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Short communication

Efficient purification and preconcentration of erythropoietin in human urine by reusable immunoaffinity column

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Abstract

In this paper, an efficient method is proposed for purification and preconcentration of erythropoietin (EPO) in human urine samples. The EPO-specific immunoaffinity column (IAC) was generated by covalent immobilization of anti-EPO polyclonal antibodies on Sepharose 4B support. The EPO-binding capacity of the IAC was found to be about 2.0 μg (6.6 IU) per 1.5 mL of gel and the activity recoveries of EPO at low concentrations of 7.8, 10 and 120 mIU/mL by the IAC were between 78 and 86%. Substantial cleanup effect was observed when the IAC was applied to human urine samples.

Keywords: Erythropoietin (EPO); Immunoaffinity column (IAC); Purification; Preconcentration

1. Introduction

As a glycoprotein produced primarily by the kidney [1], erythropoietin (EPO) regulates the proliferation of erythroid progenitor cells and induces their differentiation into mature red blood cells. Recombinant human erythropoietin (rHuEPO) produced by DNA recombinant technology in 1985 [2,3] has identical effects on erythropoiesis and appears to be biologically equivalent to EPO [4]. In clinical treatment, EPO or rHuEPO can be used to treat anemia in the patient with chronic renal failure and chemotherapy- and malignancy-induced anemia [5–7].

To obtain EPO or rHuEPO as drugs, great efforts have been made to isolate EPO from the urine of anemia patients [8–13]. Most of these methods were based on immunoaffinity chromatography and proved to be applicable to samples such as the urine of anemia patients or cell culture supernatants that contained high-abundance of EPO (or rHuEPO) (>0.5 IU/mL). However, few studies have been carried out on the purification of normal urine samples with low concentration of EPO. In recent years, the ability of EPO to increase red blood cells and therefore improve the amount of oxygen the blood can carry to the muscles leads to its misuse among endurance athletes [14,15]. And rHuEPO has been used more widely than EPO for its cheaper price and easier production. To detect and control the abuse of rHuEPO, a step of purification of rHuEPO from normal urine or urine of drug users is often required for further qualification and quantification.

In the currently available method to detect EPO in doping control adopted by International Olympic Committee (IOC) and the World Anti-Doping Agency (WADA) [16], two ultrafiltration steps are used to concentrate urine proteins with molecular weight more than 30 kDa. However, the remaining impurity proteins sometimes affect the results of following analysis of double-blotting and isoelectric focusing. A lectin immunoaffinity column (IAC) has been proposed for further cleanup of the samples in between or after the two ultrafiltration steps [17]; but as a kind of ligand specific for carbohydrates, lectin can also interact with other glycoproteins with similar structures which might influence the following detection. Therefore, a ligand that selectively combines with EPO should be a better alternative to the lectins.

In our previous work, polyclonal antibodies against rHuEPO were produced with an affinity constant of 3.2×10^6 M [18]. In

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this paper, the antibodies were immobilized on CNBr-activated Sepharose 4B to generate an EPO-specific immunoaffinity column. The operating conditions of the obtained IAC were optimized and the EPO-binding capacity and activity recovery of the IAC were both investigated. The IAC showed satisfactory cleanup effects of human urine samples, which might be useful to improve the current procedures of rHuEPO detection.

2. Experimental

2.1. Chemicals and reagents

Recombinant human erythropoietin was purchased from Beijing Bio-EPO Biotechnology Co. Ltd. Each sample vial contained about 1 mL of rHuEPO (1.5 mg Epoetin alfa in 1 mL of sodium citrate/citrate buffer), which was purified from a CHO cell line into which the gene coding for human erythropoietin has been inserted. European Pharmacopoeia Biological Reference Preparation (BRP-EPO, produced by European Directorate for the Quality of Medicines, France) was obtained from Guo'an Biotechnology Co. Ltd. Each sample vial contained 250 μg (32,500 IU) of a 50:50 blend of epoetin α and epoetin β, 0.1 mg of Tween-20, 30 mg of trehalose, 3 mg of arginine, 4.5 mg of NaCl, and 3.5 mg of Na_2HPO_4. CNBr-activated Sepharose 4B was obtained from Pharmacia Biotech (Sweden). Human serum albumin (HSA) was purchased from Sino-American Biotech (China). Bovine serum albumin (BSA), Ovalbumin (OVA), 3,3′,5,5′-tetramethylbenzidine (TMB), tris-(hydroxyl-methyl)-amine-methane (Tris) and horseradish peroxidase (HRP) labeled goat anti-rabbit antibody were products of Sigma (St. Louis, MO, USA). *N*-Hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-dimethylaminopropy)carbodimide (EDC), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and carboxymethyl dextran (CM5) sensorchip were purchased from Biacore Inc. (Uppsala, Sweden). rHuEPO was modified by oxidizing the carbohydrate portion with sodium periodate and keeping the polypeptide chain intact for specificity test [19].

