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Immuno-magnetic beads-based extraction-capillary zone electrophoresis-deep UV laser-induced fluorescence analysis of erythropoietin

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ABSTRACT

Erythropoietin (EPO) is an important glycoprotein hormone. Recombinant human EPO (rhEPO) is an important therapeutic drug and can be also used as doping reagent in sports. The analysis of EPO glycoforms in pharmaceutical and sports areas greatly challenges analytical scientists from several aspects, among which sensitive detection and effective and facile sample preparation are two essential issues. Herein, we investigated new possibilities for these two aspects. Deep UV laser-induced fluorescence detection (deep UV-LIF) was established to detect the intrinsic fluorescence of EPO while an immuno-magnetic beads-based extraction (IMBE) was developed to specifically extract EPO glycoforms. Combined with capillary zone electrophoresis (CZE), CZE-deep UV-LIF allows high resolution glycoform profiling with improved sensitivity. The detection sensitivity was improved by one order of magnitude as compared with UV absorbance detection. An additional advantage is that the original glycoform distribution can be completely preserved because no fluorescent labeling is needed. By combining IMBE with CZE-deep UV-LIF, the overall detection sensitivity was 1.5×10^{-8} mol/L, which was enhanced by two orders of magnitude relative to conventional CZE with UV absorbance detection. It is applicable to the analysis of pharmaceutical preparations of EPO, but the sensitivity is insufficient for the anti-doping analysis of EPO in blood and urine. IMBE can be straightforward and effective approach for sample preparation. However, antibodies with high specificity were the key for application to urine samples because some urinary proteins can severely interfere the immuno-extraction.

1. Introduction

Erythropoietin (EPO) is a glycoprotein hormone produced primarily by kidney, which stimulates red blood cell production. Since being commercially available in 1989, recombinant human erythropoietin (rhEPO) has become one of proteins with high economic interest due to its clinical use for the treatment of anemias [1–3]. Besides, rhEPO can be used as a doping agent to help athletes to improve performance in endurance sports. As misuse of rhEPO may lead to polycythemia [4], the use of rhEPO in sports has been prohibited by the International Olympic Committee (IOC) since 1990. The analysis of EPO particularly in sports has brought about a series of huge challenges to analytical scientists. EPO exists in hundreds of isoforms with small differences in the glycan structures while different glycoforms exhibit different biological activities [5–7]. High resolution separation of these glycoforms is the key for quality control in biopharmaceutical industrial and an essential marker to distinguish natural and recombinant EPO in anti-doping analysis. Besides, human EPO is present at very limited concentration in blood (10^{-11}–10^{-12} M) and urine (10^{-13}–10^{-14} M) of healthy individuals [7], ultrahigh sensitive detection is one of the keys for reliable assay in sports. Sensitive detection is also an important asset for pharmaceutical analysis, because EPO is instable at high concentration. Furthermore, the sample matrix has dramatic effect. Even for commercial rhEPO drugs and standards, matrix effect is also rather apparent since they contain a large amount of excipients [8,9]. Sample pretreatment is crucial for EPO assay in sports and biopharmaceutical fields.

EPO assay in sports had been well established by Lasne et al. [10,11]. rhEPO is differentiated from natural analogues according to glycoform profiling by gel isoelectric focusing (gel IEF). Urine samples are clean up and enriched using ultrafiltration while chemiluminescence detection (CL) is used to provide sufficient sensitivity. Although being officially approved by IOC, this approach is associated with apparent drawbacks: labor-intensive, time-consuming and cost-inefficiency [12]. Khan et al. [13] proposed a two-dimensional gel electrophoresis (2DGE) method for the differentiation of rhEPO from its endogenous analogues in urine (urinary human EPO, uhEPO). The approach is also associated with similar drawbacks as the official approach. Therefore, further efforts to overcome these disadvantages are very necessary.

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For high resolution profiling of EPO glycoforms, gel IEF has been the workhorse in anti-doping analysis while many efforts have been focused on the possibility of substituting gel IEF with capillary electrophoresis (CE) for fast glycoform profiling. Rapid high-resolution separation of EPO glycoforms has been realized by CE in both capillary zone electrophoresis (CZE) [14–20] or capillary isoelectric focusing (CIEF) format [9,21,22]. While maintaining similar glycoform profile, CE can reduce the separation time from hours required by gel IEF to 20–60 min, and even to 5 min [9]. So far, CZE has been the core for pharmaceutical analysis, particularly an official method for EPO analysis had been approved by the European Pharmacopeia [16]. Moreover, CZE has proved to be able to differentiate the glycoform profiles of exogenous and endogenous EPO [17].

