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Evaluation of Methods for Purifying Erythropoietin from Urine using Immunoaffinity Applications

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ABSTRACT

Erythropoietin (EPO), a hormone that regulates the synthesis of red blood cells, is frequently abused by athletes. Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) is an essential analytical technique in all anti-doping laboratories in order to detect the abuse of EPO. An immunoaffinity purification step is now considered essential for the pre-treatment of urine samples to isolate EPO prior to gel electrophoresis. In this study, we have compared the performance of two immunoaffinity purification techniques in EPO anti-doping analysis, i.e., the anti-EPO micro well plate and anti-EPO monolith column. The anti-EPO monolith column is efficient in removing undesirable proteins except for the Bovine Serum Albumin (BSA) as seen on SDS-PAGE. The BSA was eventually removed from the protocol and the undesirable protein band was eliminated without affecting the performance of the method. Throughout the study, the anti-EPO monolith column emerged as a better option, as it provided a higher sensitivity and higher throughput analysis when compared to the anti-EPO micro well plate. The anti-EPO monolith column has shown consistent results with the EPO recovery rate of 72%, while the limit of detection is as low as 0.5 mIU mL^{-1}.

Key words: Erythropoietin, immunopurification, anti-EPO monolith column, anti-EPO micro well plate, urine

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone that is responsible for producing red blood cells (Carbonell-Estrany and Figueras-Aloy, 2001; Dehnes *et al*., 2010). The recombinant human EPO (rhEPO) was produced successfully using DNA recombinant technology since 1985 (Jacobs *et al*., 1985). Unfortunately, the existence of rhEPO has led to its abuse in sports, where some athletes chose to apply rhEPO illegally to boost their performance. Since 1990, the International Olympic Committee (IOC) has prohibited rhEPO and athletes are required to be regularly tested to ensure a fair competition in sports (Delanghe *et al*., 2008). Since then, several techniques have been introduced to detect EPO abuse in the world anti-doping laboratories, including iso electric focusing (IEF) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Guan *et al*., 2008; Lasne *et al*., 2002; Morkeberg *et al*., 2014). However, the detection of rhEPO in anti-doping analysis remains a challenge for scientist, as the detection of EPO misuse can be tricky at times.

The SDS-PAGE has been shown to be able to separate rhEPO from urinary human EPO (uhEPO) based on their molecular weights (Skibeli *et al*., 2001). However, a good purification step is required prior to SDS-PAGE in order to remove other impurities that can interfere with the

subsequent immunoblotting procedure. Scientists have migrated the purification technique from using conventional ultrafiltration to immunoaffinity purification (Lonnberg *et al*., 2010). One of the advantages of EPO immunoaffinity purification includes the ability to minimize the interference by non-specific proteins from the sample matrix (Beullens *et al*., 2006). Reihlen *et al*. (2012) has mentioned that immunoaffinity purification of urine samples is able to improve signal to noise ratio and avoid cross-reactivity. There are quite a number of EPO immunoaffinity purification techniques or kits that have been introduced in the market (Guan *et al*., 2008; Lonnberg *et al*., 2010; Reihlen *et al*., 2012; Lasne *et al*., 2007; Reichel *et al*., 2009), which include the anti-EPO monolith column and the customized anti-EPO micro well plate that are widely used in many anti-doping laboratories.

The monolith column has the anti-EPO antibody is bound to a monolith layer in the monolith column, which gives an advantage of a rapid transfer of solution with efficient convective flow (Rucevic *et al*., 2006). This purification technique has shown to be able to minimize the interference by non-specific proteins (Beullens *et al*., 2006). However, the column is only allowed for a single usage in order to avoid contaminations (Lonnberg *et al*., 2010), which may increase the cost of analysis. On the other hand, anti-EPO micro well plate applies the concept of purifying EPO from urine using a commercially made EPO-specific immunoaffinity micro well plate that is meant for enzyme-linked immuno sorbent assay (ELISA). With a slight modification on the approach, EPO in the urine samples can be captured by the anti-EPO monoclonal antibody that is pre-coated on the micro well plate. The plate is disposable, easy to use and could easily fit in many samples at the same time (Mallorqui *et al*., 2010). Despite the advantages mentioned, longer preparation time is required as urine samples need to be pre-concentrated using the conventional ultrafiltration steps before loading into the micro well plate.

