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Determination of epitestosterone in human urine by off-line immunoaffinity solid-phase extraction coupled with high performance liquid chromatography

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ABSTRACT

Epitestosterone (ET) has been used as a masking agent and prohibited by the World Anti-Doping Agency (WADA) because its administration will decrease the urinary T/ET ratio, a marker of testosterone (T) administration. In this study, an off-line immunoaffinity extraction coupled with high performance liquid chromatography (HPLC) was developed to quantify the endogenous steroid ET in human urine. The immunoaffinity column (IAC) was prepared by immobilizing the anti-ET monoclonal antibodies on CNBr-activated Sepharose 4B, which can remove the contaminations and non-target compounds from matrix to enrich the target analyte ET. The mobile phase was ammonium acetate (10 mM, pH 4.0)/acetonitrile (45/55, v/v) at an isocratic flow of 1.0 mL/min and the UV absorbance detection wavelength was 244 nm for the detection of ET. The IAC showed good reliability and durability since it had been used for more than 100 runs in a year. The limit of quantification (LOQ) was 1 ng/mL. Satisfied repeatability and precision of the day-to-day and within-day were obtained with the RSD values less than 10%. Results of the recovery of the urine samples were ranged from 98% to 102% with repeatability less than 9%, indicating that the method developed can be used for the real urine sample analysis.

1. Introduction

Epitestosterone (ET, 17α-hydroxy-4-androsten-3-one), the 17α-hydroxy epimer of testosterone (T), is a natural steroid which was first found in 1947 [1] and isolated from human urine in 1964 [2,3]. The structure of ET is shown in Fig. 1. It originates mainly from the testes and a little of it probably comes from the ovaries and adrenals in the human body [4]. Its physiological function is still unclear. ET has been found in human urine [2,3,5,6], plasma [7], hair [8] and tissues such as human testes and hyperplastic prostates [9,10]. The concentration of ET in urine is much higher than that in plasma [11]. The excretion of ET in human is influenced by many factors such as gender, age [12], race, health [13] and diet [14]. Free ET is hardly detected in human urine since it is excreted in the form of glucuronide and sulfate, mainly as glucuronide form [15].

The concentration of ET in urine is close to that of T [16], nearly keeps constant for male from puberty to senescence [17] and cannot be influenced by the exogenous administration of T. The constant ratio of T to ET in urine is of great interest for the doping control of the administration of T since T can be abused to improve physical performance, muscle strength and balance in the catabolic condition of the body after the stress. ET is commonly used as a masking agent to rapidly decrease the T/ET ratio if one has T administration. The World Anti-Doping Agency (WADA) indicated that if the T/ET ratio was equal or above 4 or the concentration of ET was higher than 200 ng/mL in the screening procedure, a confirmation procedure would be needed [18]. For this reason, the IOC (International Olympic Committee) Medical Commission classified ET as a urine-manipulating agent and set 200 ng/mL (694 nmol/L) as the threshold. Therefore the accurate and fast determination of ET in the biological fluid is very important.

Various analytical techniques have been established, such as radioimmunoassay [19], high performance liquid chromatography (HPLC) [6,20], two-dimensional gas chromatography coupled with flame ionization detector (GC/GC–FID) and time-of-flight mass spectrometry (GC/GC–TOF) [21], gas chromatography/mass spectrometry (GC/MS) [22,23], gas chromatography/thermal conversion/isotope ratio mass spectrometry (GC/TC/IRMS) [24], gas chromatography/combustion/isotope ratio mass spectrometry GC/C/IRMS [4], HPLC/MS [25–28], LC/Q-TOF mass spectrometry [16], capillary electrophoresis (CE) [29] and CE/MS [30].

Since the matrix of the biological fluid is very complicated, it is very important to apply the cleanup methods to minimize the interferences before analysis. The cleanup methods include solid-phase extraction (SPE) [31,32], liquid–liquid extraction (LLE) [33],

Abbreviations: ET, epitestosterone; T, testosterone; IAC, immunoaffinity column.