Many attempts have been made toward high-sensitivity detection of EPO, which mainly include laser-induced fluorescence (LIF) [23–25], mass spectrometry [26–35], immunochromatographic assay [36] and CL [37]. Lopez-Soto-Yarritu et al. [23] examined the compatibility of the running buffer with LIF detection with on-column derivatization. Affinity CE-LIF assays have also developed, in which fluorescent labeled antigen-binding fragments [24] and aptamer [25] were added to the running buffer as affinity probes. Although the sensitivity was improved, resolution of the glycoforms was poor due to the presence of the probes. MS has become a powerful tool for sensitive detection and identification of EPO [26–35]. Particularly, a liquid chromatography (LC)-MS based proteomic approach has been successfully applied to detect EPO in real-world equine plasma samples, and it can be a promising tool in the fight against blood doping with rhEPO in the horse racing industry [30,31]. Capillary electrophoresis–mass spectrometry (CE–MS) has become a powerful hyphenated technique for the separation of a great number of biomolecules enabling characterization of analytes in complex samples by molecular mass information [32–35]. The improved methods for intact rhEPO glycoform characterization by capillary zone electrophoresis electrospray ionization-time-of-flight mass spectrometry (CZE-ESI-TOF MS) [33] and capillary electrophoresis-electrospray ionization-ion trap mass spectrometry (CE-ESI-IT MS) [34] have been developed, the main glycoforms of rhEPO were identified by the two methods. In addition to the analysis of intact protein, Gimenez et al. [35] demonstrated that detection of a specific peptide marker by immunoaffinity capillary electrophoresis-mass spectrometry (IA-CE-MS) could be used to confirm the presence of rhEPO in solution. Recently, Lönnberg et al. [36] represented an ultra-sensitive immunochromatographic approach for quantitation of EPO with a detection limit of 1.2×10^{-15} M. CL can also provide extremely high detection sensitivity. Zhang and co-workers [37] developed a silica dioxide nanoparticles-mediated CE immunoassay with enhanced chemiluminescence detection and quantification of EPO in human serum (shEPO), the detection limit was 0.9 ng/mL. Besides, to improve the detection sensitivity of CE, an on-line sample concentration technique (OLSC) has been developed, which can provide a sensitivity improvement of 50–100 folds as compared with conventional UV absorbance detection [38].

Much attention has also been focused on the sample preparation aspect for the analysis of EPO. Immuno-affinity extraction with anti-rhEPO antibodies has proved to be an effective sample preparation procedure to provide purified and enriched EPO for LC–MS separation and detection [30,31]. Recently, an immunoaffinity monolith-based extraction approach has appeared, which exhibited excellent specificity and extraction efficiency [39,40]. It can be a very promising method for sample preparation of EPO in doping control analysis.

In this study, our goals are aimed at new possibilities of sensitive detection and effective sample preparation. A deep UV-LIF detection system was built up to detect the native fluorescence of EPO. And an immuno-magnetic beads-based extraction approach (IMBE) was established for facile immuno-affinity purification and enrichment of EPO, followed with CZE-deep UV-LIF analysis.

2. Experimental

2.1. Instrumentation

All CE separations were performed on a P/ACE MDQ system (Beckman Coulter, Fullerton, CA, USA) equipped with a dual-channel LIF detector. An in-lab built deep-UV-LIF detection system was established according to the description by Britz-McKibbin and co-workers [41] with slight modification. A 266 nm diode-pumped solid-state Q-switched Nd-YAG laser (CryLaS GmbH, Berlin, Germany) was used as the excitation light source, which was coupled to the CE instrument with a UV multimode fiber patchcord (Oz Optics, Carp, Ontario, Canada). Detection of native fluorescence was performed at 340 nm using a narrow bandpass (Full width at half maximum, 25 nm) emission filter (Andover Corporation, Salem, USA). A bare fused-silica capillary (Yongnian Optical Fiber Factory, Hebei, China) of 50 μm i.d. × 60 cm (50 cm effective length) was used as the separation column. Each day the capillary was first preconditioned with 0.1 M NaOH for 60 min, followed with the running buffer for 60 min. Between runs, the capillary was rinsed with 0.1 M NaOH for 5 min and then with the running buffer for another 10 min. All solutions were filtered through hydrophilic membrane filters with 0.45 μm pore sizes. The capillary was thermostated at 35 °C. Samples were injected by pressure at 3.45 kPa for 15 s. The separation voltage was 15 kV. The running buffer is selected according to the official method of the European Pharmacopeia [16] with minor modification.