Although, various work have been done to show the capability of each of these techniques, there has been no effort to evaluate and compare these methods side by side in order to determine, which performs better for the purpose of anti-doping analysis. This is crucial to ensure that users are not jeopardizing the accuracy of analysis and losing valuable information from the samples. In this study three different elution buffers for anti-EPO monolith column were evaluated to identify the buffer that gives the highest recovery of EPO. Subsequently, the performances of anti-EPO micro well plate and anti-EPO monolith column were evaluated and compared by applying urines with different concentrations of uhEPO.

MATERIALS AND METHODS

Materials: Consumables applied in the research were purchased from Thermo Scientific (Rockford, IL, USA), unless stated otherwise.

Sample preparation: Five liters of urine samples received from healthy volunteers were pooled and stored at -20°C in 20 mL aliquots until used. The urine samples were pre-spiked with designated concentration of uhEPO before proceeding to purification step. The uhEPO standard was purchased from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, United Kingdom).

EPO purification

Ultrafiltration and purification using anti-EPO micro well plate: Ultrafiltration of urine specimens was performed prior to the application of anti-EPO micro well plate in order to concentrate the samples to a smaller volume. The ultrafiltration device (Millipore, Billerica, MA,

USA) with a Molecular Weight Cut-Off (MWCO) at 30 kDa was used. For the 20 mL urine sample, 20 μL of protease inhibitor cocktail and 2 mL of 3.75 M Tris buffer pH 7.4 were added. The urine mixture was centrifuged for 10 min and the supernatant was filtered through a 0.45 μm syringe filter. The 15 mL filtrate was applied to an Amicon Ultra-15, MWCO 30 kDa and after 10 min of centrifugation the remaining filtrate was added and centrifuged for another 10 min. The retentate was washed with 15 mL of 50 mM Tris pH 7.4 buffer supplemented with protease inhibitor cocktail thrice, then concentrated to approximately 500 μL. Later, the retentate was transferred to an Amicon Ultra-0.5, MWCO 30 kDa and centrifuged to obtain the retentate with the final volume of approximately 50 μL. The filter was then transferred into a new Eppendorf tube and reverted in a centrifuge to recover the retentate. The protocols of using anti-EPO micro well plate (Stemcell Technologies, Vancouver, Canada) were performed according to Reihlen *et al*. (2012) with minor modifications. The concentrated sample was transferred into the anti-EPO micro wells and covered with parafilm and aluminium foil. Later, it was incubated overnight at 4°C. The wells were washed with Phosphate Buffered Saline (PBS) several times and tapped dry later. The 4.4% of 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), LDS sample buffer and a self-prepared sample buffer were then added to the wells, respectively, followed by incubation at 80°C for 20 min. The eluted sample was then cooled to room temperature and centrifuged to a new Eppendorf tube. The eluate was kept at -20°C until further analysis.

Purification using anti-EPO monolith column: The procedure was carried out as described in the instructions in the MAIIA EPO Purification Kit (Art. No. 0250) (MAIIA Diagnostics, Uppsala Sweden). Briefly, 20 mL urine was mixed with the Urine Precipitate Dissolvation (UPD) buffer was added with the proportions of 10 parts of urine and 1 part of UPD buffer. The UPD-urine mixture was then heated to approximately 82-85°C, as specified in the protocol provided and thereafter rapidly cooled by immersing the tubes in a cold water bath. The urine-UPD mixture was diluted 1:1 by adding dilution buffer (dilution in water from the supplied Detergent aid and Exposure aid to 2%, respectively). The urine mixture was then filtered through a 0.45 μm high flow syringe filters. The anti-EPO column (7 mm diameter monolith, height 0.15 mm), which was provided along with the kit was washed with 1 mL of washing buffer. The sample was then passed through the anti-EPO column, using the vacuum manifold, at a flow rate approximately 1 mL min^{-1}. Next, the column was centrifuged to remove excessive water in it. The EPO was eluted by adding the desorption buffer to the column and centrifuging the column in a new vial containing pH adjustment buffer supplemented with BSA and detergent. The eluate was kept at -20°C until further analysis.

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on Tris HCl gel (10% T, 1.0 mm, 10 wells, Tris/Glycine/SDS running buffer) handcasted according to the protocol of Laemmli (1970) using chemicals from Bio-Rad Laboratories (Bio-Rad, Hercules, CA, USA). Samples were heated at 95°C for 5 min, followed by 5 min of cooling in a cool bloc. Electrophoresis was performed at voltage of 80 V for the first 10 min, followed by 120 V until the solvent reach to the end of the gel.