2.2. Instruments

A Cary 1E UV–vis spectrophotometer from Varian (USA) was used to measure the absorbance of the protein solution, and a HDJ-2B nucleic acid/protein detector from Nanjing University (China) was utilized to monitor the on-line UV signals of proteins eluted from the immunoaffinity column. The affinity constant of polyclonal antibodies was measured by the surface plasmon resonance (SPR) method using Biacore 3000 (Biacore Inc., Uppsala, Sweden). HZQ-F incubator shaker (Harbin Donglian Electronic Technology Development Co., China) was used for the coupling reaction. Enzyme-linked immunosorbent assay (ELISA) was performed with 96-well microtiter plates (costar, USA). Low concentration of EPO was detected by IMMULITE 1000, an automated quantitative immunoassay analyzer (Diagnostic Products Corporation, Los Angles, CA, USA), using the immulite chemiluminescent immunoassay system (DPC).

2.3. Evaluation of the polyclonal antibodies

The production and purification of polyclonal antibodies against rHuEPO (Epotin alfa) were performed as described in our previous work [18]. The affinity constant of the polyclonal antibodies was measured by SPR method. Sodium acetate buffer (10 mmol/L) (pH 4.0) was used to dilute rHuEPO to the concentration of 0.75 mg/mL. Then the rHuEPO was covalently immobilized on the surface (the second path) of a CM5 sensorchip which was activated by NHS/EDC. For measurement of the affinity constant, various concentrations (100, 200, 300, 400, 500 nmol/L) of polyclonal antibodies against rHuEPO were injected at a flow rate of 10 μL/min. Mass accumulation was recorded in real time and displayed as response units (RU). Biacore sensorgram curves were evaluated by Biaevaluation software 4.0. The affinity constant (K_{aff}) was defined as the ratio of the association rate constant (K_a) divided by the dissociation rate constant (K_d). The first path without rHuEPO immobilized was used as negative control.

To evaluate the selectivity of the antibody and determine the antibody-binding region on EPO molecule, cross-reactions with antibodies caused by BSA, OVA, HSA, BRP-EPO and $NaIO_4$-oxidized rHuEPO were studied by competitive ELISA.

One hundred microliters of 0.05 M pH9.6 carbonate–bicarbonate buffer containing 0.5 μg/mL rHuEPO was coated in each well of microtitre plate at 4 °C overnight. After washed with 0.05% Tween-20 in phosphate buffer saline (PBS, 0.01 M pH 7.4) for three times, the wells were blocked by 2% (w/v) skimmed milk in PBS for 2 h at 37 °C. BSA, OVA, HSA and the $NaIO_4$-oxidized rHuEPO at concentrations of 200, 100, 50, 25, 2.5, 0.25, 0.025 μg/mL were mixed with 200 μg/mL polyclonal antibodies (50 μL + 50 μL), respectively and the mixtures were added to the wells and incubated at 37 °C. An hour later, 100 μL of 1:500 HRP labeled second antibody was added and incubated for 1 h at 37 °C. Finally the substrate (TMB) was added and the color development was stopped by 60 μL of 2 M H_2SO_4. Serially diluted standard rHuEPO solutions were used as positive control.