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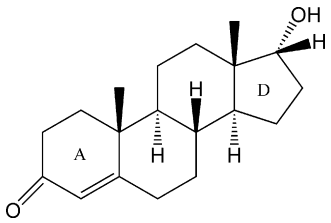

Fig. 1. Structure of epitestosterone.

and stir bar sorption extraction [34]. Immunoaffinity extraction is a special SPE based on the highly specific interaction between antigen and antibody. Immunoaffinity extraction (IAC) can efficiently eliminate the matrix contaminations and non-target compounds to enrich the target analyte. It has been applied in environmental monitoring [35], pharmaceutical and biomedical analyses [36], and food analysis [37]. As a cleanup and separation technique, IAC has been successfully used to enrich ET in the biological fluid prior to CE detection [38]. In this paper a simple and more sensitive method to determine the ET in urine by IAC coupled with HPLC was developed.

2. Experimental

2.1. Chemical and reagents

ET, T and β-glucuronidase from *Escherichia coli* (type IX-A) were purchased from Sigma (St. Louis, MO, USA). Methanol and acetonitrile were from Fisher Scientific (Fair Lawn, NJ). Sodium acetate was from Beijing Yili Fine Chemicals (Beijing, China). Hydrochloric acid, sodium chloride, sodium dihydogen phosphate, disodium hydrogen phosphate, glacial acetic acid, ammonium acetate, sodium bicarbonate and sodium hydroxide were obtained from Beijing Chemicals (Beijing, China). Potassium dihydogen phosphate and tris(hydroxymethyl)aminomethane were supplied by Sinopharm Chemical Reagent (Beijing, China). Potassium chloride was from Beijing Shuanghuan Weiye Reagent Co. Ltd. (Beijing, China). CNBr-activated Sepharose 4B was purchased from Pharmacia Biotech (Uppsala, Sweden). Anti-epitestosterone monoclonal antibodies were prepared in Immunological Laboratory in College of Life Science, Peking University [37]. All reagents were of ≥99.0% purity unless stated otherwise. Water was purified in a Milli-Q Integral 3 A10 water purification system (Millipore, Bedford, MA).

2.2. Instruments

HPLC analysis was carried out on a Shimadzu Prominenece LC-20A HPLC system (Kyoto, Japan) consisting of a LC-20AT Solvent Delivery Unit, a SPD-M20A photodiode array detector, a SIL-20A autosampler, and a DGU-20A5 on-line degasser. Data acquisition and processing/post-run analysis were accomplished with Shimadzu LC solution software, version1.23 Workstation. UV detection was performed with Cary 1E UV–vis spectrophotometer (Varian Instruments, USA). HPLC/LC/MS was performed by Agilent 1100 HPLC system (Santa Clara, CA, USA) coupled with ESQUIRE-LC ion trap mass spectrometry (Bremen, Germany). Experiments on immunoaffinity column were performed at 4 °C in a chromatography refrigerator (Beijing Freezing Equipment Factory). GS-15R multi-purpose refrigerate centrifuge was supplied by Beckman (Fullerton, CA, USA). HZQ-Q incubator shaker was purchased from Harbin Donglian Electronics Co. (Harbin, China).

2.3. HPLC condition

HPLC was performed on a Gemini C18 reversed-phase column (250 mm × 4.6 mm i.d., 5 μm particle size, 110 Å pore diameter, Phenomenex, Torrance, CA). A C_{18} guard column (4 mm × 3.0 mm, Phenomenex, Torrance, CA) was added prior to the analytical column. The mobile phase composition (acetonitrile:10 mM ammonium acetate) was 55:45 (v/v), the flow rate was 1.0 mL/min and the injection volume was 20 μL. The detection wavelength was 244 nm.

2.4. HPLC/MS/MS condition

HPLC was performed with the same condition described above. The MS instrument was operated in scan mode with high purity nitrogen as dry gas and nebulizer gas which was set at 12 L/min and 40 psi, respectively. Spray voltage of 4.0 kV was used in positive mode; capillary exit voltage and temperature was 115 V and 300 °C, respectively; skimmer 1 and trap was set at 25 V and 32 V, respectively.