Western blotting: After SDS-PAGE, the gels were equilibrated for 2 min in a 25 mM Tris, 192 mM glycine mixture buffer and then blotted (1.52 mA cm^{-2}) on PVDF membranes for 45 min using the Multiphor II Electrophoresis System (Amershem Biosciences, Amersham, United Kingdom). Four sheets of thick blotting paper were used on each side of the blotting sandwich. After the transfer

the membrane was incubated in a solution of 5 mM Dithiothreitol (DTT) in PBS at 37°C for 60 min in order to increase the sensitivity of the method. Subsequently, the membrane was blocked in 5% non-fat milk (Sunlac, Penang, Malaysia) in PBS for 60 min, incubated in a solution of primary anti-EPO antibody (R and D Systems, Minneapolis, USA) in 1% non-fat milk for 2 h, then washed in 0.5% non-fat milk in PBS (3×10 min). Later the membrane was transferred to a solution of streptavidin Horse Radish Peroxidase (HRP) in 1% non-fat milk for overnight incubation. The next day it was washed with PBS (3×10 min). Enhanced chemiluminescence was achieved by incubation of the blot in the Super Signal ELISA Femto Maximum Sensitivity Substrate solution (Thermo Scientific, Rockford IL, USA). The image of the SDS-PAGE gel was captured using the Luminescent Image Analyser LAS-3000 (Fujifilm, Tokyo, Japan). Multi Gauge version 3.0 software (Fujifilm, Tokyo, Japan) was used to analyze the bands of EPO. The exposure time was adjusted according to the initial signal intensity.

RESULTS

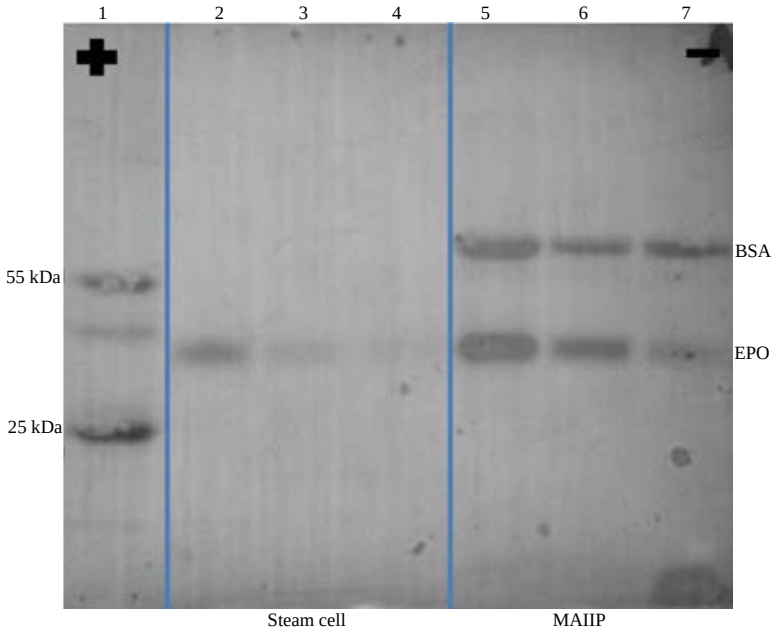
Elution of EPO in anti-EPO micro well plate using CHAPS and LDS sample buffer: An EPO quantitative test was performed using the Immulite 1000 immunoassay on three elutions from anti-EPO micro well plate, which used three different buffers, namely the LDS sample buffer, a self-prepared sample buffer and CHAPS. As seen in Table 1, the result showed that the average concentration of EPO eluted using LDS sample buffer was 3.33 mIU mL^{-1}. On the other hand, the self-prepared buffer showed an average of 1.23 mIU mL^{-1}, 63% lower than the EPO LDS sample buffer elution. However, EPO recovered using CHAPS elution buffer showed 86% higher reading than LDS sample buffer to an average of 6.17 mIU mL^{-1}.