2.2. Chemicals and materials

The rhEPO was purchased from the European Pharmacopoeia as a biological reference product (BRP), which was an equimolar mixture of epoetin-α and epoetin-β. EPO injection solution (10,000 IU/mL, equals to 83 μg/mL epoetin-α), was purchased from Sansei Pharmaceutical (Shenyang, Liaoning, China). Ultrafiltration cartridges, including Amicon Ultra-0.5 (MWCO 10,000 Da), Amicon Ultra-15(MWCO 10,000 Da) and Ultra-0.5 (MWCO 50,000 Da), were products of Millipore (Beverly, MA, USA). Complete protease inhibitor cocktail (p8340), tricine, putrescine, urea and rabbit monoclonal anti-EPO antibody (IgG) were obtained from Sigma (St. Louis, MO, USA). Mouse monoclonal anti-EPO antibody (IgG, clone 4G7) was bought from Abnova (Taipei, Taiwan, China). Rabbit polyclonal anti-EPO antibody (IgG) was provided by Bioss Company (Beijing, China). xMag^{TM} protein A Magnetic Beads are purchased from Shaanxi Lifegen Co., Ltd. (Xi'an, Shaanxi, China). All other reagents used were of analytical grade or higher. Ultrapure water was produced by a Milli-Q Advantage A10 System (Millipore, Milford, MA, USA).

2.3. Immuno-magnetic beads-based extraction

Two routes were used to extract EPO by immuno-magnetic beads, which are illustrated in Fig. 1. Route 1: anti-EPO antibody was first orientedly immobilized onto the protein A magnetic beads in accordance with the manufacturer's instructions, then the anti-EPO antibody immobilized protein A magnetic beads were incubated with an EPO-containing sample; finally, after washing away non-specifically adsorbed species with an appropriate cleanup solution, the anti-EPO antibody and EPO were eluted from the protein A magnetic beads with an appropriate elution buffer. Route 2: anti-EPO antibody was first incubated with an EPO-containing sample to form immune complexes, then the immune

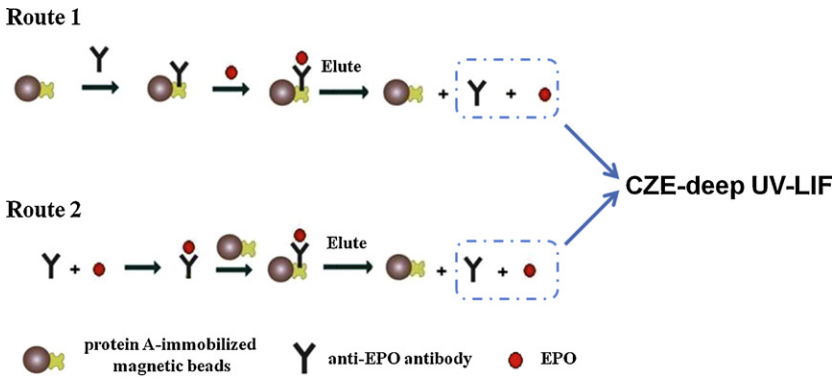

Fig. 1. Schematic of the two extraction routes.

complexes were bond onto the protein A magnetic beads through interaction between the Fc region of the anti-EPO antibody and protein A; finally, after washing away non-specifically adsorbed species with an appropriate cleanup solution, anti-EPO antibody and EPO were eluted from the protein A magnetic beads with an appropriate elution buffer. The reaction, extraction and elution in both routes were all conducted in a 200 μL plastic microcentrifugal tube and agitated on a rotator. All the steps in both routes could be easily handled through magnetic manipulation of the magnetic beads. The eluates were injected for CZE-deep UV-LIF analysis.

2.4. Preparation of BRP standard

One vial of the EPO BRP sample, which contained 250 μg of EPO, was dissolved in 250 μL of water to give a 1 mg/mL solution of rhEPO. The solution was ultrafiltered according to the procedure described in Ref. [21]. Briefly, 250 μL EPO BRP was first diluted into 500 μL. Then the sample was passed through an Amicon Ultra-0.5 (MWCO 10,000 Da) and centrifuged for 45 min at 14,000 × *g*. The retentate was washed five times with 470 μL water under the same centrifugal force. The residue was recovered by inverting the cartridge and centrifuging it at 2000 × *g* for 3 min into a new vial. Finally, sufficient water was added to adjust rhEPO concentration to 1.0 mg/mL. The prepared stock solutions were preserved at 4 °C.