2.5. Purification of the monoclonal antibodies

Monoclonal antibodies (anti-ET) were collected from mouse ascites and were further purified based on the modified caprylic acid–saturated ammonium sulfate method [39]. Emulsion of 762 μL of caprylic acid and 11.55 mL of acetate buffer (0.06 M, pH 4.8) was slowly dropped to 7.7 mL of 3D7 ascites. The solution was stirred for 30 min and then centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was adjusted to pH 7.4 with 1.0 M NaOH and cooled down at 4 °C. Then saturated ammonium sulfate (pH adjusted to 7.4 by ammonia solution) of the same volume was added slowly under stirring. The mixed solution was left at 4 °C overnight and was centrifuged at 13,000 rpm for 30 min at 4 °C. The precipitation was dissolved in 0.5 mL of PBS (0.01 M phosphate buffer, 0.8% NaCl, 0.02% KCl, pH 7.4). The solution was dialyzed against PBS for 24 h, and then stored at −20 °C. The concentration of purified antibody was determined by UV method using the Lowry–Kalckar formula: $C_{protein}$ (mg/mL) = $1.45A_{280} - 0.74A_{260}$, where A_{280} and A_{260} represent the ultraviolet absorbance at 280 nm and 260 nm, respectively.

2.6. Preparation of immunoaffinity column

The IAC was prepared according to the method reported before with little modification [40]. 0.3 g of CNBr-activated Sepharose 4B was swelled and washed with 1 mM HCl, and then mixed with 10 mL coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) containing 9.4 mg of the purified monoclonal antibody. The coupling reaction proceeded in an incubator shaker at a shaking speed of 110 rpm for 2.5 h at 20 °C. After the coupling reaction, the gel was transferred into a glass column (100 mm × 10 mm i.d.). The uncoupled antibodies were removed from the column by elution with the coupling buffer.

The unreacted group in the gel was blocked with Tris–HCl buffer (0.1 M, pH 8.0) for 2 h. The gel was then washed 3 times alternatively with 10 mL HAc–NaAc buffer (0.1 M, pH 4.0, containing 0.5 M NaCl) and 10 mL Tris–HCl buffer (0.1 M, pH 8.0, containing 0.5 M NaCl). Finally, the column was equilibrated with 0.01 M PBS. The column should be preserved in 0.01 M PBS containing 0.02% NaN_3 at 4 °C for long time storage.

2.7. Sample preparation

1 mL PB buffer (75 mM phosphate, pH 6.8) was added to 1 mL urine sample, then 300 μL β-glucuronidase (5 mg/mL in PB buffer) was added to the mixture. After vortex, the sample was incubated at

Table 1
Results of day-to-day and within-day for ET in spiked urine.

Concentration added (ng/mL)	Day-to-day		Within-day	
	Concentration found (ng/mL)	RSD (%)	Concentration found (ng/mL)	RSD (%)
20	19.3 ± 4.1 (n = 16)	6.7	19.9 ± 1.1 (n = 6)	3.5
200	186 ± 56 (n = 16)	7.6	185 ± 29 (n = 5)	7.1
1000	1016 ± 72 (n = 16)	3.7	1154 ± 41 (n = 6)	2.9

37 °C for 24 h. Following glucuronidase hydrolysis, the urine sample was cooled down and filtrated through a 0.22 μm filter (Jinteng, China), then the filtered sample was loaded on the immunoaffinity column which had already been conditioned with 0.01 M PBS. The column was washed with 3 mL 0.01 M PBS to remove the nonspecifically bounded analytes. Subsequently, it was washed by 1 mL water and later eluted by methanol/water (80/20, v/v). The collected effluent was dried by high purity nitrogen gas and the residues were redissolved in 100 μL methanol/water (80/20, v/v) which was applied to HPLC to determine the concentration of ET.

2.8. Specificity of the monoclonal antibodies

The 10 mL T or ET standard solution in PBS (1 μg/mL) was separately loaded onto the immunoaffinity column conditioned previously, then the column was washed by 1 mL water and later was eluted by methanol/water (80/20, v/v). 1 mL mixture of T and ET standard solution (each 10 μg/mL in PBS) was treated as the same procedure above. The concentration was determined by HPLC. T and ET were separately dissolved in methanol as a stock solution (1 mg/mL) and stored at −20 °C, then stock solution was serially diluted to 10 μg/mL, 5 μg/mL, 2 μg/mL, 1 μg/mL, and 0.5 μg/mL by methanol/water (80/20, v/v). The calibration curves for ET and T of the peak area (A) versus concentration (C) were as follows respectively:

$$\text{ET: } A = 8.3 \times 10^4 C - 2020 \qquad R^2 = 0.9996$$
$$\text{T: } A = 5.3 \times 10^4 C + 1290 \qquad R^2 = 0.9975$$

2.9. Method validation

The spiked steroid-free urine was used for validation studies. It was obtained by passing 100 mL urine through a column packed with 25 g of XAD-2 resin (Serva, NY, USA) which was previously conditioned with ethanol and water. The first 20 mL was discarded. All spiked urine proceeded as described in sample preparation (Section 2.7) except enzyme hydrolysis step.

