates from buffer spiked with either BRP and NESP or NIBSC, is shown side by side with standard loaded directly on the gel.

3.2. Reproducibility

The reproducibility of the sample preparation procedure using IAC was both satisfactory and improved when compared to UF (Fig. 4). For each sample extracted in triplicate by both concentrating methods, the PBI (respectively, PAI in NESP-enriched samples) was calculated from the corresponding IEF profiles. The improved inter-assay and inter-laboratory reproducibility using IAC can at least partly be explained by the increased resolution of the gels that is achieved with the columns after removal of most urinary proteins (see Figs. 1 and 2). The results obtained in both laboratories were very similar, independently of the SG, TPC and EPO concentration of the different urine samples (Fig. 4).

3.3. Specificity

For each individual sample, the result was the same when applying the WADA identification criteria (TD2009EPO), independently

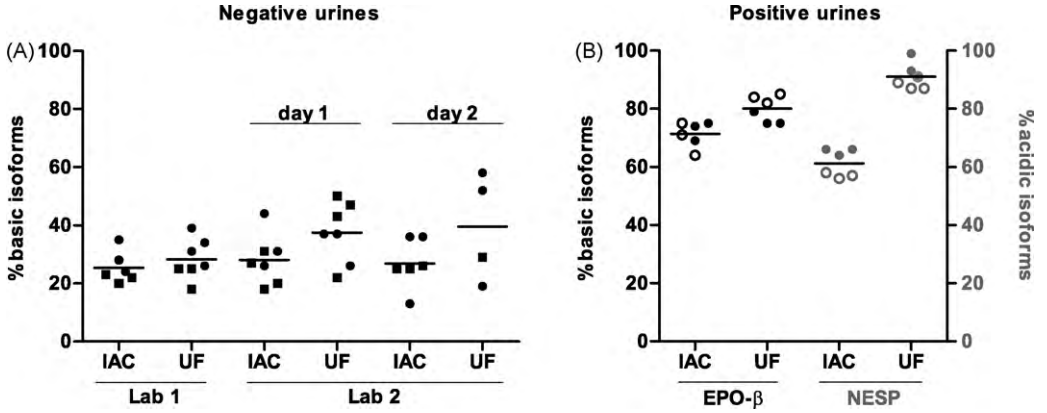

Fig. 4. Reproducibility and repeatability of PBI and PAI. (A) The analysis of the seven detectable negative samples, each presented with its triplicate mean prepared by UF and IAC in both labs, where filled circles indicate normal urines and filled squares indicate concentrated urines. One sample (N2) was below the limit of quantification. The procedure was repeated on a different day by a different technician in lab 2. (B) PBI for the EPO-β positive urine and PAI for the NESP-positive urine, shown with triplicate measurements from lab 1 (open circles) and lab 2 (filled circles).

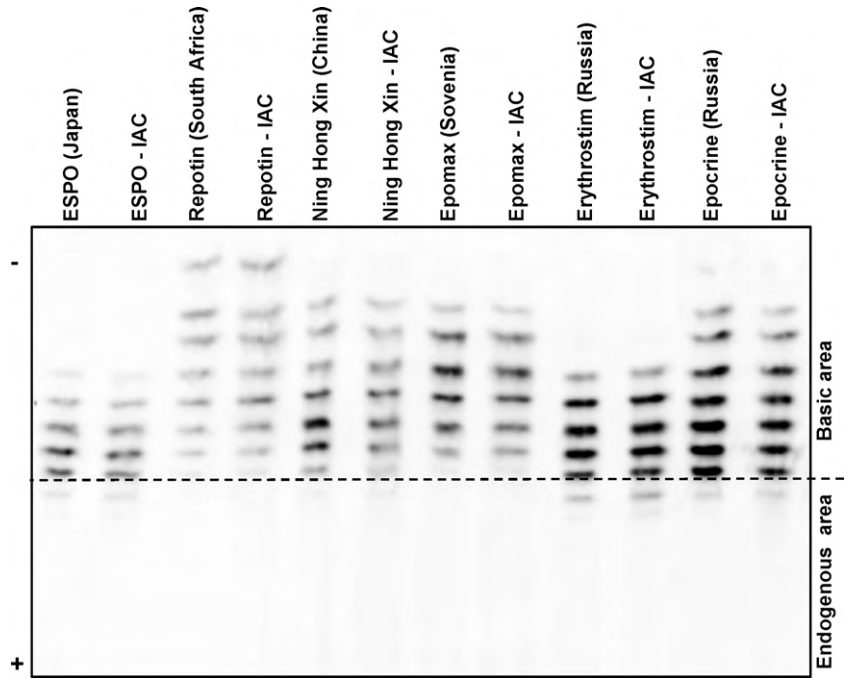

Fig. 5. IEF gel representing eluates of buffers spiked with various rEPOs (IAC), and standards loaded directly on the gel. The standards were loaded in the following amounts: 0.3 ng ESPO; 0.2 ng repotin; 0.3 ng Ning Hong Xin; 0.3 ng Epomax; 0.25 ng Erythrostim and 0.25 ng Epocrine. The amount of standards in the eluates was estimated to be 0.24 ng, assuming a recovery of 0.7 after IAC.

of the preparation method. No false-positive or false-negative result was observed following the use of IAC. It should however be mentioned that the NESP-positive sample was returned as "suspicious" by one of the laboratories because it failed to fulfill the 3rd criterion after both IAC and UF, a fact that was explainable by a poor band resolution in the acidic domain of the gel in question and not the sample preparation procedure.