2.4. Production of immunoaffinity column

The immunosorbent was produced according to the manufacturer's instructions and related literatures [20–22]. Briefly, polyclonal antibody was purified by a modified caprylic acid-saturated ammonium sulfate method [23]. After purification, the antibody was dialyzed against the coupling buffer (0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl) overnight and the concentration was then adjusted to 10 mg/mL for the coupling reaction. CNBr-activated Sepharose 4B (0.4 g) was suspended in 10 mL of 1 mM HCl and the solution was transferred to a G-4 glass filter. The gel was washed with 200 mL of 1 mM HCl and mixed with the antibody solution prepared as described above with the outlet of filter sealed. The mixture was then transferred to a flask. The coupling reaction was kept for 2 h under shaking at a speed of 150 rpm at 20 °C.

The resulting immunosorbent was packed into a glass column cartridge (0.5 mm × 10 mm) and the effluent was measured

with an on-line UV detector. After the gel beads settled down, the coupling buffer was applied to wash the column until the absorbance at 280 nm reached zero. The protein content in the collected effluent was measured by a Cary 1E UV–vis spectrophotometer to estimate the coupling efficiency [20]. Then 0.1 M, pH 8.0 Tris–HCl was used to block the excess active group on the immunosorbent and the saturation procedure was kept for 2 h at room temperature. After washing alternatively with five column volumes of 0.1 M pH 4.0 acetate buffer containing 0.5 M NaCl and five column volumes of 0.1 M pH 8.0 Tris–HCl buffer containing 0.5 M NaCl for three cycles, the column was washed with 0.01 M pH 7.4 phosphate buffered saline (PBS) and stored at 4 °C for further use. A blank control column was prepared in the same way as above without addition of the antibodies.

2.5. Optimization of the eluting conditions of affinity-bound EPO on the immunoaffinity column

An aliquot of 500 μL of PBS containing 0.5 μg rHuEPO was loaded onto the column. After washing with 10 mL of PBS, the affinity-bound rHuEPO was eluted by 6 mL of PBS containing 2 M NaCl or 0.2 M pH 2.5 Glycine–HCl (Gly–HCl) for comparison. All experiments were performed at room temperature. The effluent was collected in fractions of 1 mL with Eppendorf tubes. The first tube of effluent started to be collected after the sample solution all entered the gel. The effluent of Gly–HCl was neutralized to pH 7.0 immediately by 1 M Tris. All the effluents were analyzed by ELISA.

For comparison of the eluting efficiency of different eluting solutions, 100 μL of the above effluent was coated on the well of 96-well microtiter plate at 4 °C overnight. After blocking with 300 μL of 2% (w/v) skimmed milk for 2 h at 37 °C, 100 μL of 100 μg/mL polyclonal antibody against rHuEPO was added and incubated for 1 h. Then 100 μL of 1:500 HRP labeled second antibody was added and incubated at 37 °C. An hour later, TMB was added as the substrate and the reaction was stopped by 60 μL of 2 M H_2SO_4 after 15 min. All data of ELISA were recorded by the Microplate Reader. Calibration curves were obtained by measuring serially diluted rHuEPO standard solutions (1.5, 0.15, 0.015 μg/mL) along with the samples under the same operating conditions.

2.6. EPO-binding capacity and recovery test

The EPO-binding capacity of the column was determined by continuous sample loading. Ten milliliters of 500 ng/mL rHuEPO was loaded on the column and the effluent was collected as soon as the sample began to enter the gel. The detection of EPO was performed by ELISA and the same experiment was done on the blank control column for comparison.

The recovery of the immunoaffinity column was measured by loading three different concentrations of rHuEPO standard solutions: 7.8, 10 and 120 mIU/mL of rHuEPO in 0.01 M PBS. Six milliliters of PBS was used to wash the non-specific retained components and 8 mL Gly–HCl was used to elute the affinity-bound rHuEPO. The determination of EPO in the effluent was performed by chemiluminescent immunoassay using IMMULITE 1000.

2.7. Urine samples test

Urine samples (10 ml) were obtained from healthy volunteers (male, between 20 and 40 years old) and stored in tubes at −20 °C immediately after collection. Upon use, the samples were thawed at room temperature and filtered to remove the precipitates. The obtained urine filtrate was diluted 1:2 with 0.01 M PBS and mixed with rHuEPO at a concentration of 7.8 mIU/mL. Ten milliliters of the mixture was loaded onto the immunoaffinity column that has been preconditioned with 0.01 M PBS. PBS (0.01 M) was used to wash the impurity until the absorbance at 280 nm of the effluent declined to zero. Then 8 mL of 0.2 M pH 2.5 Gly–HCl was added to elute the EPO on the column and the effluent was collected as described above. All the results were measured by IMMULITE 1000 and the Cary 1E UV–vis spectrophotometer.