2.5. Ultrafiltration of urine

Urine samples were obtained from two healthy volunteers. The urine samples were kept frozen at −20 °C according to a previously reported method [11] with minor modification. Briefly, after thawing at room temperature, 2 mL of 3.75 M Tris/HCl, pH 7.4, and 0.5 mL of complete protease inhibitor cocktail were added to 20 mL urine. The sample was heated at 82–85 °C in hot water bath for 10 min, and thereafter was immediately cooled in cold water for approximately 10 min. After centrifugation at 2700 × *g* and 20 °C for 20 min, the supernatant was filtered through hydrophilic membrane filters with 0.22 μm pore sizes. Then an appropriate amount of the BRP standard was added. The EPO-spiked urine sample was then submitted to a 15 mL Amicon Ultra-15 (MWCO 10,000 Da), followed with centrifugation at 5000 × *g* and 20 °C for 30 min. The retentate was then washed with 20 mL of 50 mM Tris/HCl, pH 7.4, and 0.5 mL complete protease inhibitor cocktail in the same Amicon Ultra-15 by centrifugation under the same conditions. The washed retentate (about 100 μL) was then recovered as instructed by the manufacturer and transferred to an Amicon Ultra-0.5 (MWCO 50,000 Da) to remove urinary cross-reacting proteins with anti-EPO antibody. Filtrate was collected. Finally, EPO in the spiked urine samples was enriched by protein A magnetic beads.

3. Results and discussion

3.1. Deep UV-LIF detection

Deep UV-LIF detection takes advantage of the intrinsic fluorescence of analytes such as tryptophan, tyrosine and phenylalanine to eliminate the need for derivatization [42–46]. Endogenous EPO has 3 tryptophan, 4 tyrosine and 4 phenylalanine residues while exogenous EPO has 5 tryptophan, 4 tyrosine and 4 phenylalanine residues [7]. Therefore, deep UV-LIF is very suitable for the detection of EPO. The performance of the deep UV-LIF detector was first investigated and compared with the UV absorbance detector equipped with the MDQ instrument. As shown in Fig. 2, under identical conditions, deep UV-LIF detection improved detection sensitivity, both the peak area and peak height increased by 1 order of magnitude as compared with UV absorbance. Due to the enhanced detection sensitivity, more peaks could be observed by deep UV-LIF; eight peaks were detectable by deep UV-LIF, but only seven peaks were observed by UV absorbance detection. Although the sensitivity enhancement of the native fluorescence was not so significant as compared with fluorescent dye labeling, an advantage of native fluorescence detection is that the glycoform profile of the protein was completely preserved (see Fig. 2), which is essential for the differentiation of endogenous and exogenous EPO. Unlike LIF detection with fluorescent labeling, deep UV-LIF detection avoids not only tedious labeling procedure which even involves toxic chemicals but

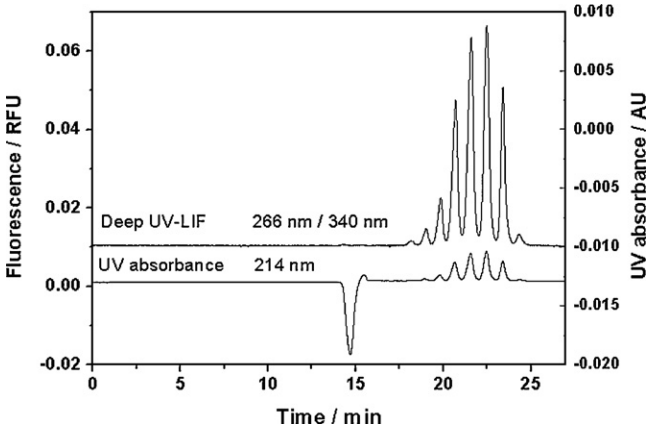

Fig. 2. Comparison of deep UV-LIF with conventional UV absorbance detection. Sample: 1.0 mg/mL rhEPO dissolved in ultrapure water. CE conditions: Capillary, bare fused-silica capillary with 60 cm total length (50 cm effective length) and 50 μm i.d.; capillary temperature, 35 °C; separation voltage, 15 kV; running buffer, 10 mM sodium acetate buffer containing 7 M urea, 10 mM Tricine, 3.9 mM putrescine, and 100 mM NaCl at pH 5.50; UV absorbance detection wavelength, 214 nm; deep UV-LIF detection: detection at 340 nm with an excitation wavelength of 266 nm.