Comparison of anti-EPO monolith column and anti-EPO micro well plate: A set of samples evaluated using anti-EPO monolith column and anti-EPO micro well plate with ultrafiltration methods was presented in Fig. 1. Lanes 2-4 were samples purified using anti-EPO micro well plate whereas lanes 5-7 were samples purified using anti-EPO monolith column. Urine samples were pre-spiked with erythropoietin as followed, 20 mIU mL^{-1} (Lanes 2 and 5), 10 mIU mL^{-1} (Lanes 3 and 6) and 5 mIU mL^{-1} (Lanes 4 and 7). The molecular weight of EPO is estimated to be around 30.4 kDa (Fisher, 2003). Based on the figure shown, there are no foreign bands or other noise signal visible within the two protein markers reflecting 55 and 25 kDa (Lane 1). The bands reflected on Lanes 5-7 were generally, more intense compared to bands on Lanes 2-4, respectively. However, there is a foreign band detected on Lanes 5-7, respectively, the samples, which were purified using anti-EPO monolith column. As indicated in Fig. 1, the band reflected the protein weights of around 60-70 kDa.

Table 1: Quantitative analysis of EPO recovered from the urine sample pre-spiked with 5 mIU mL^{-1} of uhEPO in the stem cell purification. Three different elution buffers were used, namely the LDS sample buffer, self-prepared sample buffer and CHAPS. EPO recovered in the self-prepared sample buffer is 63% lower than the LDS sample buffer and the CHAPS buffer is 86% higher than the LDS sample buffer

	EPO concentrations (mIU mL^{-1})		
Elution buffer	1st	2nd	Average
LDS sample buffer	3.07	3.59	3.33
Self-prepared sample buffer	1.11	1.34	1.23 (63%↓)
CHAPS	5.87	6.46	6.17 (86%↑)

CHAPS: 3-[(3-cholamidopropyl) dimethyamino]-1-propane sulfate, LDS: Lithium dodecyl sulfate and EPO: Erythropoietin



Lane	2	3	4	5	6	7
EPO concentration (mIU mL^{-1})	11.8	8.8	4.4	25.4	17	14.4

Fig. 1: SDS-PAGE of urine samples using anti-EPO micro well plate (stem cell purification with ultrafiltration) and anti-EPO monolith column (MAIIA purification), Lane 1: Protein markers, Lanes 2-4: Pre-spiked urine samples with 20, 10 and 5 mIU mL^{-1} of uEPO, respectively and purified with anti-EPO micro well plate (stem cell purification with ultrafiltration) method, Lanes 5-7: Pre-spiked urine samples with 20, 10 and 5 mIU mL^{-1} of uEPO, respectively and purified with anti-EPO monolith column (MAIIA purification) method. The table shows the quantitative analysis of EPO recovered from each samples, UF: Ultrafiltration

A quantitative analysis has also been conducted to the immunopurified samples using Immulite 1000 immunoassay system to determine the concentration of purified EPO. Based on the results obtained from Immulite 1000 immunoassay system, the amount of EPO recovered by using the anti-EPO monolith column were generally higher for all concentrations of spiked uhEPO compared to purification using anti-EPO micro well plate. For a concentration of 5 mIU mL^{-1} of pre-spiked EPO in the urine sample, the anti-EPO monolith column recovered twice the amount of uhEPO compared to anti-EPO micro well plate.

Recovery rate and consistency of using anti-EPO monolith column: To confirm on the recovery rate of uhEPO by using the anti-EPO monolith column, another set of tests were conducted (Fig. 2). The 10 mIU mL^{-1} of uhEPO standard were loaded into Lanes 2-4, respectively, whereas Lanes 5-9 were loaded with purified urine samples pre-spiked with 10 mIU mL^{-1} uhEPO using the anti-EPO monolith column. The recovery rate of uhEPO was calculated using the Multi