3. Results and discussion

3.1. Determination of affinity constant

The sensorgrams of SPR can provide real time information on binding affinity, specificity and kinetics of the antibodies to rHuEPO. Based on the results obtained from different concentrations of the polyclonal antibodies, K_a and K_d were found to be 1.15×10^4 L/mol/s and 1.36×10^{-3}/s. Thus the K_{aff} was 8.46×10^6 L/mol. This indicated that the polyclonal antibodies can specifically recognize rHuEPO and have suitable affinity for further application as immobilized ligands.

3.2. Selectivity of the anti-rHuEPO polyclonal antibodies

Since antibodies were the main functional ligands of an IAC, the selectivity of antibodies would have great influence on the purification and enrichment property of the obtained IAC. In this study, some common proteins (BSA, OVA and HSA) were chosen for investigating the specificity of antibodies against rHuEPO. To further confirm the binding efficiency of the polyclonal antibodies to rHuEPO from different sources, BRP-EPO, a typical rHuEPO product which contained a 50:50 blend of epoetin α and epoetin β, was also applied in the cross-reaction test. From the results shown in Fig. 1, the competitive binding ability of the two different types of rHuEPO was obvious, while the other three proteins (BSA, OVA and HSA) did not show any decline of absorbance value resulting from competition. The results proved the selectivity of the antibodies towards rHuEPO in comparison with other abundantly existing irrespective proteins. BRP-EPO showed strong prohibitive ability in competitive ELISA. That meant epoetin β could also be recognized by the antibodies since the competitive effects were almost identical between the rHuEPO produced by China (epoetin α) and BRP-EPO produced by France (epoetin α and epoetin β). In fact, many rHuEPO products, such as EPREX, NEORECORMON and ERYTHRO, all belong to epoetin α or epoetin β and have

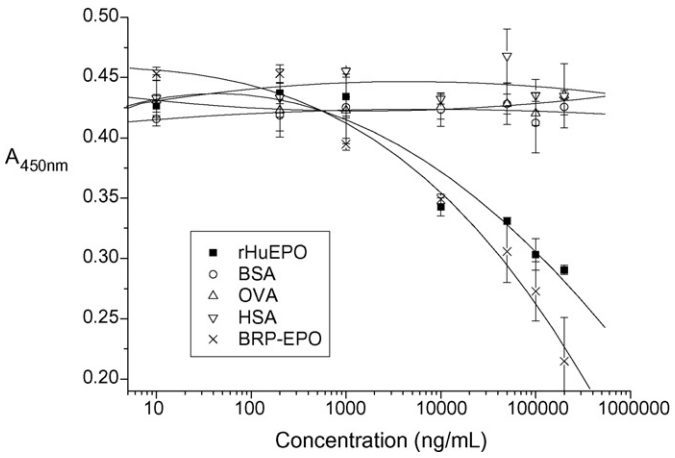

Fig. 1. Cross-reaction of some proteins by competitive ELISA. The concentration of coated rHuEPO was 0.5 μg/mL. The concentrations of the competitive proteins of rHuEPO, BSA, OVA HSA and BRP-EPO were 100, 50, 25, 12.5, 1.25, 0.125, 0.0125 μg/mL in each well, respectively. The concentration of antibodies was 100 μg/mL.

the same structure of peptide portion, so the polyclonal antibodies we produced are able to react with all these products in samples.