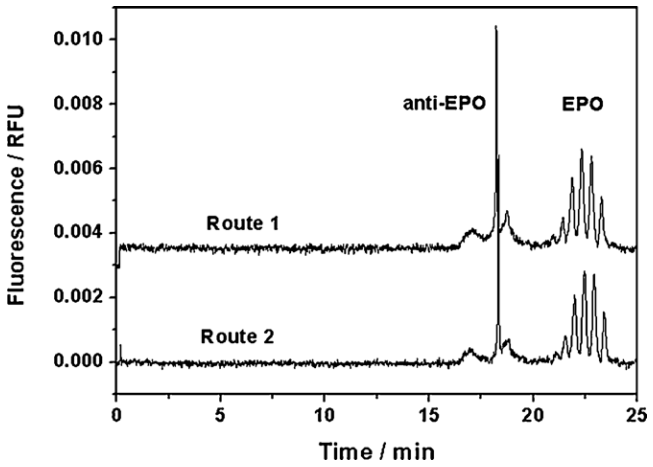

Fig. 3. Comparison between the two different extraction routes. CE conditions are identical as in Fig. 2. Sample: 0.5 mg/mL rhEPO dissolved in 100 mM phosphate buffered solution (PBS), pH 7.4. Extraction and elution conditions: 0.5 mg protein A magnetic beads; 30 μL of 1 mg/mL anti-EPO antibody dissolved in 100 mM PBS, pH 7.4; 20 μL of 0.5 mg/mL EPO dissolved in 100 mM PBS, pH 7.4; incubation temperature: 37 °C; incubation time: 3.5 h; elution solution: 10 μL of 10 M urea; elution time: 2 h.

also distorted glycoform distribution due to changes in properties caused by labeling.

3.2. The extraction procedures

The two extraction routes shown in Fig. 1 were compared under otherwise identical conditions. As predicted in Fig. 1, peak for the antibody will appear in the CZE analysis. As shown by Fig. 3, both EPO and the antibody were detected. As the antibody was well resolved from EPO under the conditions used, the presence of the antibody in the obtained sample did not affect the separation and detection of EPO glycoforms at all. Besides, identical profiles and identical signal intensities were obtained for both EPO and the antibody with the two different routes, which indicates equal efficiency of the two routes. In route 1, the protein A magnetic beads were first coated with the anti-EPO antibody, thus antibodies in real samples (such as blood samples) cannot bind with the protein A. As a comparison, in route 2 antibodies in real samples can bind with the protein A on the magnetic beads and thus they will degrade the specificity and reduce the extraction efficiency. Thus, route 1 was chosen for the later experiments in this study.

3.3. Optimization of the immuno-extraction conditions

Antigen–antibody reactions can be influenced by the experimental conditions. In this study, the immuno-extraction conditions were optimized, including elution solution, elution time, incubation temperature, incubation time, pH and ionic strength.

3.3.1. Effect of elution solution and elution time

In order to efficiently elute EPO from the immuno-magnetic beads, two typical elution buffers that have widely been used for disruption of antigen–antibody complex were compared: (1) 0.1 M glycine–HCl (pH 2.8) [47–49], and (2) aqueous solution of urea [50–52]. Usually, urea concentration used for this purpose ranges from 6 to 8 M. However, because two kinds of complexes co-existed (i.e., the EPO–antibody complex and the EPO–antibody–protein A complex), a higher concentration (10 M) was chosen in this study. It was found that the urea solution was much more effective than the glycine–HCl buffer (Fig. 4A). Therefore, 10 M urea was adopted as the elution solution in the later experiments. The effect of elution time was also investigated, which ranged from 1, 2 and 3 h. It was found that the extracted amount was nearly the same if the elution time was not less than 2 h. Therefore, an elution time of 2 h was used for the later experiments.

3.3.2. Effect of incubation temperature

The antibody immobilized protein A magnetic beads incubated with EPO for 2.5 h at 25 °C and 37 °C, respectively. It was found that the enrichment efficiency of EPO was greatly improved when 37 °C was used (Fig. 4B), which indicates that the extraction efficiency increased with the increase of the incubation temperature. However, as reported in literature [53] that once the incubation temperature exceeds 40 °C, anti-EPO antibody may be inactivated, higher temperature was not employed. Thus, 37 °C was chosen for the later experiments.

3.3.3. Effect of incubation time

Incubation time is an important factor that determines the extraction efficiency. In this study, three time points, including 1.5, 2.5 and 3.5 h, were compared under otherwise identical conditions. As shown in Fig. 4C, incubation for 3.5 h provided the most intense signal for all the glycoforms of EPO, which suggests that longer extraction duration favors the extraction efficiency. However, considering extended incubation time might result in degradation of EPO (the half-life of rhEPO in blood is 5–6 h after injection [7]), longer incubation time was not investigated and 3.5 h was selected for the later experiments.