In order to evaluate the method, the calibration curve was accessed by analyzing spiked urine samples in the range of 1–800 ng/mL for ET by IAC coupled with HPLC. The day-to-day and within-day repeatability and the precision were obtained by analyzing spiked urine at 20 ng/mL, 200 ng/mL and 1 μg/mL, respectively.

2.10. Application to real urine samples

In order to evaluate the applicability of the method for the routine analysis, ET in the urine samples of three mature males and three male infants were detected, respectively. The recovery and repeatability were also evaluated.

3. Results and discussion

3.1. Evaluation of the immunoaffinity column

The purified anti-ET MAbs were immobilized onto the Sepharose gel by forming a stable amide bond between the free amino groups of the monoclonal antibodies and CNBr-activated Sepharose 4B, which had been proved to be an effective method [40]. The coupling process led to the coupling yield of 3.89 mg MAbs for 0.3 g (13 mg/g) CNBr-activated Sepharose 4B. The final gel volume of IAC was 0.8 mL, and the column capacity varied from 4 μg to 5 μg for ET in different loading concentrations of ET and different detection times. It is calculated from the column that only about 4 μg (13.9 nmol) ET can be bound to the 3.89 mg immobilized MAbs (25.9 nmol) assumed that MAbs binding to the Sepharose gel homogeneously. Since IgG has two identical binding sites for the target analyte, about the 27% of the binding sites on antibodies seemed to act as receptors for the ET. This result is reasonable since the antibodies conjugated to the Sepharose gel would lose some activity due to the random orientation and steric hindrances. Therefore the reactive binding sites for ET on the attached antibodies were limited. However, compared to polyclonal antibodies (PcAbs), the MAbs bind antigen effectively, since there were much more effective binding sites in MAbs than those in PcAbs [41]. The IAC was very stable and could be used repeatedly. It was still effective and the column capacity remained 4 μg after more than 100 loading runs in about one year.

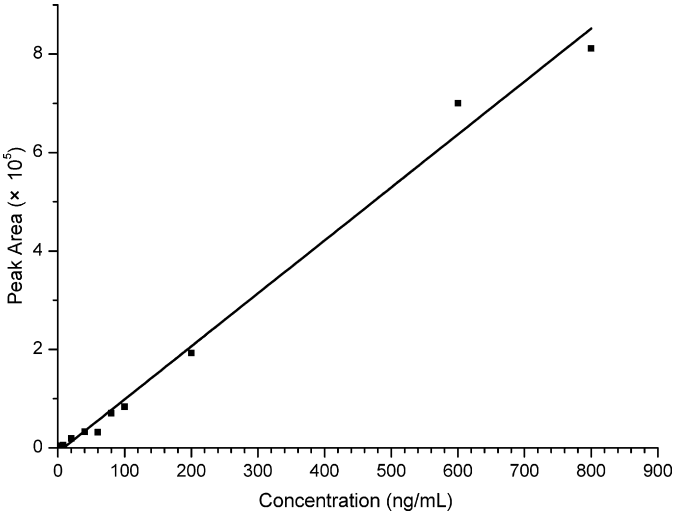

Fig. 2. Calibration curves for ET by IAC–HPLC. The separation was performed on a Gemini C18 reversed-phase column (250 mm × 4.6 mm i.d., 5 μm) under an isocratic elution of acetonitrile (ACN)/10 mM ammonium acetate at a flow rate of 1.0 mL/min, the detection wavelength was 244 nm.

Table 2
Analytical precision and reproducibility for ET in spiked urine.

Samples	Concentration added (μg/mL)	Concentration found (μg/mL)	Recovery (%)	RSD (%)
1	1.0	0.952 ± 0.008 (n = 3)	95.2	0.8
2	2.0	1.669 ± 0.018 (n = 3)	83.5	0.6