Since EPO is a kind of glycoprotein and consists of peptide portion and carbohydrates, $NaIO_4$ can be used to destroy the structure of carbohydrates via oxidation and keep the peptide portion intact [19]. To determine the specific part of EPO that the antibodies selectively bound to, cross-reaction of $NaIO_4$-treated rHuEPO was investigated and the results were shown in Fig. 2. In Fig. 2, both the oxidized and intact rHuEPO could react with the antibodies, and the competitive effect of rHuEPO oxidized by $NaIO_4$ was even better than that of rHuEPO. The data indicated that most of the polyclonal antibodies were against the peptide portion of EPO, and the binding sites were probably near the conjugation point of the peptide and carbohydrates. Considering the fact that rHuEPO and EPO have the same structure of peptide

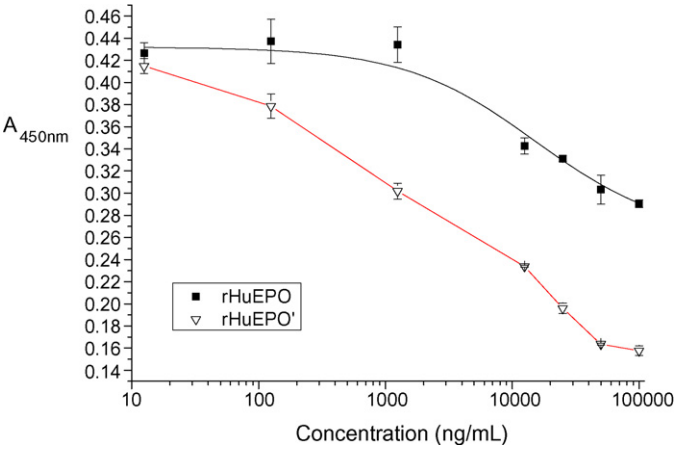

Fig. 2. The result of competitive ELISA between rHuEPO and rHuEPO′. rHuEPO′ represents rHuEPO oxidized by $NaIO_4$. The concentration of coated rHuEPO was 0.5 μg/mL. The concentrations of rHuEPO and rHuEPO′ were 100, 50, 25, 12.5, 1.25, 0.125, 0.0125 μg/mL in each well, respectively. The concentration of antibodies was 100 μg/mL.

portion, IAC using these antibodies can enrich both rHuEPO and EPO, which was important for further analysis in either medical research or doping control.

In recent years, a new rHuEPO product, ARANESP, was found to be used among athletes [24]. Compared with the amino acid sequence of rHuEPO (α or β), ARANESP differs at five positions (Ala 30 Asn, His 32 Thr, Pro 87 Val, Trp 88 Asn and Pro 90 Thr) [25]. But these positions were all in the connecting loops (AB loop and BC loop) of EPO's three dimensional structure [26], which were proved to have little effect on the secondary and tertiary structure [27], so the main peptide structure (four α-helices), the interaction sites with EPO receptor and proper folding of the molecule remained the same. However, since the ARANESP product is not commercially available in China, the experimental data cannot be provided in this paper.

3.3. Production of IAC

The coupling efficiency of polyclonal antibody to the CNBr-activated Sephrose support can be calculated according to the following equation [20].

$$\text{Coupling efficiency} = \frac{V_0A_0 - V_E(A_E - A_{E0})}{V_0A_0} \times 100\%$$

where A_0, A_E, A_{E0} represent absorbance values at 280 nm corresponding to the total protein solution before coupling, the effluent after coupling and the coupling buffer, respectively. V_0, V_E represent the volumes corresponding to the original protein solution and the effluent of protein unbound. In our experiments, the coupling efficiency was found to be 92% and the volume of the swollen gel was about 1.5 mL.

3.4. Optimization of the binding and eluting conditions

In our preliminary experiments, the rHuEPO standard solution was loaded onto the IAC using 0.01 M PBS (pH 7.4) as the binding buffer. Compared with the blank column, the IAC showed excellent retention property of rHuEPO (close to 100%). Since 0.01 M PBS was very similar to the body fluid condition and also beneficial to keep the activity of target proteins, it was used as the binding buffer for the later experiments.

The antigen binding process by the immobilized antibodies on the IAC is generally considered as a kinetic equilibrium between adsorption and desorption. So it is more difficult to capture low-abundance antigens in large sample volumes. Compared with monoclonal antibody, polyclonal antibody has stronger antigen-capture ability since it can bind to the protein antigens via different epitopes. That maybe the reason that the rHuEPO samples with concentrations as low as 7.8 or 10 mIU/mL were both well reserved by the IAC. But multivalent combination sometimes also results in difficult eluting [28]. So eluting conditions are very important factors to be considered when IAC made by polyclonal antibody was used to purify antigen. In our experiments, two different eluting buffers were investigated.