3.3.4. Effect of pH and ionic strength

The pH and ionic strength for EPO extraction were also investigated. The pH points examined included 6.4, 7.4, 8.4 and 9.0. It was experimentally observed that extraction at pH 7.4 exhibited the best efficiency, though the overall effect of pH was not very significant (data not shown). Phosphate buffer of five different concentrations was used as the extraction buffer, including 0.1, 0.2, 0.3, 0.4 and 0.5 M. It was found that increasing the concentration of the extraction buffer resulted in a slight decrease in the amount of EPO exacted, which is in good agreement with the literature conclusion that high ionic strength is not favorable to the formation of antigen–antibody complex [54]. Therefore, 0.10 M potassium phosphate buffer at pH 7.4 was used throughout the remainder of this study.

3.3.5. Effect of antibody sources

Three different anti-EPO antibodies were compared for their extraction performance, including a mouse monoclonal antibody, a rabbit monoclonal antibody and a rabbit polyclonal antibody. As shown in Fig. 4D, the three antibodies exhibited slight difference in the extraction efficiency, though the mouse monoclonal anti-EPO antibody provided highest efficiency. Considering the cost (the mouse monoclonal anti-EPO antibody is most expensive, about four times costly as compared with rabbit monoclonal anti-EPO antibody), the rabbit monoclonal antibody was chosen for later experiments.

Based on the above investigations, the optimal extraction and elution conditions were: extraction with rabbit monoclonal antibody, incubated in 0.1 M phosphate buffer (pH 7.4) at 37 °C for 3.5 h; elution with 10 M urea for 2 h.

3.4. The detection limit and repeatability

Under the optimized experimental conditions, the detection limit of EPO in solution was measured to be 1.5×10^{-8} mol/L (S/N = 3, calculated with the smallest peak within the five peaks observed), which is two orders of magnitude lower than conventional CZE with UV absorbance detection. Such detection sensitivity

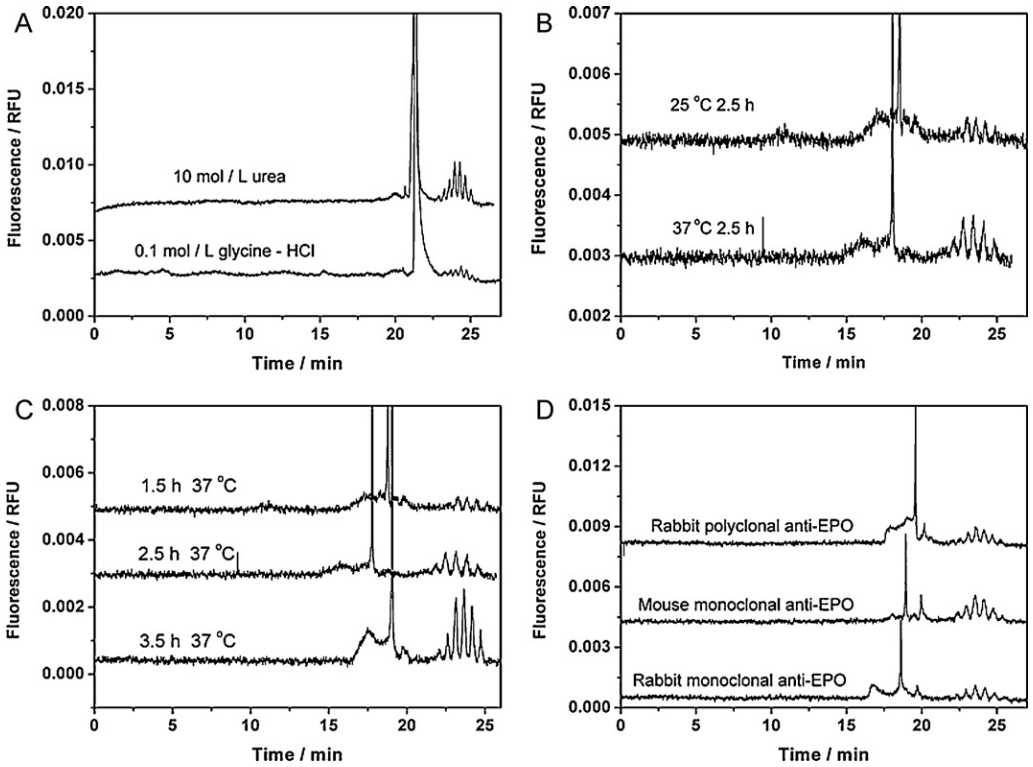

Fig. 4. Effects of experimental conditions on the extracted amount. (A) effect of elution solution, (B) effect of incubation temperature, (C) effect of incubation time, (D) effect of different commercial antibodies. Sample: 0.5 mg/mL rhEPO dissolved in 100 mM PBS, pH 7.4. Except for the specified, other conditions are the same as in Fig. 3.

is clearly far from the requirement for the detection of real samples, such as urine and blood. However, the improvement in sensitivity is an important value for other applications such as pharmaceutical analysis.