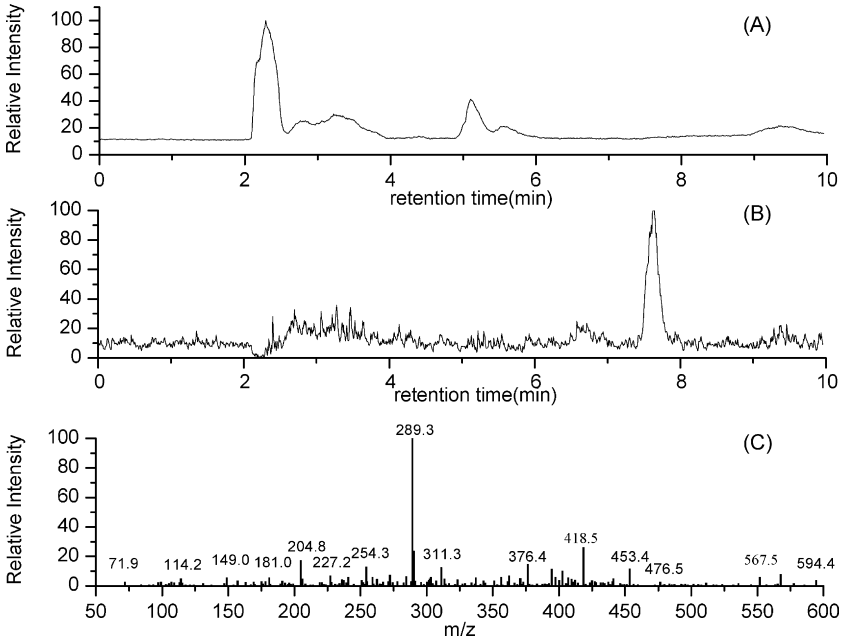

Fig. 3. TIC, EIC and mass spectra of T in the urine, (A) the total ion chromatography (TIC) of the urine pretreated by IAC, (B) extracted ion chromatogram (EIC) of mass-to-charge ratio 289 ($M+H^+$ of T and ET), and (C) mass spectra at 7.6 min which was the retention time of T.

3.2. Specificity of the column

The analyte was specifically bound to the complementarity-determination region of the antibodies. Antibodies can not only bind the target analyte but also the other analogs with the similar structures. But affinity of antibodies to different compounds is different. The stronger the affinity between the antibodies and the analyte, the higher column capacity and recovery for the analyte can be obtained. The column capacities of the IAC for T and ET were investigated respectively. When 10 μg ET and 10 μg T were loaded on the IAC column separately, the column capacity was 5.0 μg for E and only 1.0 μg for T. Different column capacity between ET and T showed that ET and T had different immunoaffinity towards MAbs. T was washed away easier than ET in the washing procedure because of the lower affinity. When 10 μg mixture of T and ET was loaded on the immunoaffinity column, the immunoaffinity column capacity was 5.0 μg for ET and only 0.1 μg for T. This was because the bound antibodies in IAC was designed and prepared for ET specifically and accordingly ET had higher affinity towards MAbs than T. Since BSA was conjugated to C3 on the A ring in ET, the specificity was directed towards the D ring. T and ET differ chemically from each other only in the configuration of the hydroxyl group on C-17 on the D ring. The monoclonal antibodies are anti-3-epitestosterone–BSA antigens, therefore the discrimination of the column capacity for T and ET in higher concentration represents the different immunoaffinity. Different recoveries also showed the different immunoaffinity. When 1 μg/mL for both of ET and T in the mixture (loading volume was 1 mL), the greater difference in recovery between them was observed. The recovery was 93.8% for ET and only 58.2% for T. Our result was consisted with the cross-reactivity result reported previously [38].

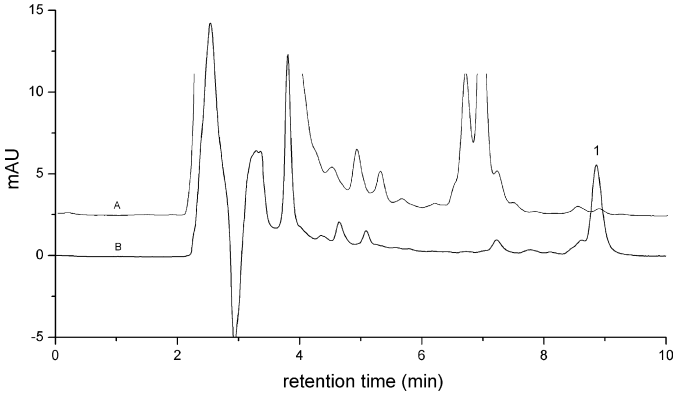

Fig. 4. Chromatography of the urine added enzyme with and without IAC, (A) the urine sample added enzyme without treatment by IAC, (B) the urine sample added enzyme with treatment by IAC. Peak 1: epitestosterone. HPLC conditions were the same as described in Fig. 2.