Since the interaction between antibody and antigen can be affected by factors such as the pH and ionic strength, solutions

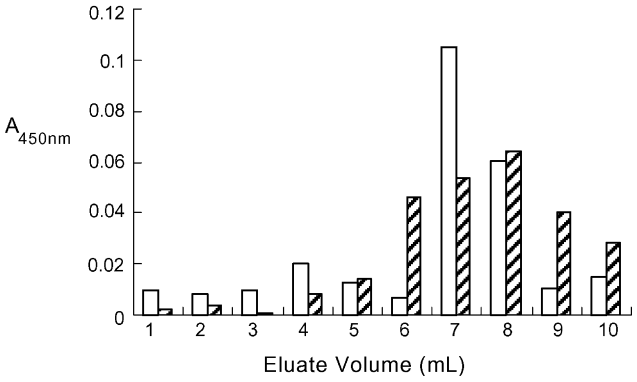

Fig. 3. Elution profiles of rHuEPO bound on the IAC by different eluting buffers. The open columns correspond to the eluate using Gly–HCl as the eluting buffer and the shaded columns are the results by 2 M NaCl.

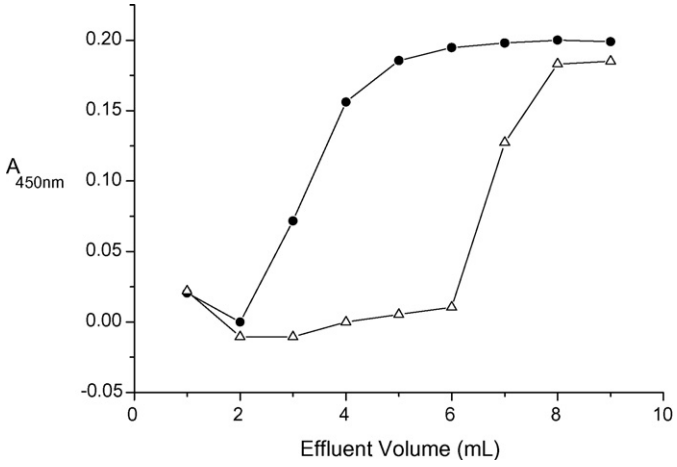

Fig. 4. Effluent curves of the continuous loading of rHuEPO (500 ng/mL) samples on the IAC (△) and blank column (●).

with high salt concentration (2 M NaCl, pH 7.4) or low pH (0.2 M pH 2.5 Gly–HCl) were tested as the eluting buffers, respectively. Fig. 3 showed the eluting profiles of rHuEPO under the two different conditions. In Fig. 3, the desorbed rHuEPO by Gly–HCl was more concentrated in smaller volumes compared with those eluted by 2 M NaCl. So Gly–HCl was chosen as the eluting buffer in further experiments. The effluent using Gly–HCl was neutralized with 1 M Tris before measured with ELISA or IMMULITE 1000 to ensure the immunochemical reaction between the antibody and antigen. From the results shown in Fig. 1, it can be calculated that the linear regression equation of the ELISA is $OD_{450\,nm} = 0.281 \log c_{EPO} - 0.332$ with the working range of 50–5000 mIU/mL and a detection limit of 50 mIU/mL. The calibration curve of the IMMULITE 1000 Analyzer was also measured and the linear regression equation and working range were found to be $CPS = 88{,}495\, c_{EPO} - 367{,}148$ and 1.0–200 mIU/mL, respectively, where the CPS represents the intensity of the chemiluminescence. The quantification by IMMULITE 1000 Analyzer is more sensitive but very expensive, so the preliminary optimization of the binding and eluting conditions at relatively high EPO concentrations were evaluated by the ELISA and the quantification of the binding capacity and recovery were all performed with the IMMULITE 1000 method in the following study.