On the basis of the calculated PBI, both labs experienced a slight shift from the basic towards the endogenous area after concentration of the samples using IAC (Fig. 4A). This shift can be explained by the disappearance of the higher background and smear in the basic area of the gel having concentrated the samples using IAC, often observed after concentration with UF (see sample C2 in Fig. 2). The same was true with NESP-enriched samples, in which PAI were slightly lower after affinity purification. As IAC was shown to have a lower affinity towards NESP compared to classical epoetins (see Section 3.5), this fact was somewhat expected. The lower affinity of the columns towards NESP did not cause any isoform discrimination; this was evident when comparing the obtained EPO profiles of negative urines after IAC with those obtained after UF (Fig. 2), and when eluates from buffer spiked with standards were compared to standards loaded directly on the gel (Fig. 3). Further, when comparing the isoelectric profiles of various EPO-analogous after IAC with the profiles obtained after direct deposit of the analogues on the IEF gel, no isoform discrimination by the columns was observable for a wide selection of available EPO-analogues (Fig. 5).

3.4. Repeatability

The repeatability of the affinity purification procedure was satisfactory and also slightly improved compared to that achieved with ultrafiltration (Fig. 4), the latter likely due to reduced background and smear (Figs. 1–3).

3.5. Recovery

In both labs, the EPO amounts measured in the eluates were lower than those measured in the retentates. The ratio $EPO_{eluates}/EPO_{retentates}$ obtained in the two laboratories was 0.7 (SD = 0.2) and 0.8 (SD = 0.2), respectively. Fig. 6 shows a two-by-two representation of the amount of $EPO_{eluates}$ against the amount of $EPO_{retentates}$ of all urine samples prepared by the two labs. Such EPO losses during affinity purification are probably due to matrix effects, with diverse urinary proteins preventing the EPO molecules access to all epitopes. This effect is not due to insufficient capacity of the column, as the antibodies are fixed in excessive amounts compared to the number of EPO molecules present in the urine samples. The fact that spiked standards generally presented a better EPO recovery following affinity purification supported the matrix hypothesis. Despite this, and thanks to the much higher signal-to-noise ratio yielded by immunopurification, only urines containing extremely low EPO concentrations were expected to avoid detection after IAC extraction. Interestingly, the columns appeared to have a lower affinity for NESP in urine when compared with classical epoetins. This fact was illustrated by a mean ratio $EPO_{eluates}/EPO_{retentates}$ of 0.4 for NESP-containing urine samples, but when applied to

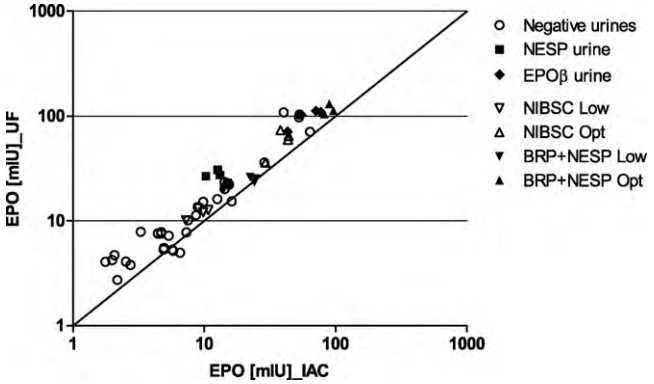

Fig. 6. Two-by-two representation of the EPO amounts [mIU] measured in the retentates (UF) and eluates (IAC) prepared from the negative urines, excretion urines (NESP and EPO-β) and spiked buffer validation samples. The open and filled triangles indicate buffer spiked with NIBSC and the BRP and NESP standards, respectively, added at either low or optimal concentration.

buffer the ratio was about 0.8. This observation explained the slight decrease observed in the PAI of NESP-containing samples extracted by IAC when compared with UF (see Fig. 4).

4. Conclusion

The new sample preparation method we tested, the EPO immunoaffinity columns, fulfilled all criteria required to fit for the purposes of anti-doping analyses. It allowed a significant improvement of the quality of IEF gels by augmenting the signal-to-noise ratio. Even if being similar to that achieved with UF, the repeatability and both inter-assay and inter-laboratory reproducibility were systematically improved following affinity purification. Finally, the slightly reduced recovery of the columns was almost fully counterbalanced by the high resolution of the IEF profiles following IAC treatment and should therefore prevent an increase in number of undetectable EPO profiles. Therefore, it can be concluded that IAC constitute a specific and powerful tool to prepare urinary EPO samples prior to IEF analysis. In addition, the fact that the columns are disposable and intended for single use prevents any carry-over effect and guarantees an excellent specificity to the product. A routine-based use of IAC has the potential of significantly improving the sensitivity of the actual EPO test.

Acknowledgments

The authors wish to gratefully acknowledge Mette Borgen at the Norwegian Doping Control Laboratory, and Rita Saugy at the Laboratoire Suisse d'Analyse du Dopage for their technical support, and to extend their gratitude to Peter Hemmersbach, Neil Robinson and Martial Saugy for helpful comments on the manuscript.

This work has been performed in the *EUREKA* project "EPO affinity tool", with financial support for affinity reagents from VINNOVA (The Swedish Governmental Agency for Innovation Systems). Séverine Lamon was supported financially by a grant from Anti-doping Switzerland.

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