Table 3
Results of the analysis of the urine samples.

Samples	ET found (ng/mL)	RSD (%)
Mature male 1	150 (n = 4)	8.9
Mature male 2	6.2 (n = 3)	2.9
Mature male 3	38.6 (n = 3)	3.7
One-month-old infant	19.3 (n = 3)	8.2
Five-month-old infant	5.0 (n = 3)	5.7
Six-month-old infant	14.1 (n = 3)	5.3

Table 4
Results of the recovery and repeatability of the urine samples.

Samples	ET found (ng/mL)	ET spiked (ng/mL)	ET found after adding (ng/mL)	Recovery (%)	RSD (%)
1	150	75 (n = 5)	230 ± 10	102	3.1
2	150	150 (n = 5)	294 ± 16	98.0	3.7

3.3. Method validation

The calibration curve (Fig. 2) was obtained and the corresponding calibration curve of the peak area (A, μV S) versus concentration (C, ng/mL) was as follows:

$$A = 107C - 9699 \qquad R^2 = 0.992$$

The results of the day-to-day and within-day were listed in Table 1. Satisfied repeatability and precision were obtained with the RSD values less than 10%. The results of repeatability and precision at different concentrations were listed in Table 2, and RSD value was below 1%. The limit of quantification (LOQ) for ET was 1.0 ng/mL, which was below the minimum required performance levels (MRPL) required (2 ng/mL) by WADA [42]. The sensitivity of IAC–HPLC was much higher than that of IAC–CE (23 ng/mL [37]), CE (85 ng/mL [29]), CEMS (1.25 μg/mL [30]) and HPLC (30 ng/mL [20]). LOQ of IAC–HPLC was equal to that of LC-Q (1 ng/mL [16]). Therefore our method shows higher sensitivity than most of others.

3.4. Analysis of urine samples

ET exists mainly in the form of glucuronide in the original urine. But ET glucuronide conjugation or sulfate conjugation was not specific towards MAb immunoaffinity column. The original urine sample was loaded on the IAC, followed by the step of sample preparation. The pretreated sample was analyzed by HPLC/MS (Fig. 3). The mass-to-charge ratio (m/z) of the main product ion of the T and ET glucuronide conjugation is 289 [25]. There were no other compounds except free T showed in extracted ion chromatogram (EIC) of the m/z 289. This meant that the conjugated compounds of T cannot be caught by IAC either. The ET cannot be detected by IAC without enzyme hydrolysis. But after the enzyme hydrolysis, the matrix became more complicated even though the original urine itself was very complex indeed (Fig. 4) without IAC treatment. The complex matrix and untargeted analytes can be removed and the target analyte can be enriched by IAC procedure. Peak at 8.9 min was corresponding to ET, which was also confirmed by HPLC/MS (data were available in Supplementary). Therefore, the sensitivity was improved and a cleaner chromatography could be obtained (Fig. 4).

The real urine samples were collected from three mature males and three boy infants. The results were shown in Table 3. The ET concentrations in male urine varied from 6.2 ng/mL to 150.0 ng/mL, and the concentrations in infants were from 5.0 ng/mL to 19.3 ng/mL with good RSD. The results are acceptable since many factors such as diet, age and sampling time could lead to the different concentration in different humans. The recoveries were performed in the male urine. Two different concentrations of ET were added to the real male urine to estimate the recovery (Table 4). Satisfied recoveries (98–102%) and good repeatability (RSD value less than 9%) of the sample were obtained, indicating that the analysis of ET by IAC coupled HPLC was appropriate to the practical detection.

4. Conclusion

This study provided a new approach to detect ET by off-line IAC coupled with HPLC. With the aid of IAC, the contaminations and non-target compounds in the complex biological matrix such as urine could be removed and the target analyte ET was enriched. This method provided a good selectivity towards ET in the complex urine matrix. LOQ of the assay was as low as 1 ng/mL, which is much higher than most of other reported works. The low LOQ was in accordance with the MRPL for ET defined by the World Anti-Doping Agency (WADA). This method could be applied to an alternative screening method. IAC showed good reliability and durability and could be used for more than 100 times in one year in this study. The IAC based on the MAbs reduces the cost of the pretreatment in analysis and could be developed to be a commercial SPE.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.03.031.

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