The concentrations of EPO tested were in the range of 1.5×10^{-8}–1.5×10^{-5} mol/L. The linear range for EPO was from 1.5×10^{-8} to 1.0×10^{-6}. Extraction repeatability was determined using EPO solution containing 1.0×10^{-6}, 3.6×10^{-7} and 1.0×10^{-7} mol/L EPO for five replicate extractions, respectively, the RSD values of peak areas were below 5%. Intraday repeatability for migration time was acceptable, with RSD values less than 2.0%.

3.5. Extraction of EPO from injection solutions

To examine the feasibility of the developed method to complex samples, commercial EPO injection solution which contained EPO-α and human serum albumin (HSA, acted as a stabilizer) were used as a model sample. For analysis of EPO in this injection solution, an ultrafiltration procedure is usually required to remove the interference of HSA. When no pretreatment step was performed, a broad peak, rather than a distinct glycoform profile, was observed for the injection solution. As a comparison, after the sample was extracted with the protein A-coated magnetic beads, a distinct glycoform finger map was observed, as can be seen in Fig. 5. Therefore, the developed method can be applied to the quality control of rhEPO in pharmaceutical industry.

3.6. Effect sample matrix in urine

As the detection sensitivity of the developed method failed to detect EPO in urine, EPO-spiked urine samples were tested to investigate the effect of sample matrix. The final concentration of BRP EPO spiked in the urine samples was 3.0×10^{-8} M. If there is no any matrix effect, the developed method should be able to detect the EPO glycoforms. However, it was found that sample matrix exhibited a very detrimental effect on the extraction. When the samples were extracted directly with the immuno-magnetic beads, no glycoforms were observed. In order to verify if low-molecular-weight components in the sample matrix interfered the immuno-extraction, an ultrafiltration step using filtration membrane with a MWCO value of 10,000 Da was carried out prior to the immuno-extraction. However, similar result was obtained. Therefore, the interference from the low relative molecular mass components was excluded. It was reported that highly abundant urinary proteins, such as Tamm Horsfall glycoprotein (M_r, 69,000) and alpha-2-thiol proteinase inhibitor (M_r, 72,000), are cross-reactive to EPO antibodies [13]. To verify the effect of high-molecular-weight components, an ultrafiltration step using

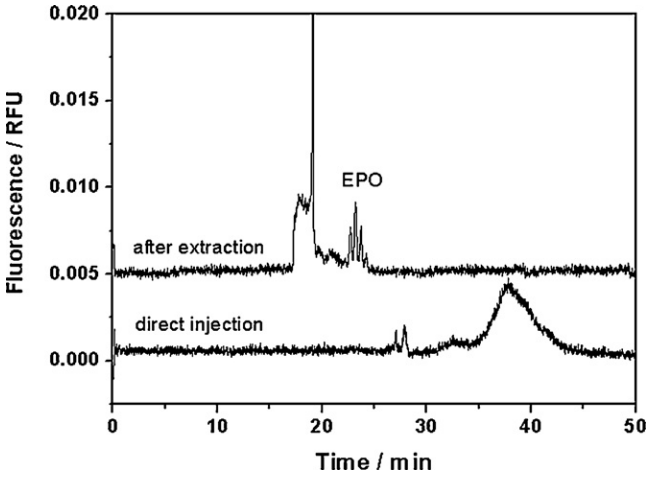

Fig. 5. CZE-deep UV-LIF analysis of EPO extracted from injection solution. Sample: 200 μL of 10,000 IU EPO injection. Other conditions are the same as in Fig. 3.

Table 1
Comparison of different methods for the separation and detection of EPO.[a]

Analytical method	Limit of detection	EPO type	Separation time	Resolution (glycoforms observed)	Ref.
CZE-deep UV-LIF	0.5 μg/mL	α, BRP, α in injection	25 min	5 for α; 8 for BRP	[This work]
CE-LIF	7.4 ng/mL	α, shEPO	9 min	1	[25]
CE-CL	0.9 ng/mL	shEPO	250 s	1	[37]
Gel IEF-CL	3.4 pg/mL	α, uhEPO	2–3 days	4–6 for α; 10–15 for uhEPO	[11]
2DGE-CL	2.5 pg/mL	BRP, uhEPO	2–3 days	5 for BRP; 6 for urine; 8 for uhEPO-spiked urine	[13]
CZE-ESI- TOF MS	1 mg/mL[b]	BRP	30 min	64	[33]
CE-ESI- IT MS	3 mg/mL[b]	BRP	45 min	17	[34]

[a] Abbreviations for EPO: α; epoetin-α; shEPO; human serum EPO; uhEPO; urinary human; BRP; biological reference product.
[b] Lowest EPO concentration used.

filtration membrane with a MWCO value of 50,000 Da was carried out prior to the immuno-extraction. With the absence of components with molecular weight higher than 50,000 Da, EPO was extracted from urine and distinct glycoform profile was observed, though the peak intensity was low (Fig. 6). Therefore, for further development of immuno-extraction techniques for purification and enrichment of EPO, matrix effect will be a major concern and antibodies with high specificity is essential.