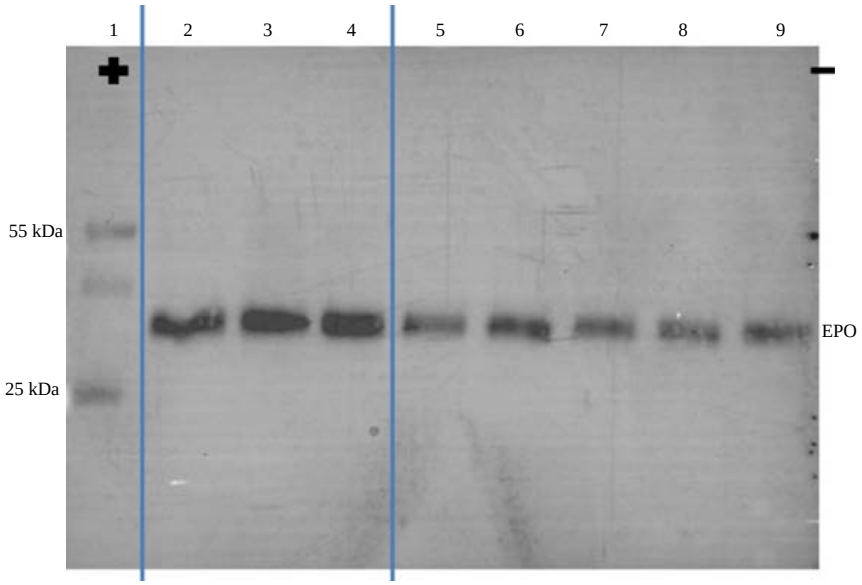


Fig. 2: Consistency and recovery of EPO purified by anti-EPO monolith column (MAIIA purification), Lane 1: marker, Lanes 2-4: uEPO standards of 10 mIU mL^{-1}, Lanes 5-9: Pre-spiked urine samples with 10 mIU mL^{-1} of uEPO and purified with anti-EPO monolith column (MAIIA purification) method

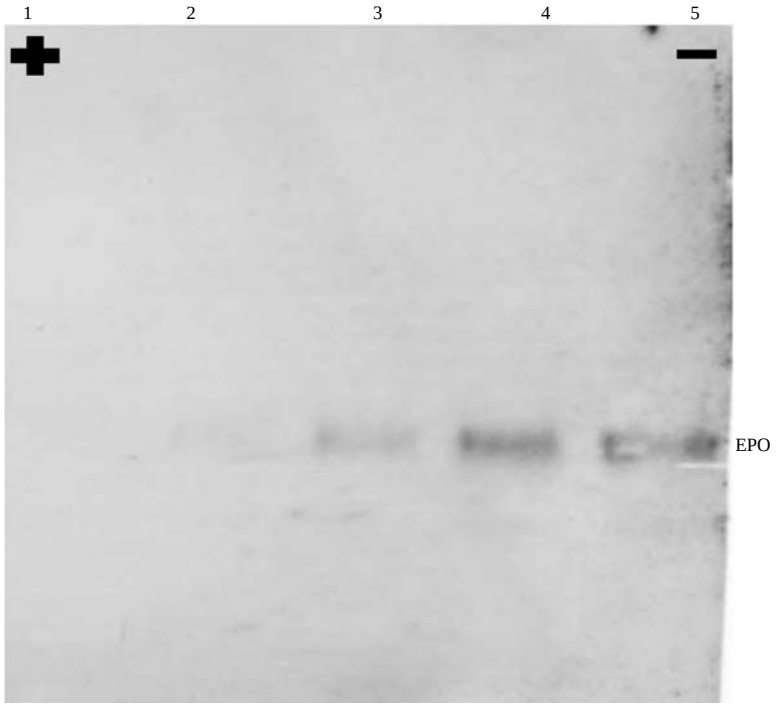


Fig. 3: Limit of detection of EPO using anti-EPO monolith column (MAIIA purification). Lanes 1-5: Pre-spiked urine samples with (from left to right) 0.1, 0.5, 1, 2 and 5 mIU mL^{-1} of uEPO, respectively and purified with anti-EPO monolith column (MAIIA purification) method

Gauge version 3.0 software. We found that the anti-EPO monolith column was able to recover 72% of uhEPO relative to the standard sample. The outcome was calculated as the average values for 5 different purifications of uhEPO.

Limit of detection for EPO using anti-EPO monolith column: Another test was conducted to discover the limit of detection of EPO using the anti-EPO monolith column, SDS-PAGE and Western blotting workflow (Fig. 3). Lanes 1-5 was loaded with pre-spiked urine samples with uhEPO of different concentrations. The lowest concentration added was 0.1 mIU mL^{-1} and highest was 5 mIU mL^{-1}. Based on the image, the uhEPO band is visible from lane with 5 mIU mL^{-1} until 0.5 mIU mL^{-1} but not on lane with 0.1 mIU mL^{-1}. The tests were conducted in triplicates (results were not shown).