3.5. Evaluation of IAC

The EPO-binding capacity of the IAC was measured by breakthrough volume test. Fig. 4 shows the results of continuous samples loading on the IAC and blank control column, respectively. It can be seen that rHuEPO was soon detected in the effluent of the blank column, while the IAC showed its ability to recognize and retain the antigen rHuEPO. The effluent curve of the IAC did not go up quickly, indicating that most of the loaded rHuEPO was captured by the specific immunosorbent. Comparing the effluent volume of the two columns before rHuEPO appeared in the effluent, the breakthrough volume of rHuEPO was about 4 mL when 500 ng/mL rHuEPO was loaded. So the maximum binding capacity of rHuEPO was estimated to be about 2.0 μg (6.6 IU).

To measure the recovery of the immunoaffinity column, different amount of rHuEPO in 0.01 M PBS were loaded onto the column and the recovery results were listed in Table 1.

3.6. Urine test

To evaluate the influence of matrices in real urine samples, 10 mL of two-fold diluted human urine was spiked with 78 mIU of rHuEPO and loaded onto the IAC. Non-spiked diluted urine was also measured for comparison. The recovery of standard rHuEPO added in the urine sample was found to be 86 ± 9% ($n = 3$), which was consistent with above test (see Table 1) and the concentration of endogenous EPO in the original urine sample was observed to be 2.2 mIU/mL ($n = 3$). So the interference of urine matrices was negligible in this purification procedure. And the UV absorbance measurement results revealed that more than 91% of the interfering protein and other UV absorbing small molecules had been removed during the washing steps. The specific activity of EPO eluted was 0.65 IU/A with a purification fold of 1377.

Table 1
The recovery results of rHuEPO on the IAC

Amount of rHuEPO added (mIU)	Amount of rHuEPO measured (mIU)	Recovery (%)		RSD (%) ($n = 3$)
		In elute fraction	In flow-through fraction	
40 (4 mL × 10 mIU/mL)	31	78	14	5.3
78 (10 mL × 7.8 mIU/mL)	64	82	12	9.5
2400 (20 mL × 120 mIU/mL)	2053	86	26	11.8

The IAC can be reused for many times. After each elution, 10 mL of 0.2 M, pH 2.5 Gly–HCl was applied to remove any remaining substances adsorbed on the column. Then the IAC was reconditioned with 0.01 M PBS for the next use. In our experiments, we found the binding efficiency of the IAC was stable after used for at least 30 times during about two months. The variation among different batches of IACs was also tested and the relative standard deviation of recoveries was found to be less than 10% ($n = 5$).

During the practical detection procedure for doping control of rHuEPO, isoelectric focusing and double blotting are used to distinguish rHuEPO from native EPO. Since the concentration of EPO in normal human urine is very low (about 10 pg/mL), normally 20 ml of crude urine sample are collected from athletes and then concentrated by two ultrafiltration steps. However, the ultrafiltration using Centricon and Centricon plus 20 from Millipore with molecular weight cut-off of 30 kDa could not remove those impurities with high molecular weight, which might result in the distortion of blotting bands. Lasne et al. [16] tried to straighten the bands using wheat germ agglutinin (WGA) to purify the retentate after ultrafiltration. The results were greatly improved with a >60% recovery of the WGA column. But as mentioned above, WGA is a ligand special to the carbohydrates with certain residues, not EPO molecule only. That means WGA column will possibly introduce the contamination of carbohydrates and other glycoproteins [29]. And the competition of these substances may be the reason for the low recovery of WGA column (about 60%) described in literatures [30–32]. In our experiments, an IAC with immobilized polyclonal antibodies against EPO was used to purify and concentrate EPO in the urine. The recognition and retention property were more selective than those of WGA. And the high recovery of rHuEPO in both PBS and urine matrix showed that the IAC was a promising tool for the pretreatment of EPO-containing samples instead of WGA. Furthermore, it might be helpful to speed up the whole procedure of rHuEPO detection.

4. Conclusions

In this study, an EPO-specific IAC was produced and used to purify and concentrate EPO in human urine samples. The EPO-binding capacity of the IAC was found to be about 2.0 μg (6.6 IU) per 1.5 mL of gel at a loading concentration of 500 ng/mL and the activity recoveries were between 78 and 86% for rHuEPO at low concentrations of 7.8, 10 and 120 mIU/mL. The satisfactory cleanup performance in pretreatment of urine samples showed the IAC a helpful tool for improving the current detection method for doping control of rHuEPO.

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