3.7. Method comparison

Table 1 shows a side-by-side comparison of the analytical performance of the present method with the other methods that combines a separation platform and highly sensitive detection, such as CE-LIF, CE-CL, 2DGE-CL and CE-MS. Although affinity probe CE-LIF [25] and CE-CL [37] provide higher sensitivity and shorter separation time as compared with the current method, they fail to differentiate the EPO glycoforms. Gel IEF-CL [11] and 2DGE-CL [13] provide the highest detection sensitivity among all the methods as well as comparable resolution as compared with the present method, but their apparent disadvantage is that the separation is time-consuming (2–3 days). Due to the powerful structural identification capability of MS, both CZE-ESI-TOF MS and CE-ESI-IT MS can detect the most glycoforms among all the methods. However, the sample concentration required is the highest and the separation time is longer than the current method. As these competing methods did not focus on the sample preparation step, comparison of the performance of all the sample preparation procedures involved is not provided. However, the performance of the IMBE approach can be compared with the sample preparation procedure used in 2DGE-CL [13], which relied on the combination of acetonitrile precipitation and ultrafiltration. The IMBE approach is much straightforward; it requires only 6.5 h totally and avoids the need of costly instruments. As comparison, the precipitation-ultrafiltration procedure takes about 15 h and needs a centrifugal machine. Thus, like the competing methods, the present method has its own advantages and disadvantages; it exhibits several advantages, including resolution, speed and simplicity, whereas relatively low sensitivity is its disadvantage.

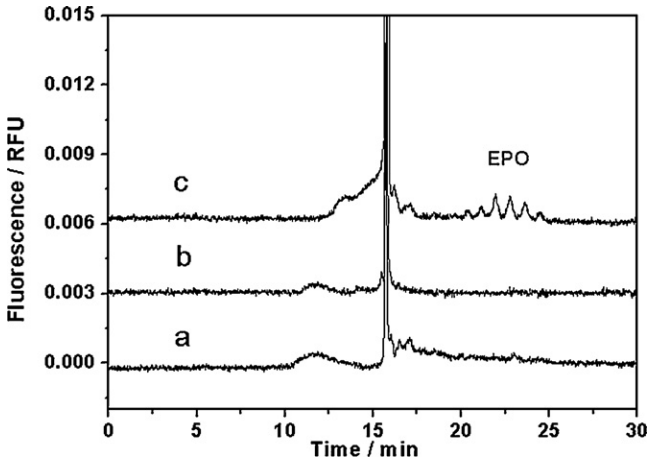

Fig. 6. Comparison of the sample pretreatment procedures on the extraction. (a) direct extraction without ultrafiltration, (b) extraction after ultrafiltration with Amicon Ultra-15 (MWCO 10,000 Da), (c) extraction after ultrafiltration with Amicon Ultra-0.5 (MWCO 50,000 Da). Sample: 20 mL urine sample spiked with 3.0×10^{-8} M rhEPO. Other conditions are the same as in Fig. 3.

4. Conclusion

We established a deep UV-LIF detector for sensitive detection of EPO and an immuno-extraction approach for convenient extraction of EPO. As compared with conventional UV absorbance detection, the detection sensitivity was enhanced by 1 order of magnitude. Different from regular LIF detection with fluorescent labeling, the deep UV-LIF detection can preserve the original glycoform distribution, allowing for differentiation of endogenous and recombinant EPO. With magnetic beads as a convenient extraction probes, the IMBE approach was effective for extraction of EPO in pharmaceutical products, but the antibody used in this study was not suitable for the extraction of urine samples due to its cross-reactions with other urinary proteins. By combining the two component approaches with CZE separation, the detection sensitivity was improved by 2 orders of magnitude as compared with conventional CZE with UV absorbance detection. Due to the speed, resolution and sensitivity, the IMBE-CZE-deep UV-LIF method can be a useful tool for quality control in pharmaceutical industry. However, the sensitivity is far from the requirement for real world anti-doping analysis, for which the current official approach is still the gold standard even it is associated with some significant drawbacks.

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