DISCUSSION

The most suitable elution buffer can enhance the recovery of antigens in immunoaffinity purification which can significantly improve subsequent analysis. For EPO anti-doping analysis, the immunoaffinity chromatography needs not only give the highest recovery but also the lowest contamination and the elution buffer has to be suitable for the subsequent SDS-PAGE or IEF analysis. Previous works have asserted that LDS and CHAPS as the most suitable sample buffers for SDS-PAGE and IEF, respectively, thus these buffers were used to elute EPO from the anti-EPO micro well plate (Reihlen *et al*., 2012; Reichel *et al*., 2009). However, the reasons and justification for such a claim was unclear. The result obtained by comparing the three different buffers reflected that the CHAPS elution buffer has the ability to recover more EPO compared to the other tested buffers. Such finding is consistent with the work of Hamada and Tsuruo (1988), which reported that CHAPS is one of the preferable options for eluting glycoproteins and is suitable to be applied subsequently in SDS-PAGE too. On the other hand, a low recovery from a self-prepared sample buffer might be due to the instability of the chemicals used to prepare the sample buffer. In order to optimize the performance of anti-EPO micro well plate in the recovery of EPO, CHAPS was applied as the elution buffer in the subsequent experiments when compared with the anti-EPO monolith column.

From present study, it was evident that the uhEPO recovered using the anti-EPO monolith column is higher than using the anti-EPO micro well plate based on the intensity of the bands formed. Lonnberg *et al*. (2010) has shown that the anti-EPO monolith column is able to remove 99.7% of undesirable proteins in urine samples. The advantage of anti-EPO monolith column includes its high specificity and selectivity antibody (Mi *et al*., 2006). Although all urine samples were pre-spiked with the same amount of EPO, the anti-EPO monolith column was able to recover more uhEPO from urine than the anti-EPO micro well plate, based on the intensity of the bands formed. We suspected that the multiple washing steps in the anti-EPO micro well plate method may result in lesser uhEPO being retained, which was also mentioned by Swanson *et al*. (2004). Besides, the prior ultrafiltration steps might be another reason that caused the low recovery of anti-EPO micro well plate. Ultrafiltration is essential as the concentration of uhEPO in the urine sample is too low for the anti-EPO antibody to capture. However, one of the disadvantages of ultrafiltration is the nonspecific retention of other proteins on the membrane filter (Lee *et al*., 2003). In addition, some of the retained EPO may get stuck excessively on the membrane filter and unable to be recovered completely during the purification process leading to low intensity of EPO on the blotted membrane. The condition of elution used can also be one of the factors that affect the

effectiveness of immunoaffinity purification using micro wells plate (Mallorqui *et al*., 2010). Thus, the outcome possibly can be further improved with different elution conditions that have yet to be evaluated.

Another incident spotted was the extra bands visible on the entire samples, which had undergone through the anti-EPO monolith column. Based on the markers, the molecular weight of the extra protein band is around 60-70 kDa, which was suspected to be bovine serum albumin (BSA) protein (66 kDa) that was supplied as one of the components of the buffer in the MAIIA purification kit. A slight modification by removing BSA from the protocol later showed that the undesirable band could no longer be detected. The efficiency of anti-EPO monolith column purification method on recovering the EPO was not affected with the removal of BSA (results were not shown). Therefore, the subsequent experiments were conducted without the addition of BSA.

The results obtained in Fig. 2 reflected the high EPO recovery rate of anti-EPO monolith column, indicating that the technique is suitable and reliable in capturing EPO from urine. The outcome is supported by the findings of Lonnberg *et al*. (2010) that showed high recoveries of different EPO compounds using this technique. The purifications were also consistent between replicates based on the intensity of the bands. This is crucial to ensure good repeatability, when re-analyzing suspect samples. Overall, the anti-EPO monolith column has clearly demonstrated its high recovery and consistency on the analysis of EPO.

The limit of detection for the immunoaffinity purification method is important in any development or modification works of immunoaffinity procedure and is even more crucial in anti-doping analysis. Anti-doping laboratories need to ensure that their immunoaffinity protocols have the required limit of detection, because they often deal with samples with very low concentrations of EPO. The ability of the anti-EPO monolith column to capture low amounts of EPO is essential to detect and identify athletes who tend to abuse small doses of EPO on a prolonged period.

After a series of experiments, among the two methods evaluated, overall the anti-EPO monolith column was shown to perform better than the anti-EPO micro well plate for the detection of EPO in urine. The anti-EPO monolith column, with a minor modification on the manufacturer's procedure, is able to remove most of the undesirable proteins and at the same time recovers a high amount of EPO. The BSA can be excluded from the protocol without significantly affecting the performance of the kit. With a high recovery rate of 72% and low detection limit of 0.5 mIU mL^{-1} for uhEPO in urine samples, the anti-EPO monolith column is proven to be competent enough to be applied as a sample pre-treatment step in the critical analysis of anti-doping. In addition, the approach presented here can also be useful in evaluating any immunoaffinity techniques for other purposes.

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