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Pseudo-homogeneous immunoextraction of epitestosterone from human urine samples based on gold-coated magnetic nanoparticles

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ABSTRACT

A pseudo-homogeneous immunoextraction method based on gold-coated magnetic nanoparticles (MNPs) for the specific extraction and quantitative analysis of epitestosterone (17α-hydroxy-4-androsten-3-one, abbreviated as "ET") from human urine samples by high-performance liquid chromatography (HPLC) has been developed. Half-IgG of anti-ET monoclonal antibodies were covalently immobilized onto $(Fe_3O_4)_{core}$-Au_{shell} (Fe_3O_4@Au) MNPs. An external magnetic field was applied to collect the MNPs which were then rinsed with distilled water followed by elution with absolute methanol to obtain ET as the analyte. The obtained extraction solution was analyzed by HPLC with UV detection (244 nm) within 12 min. The standard calibration curve for ET showed good linearity in the range of 20–200 ng mL^{-1} in phosphate-buffered saline (PBS) solutions with acceptable accuracy and precision. Limit of detection for ET was 0.06 ng mL^{-1} due to an enrichment factor of 100-fold was achieved. The results obtained by the present method for spiked human urine samples were in agreement with those from indirect competitive enzyme-linked immunoadsorbent assays (ELISAs). The antibody-conjugated Fe_3O_4@Au MNPs are novel materials for immunoaffinity extraction. Compared with the conventional technique using immunoaffinity column, the method described here for sample pretreatment was fast, highly specific, and easy to operate.

1. Introduction

An antibody is a kind of glycoprotein produced in the animal body itself in response to an antigen [1]. A typical antibody (i.e., immunoglobulin G, or IgG) contains two couples of polypeptide chains. Each polypeptide chain consists of a heavy chain, a light chain, and one antigen-binding site. The two heavy chains are linked via disulfide bonds. It has been reported that the two heavy chains could be split into two half-IgG when treated with 2-aminoethanthiol [2]. This approach was used to break the disulfide bonds of the immunoglobulin while maintaining the biological activity of the antigen-binding sites. The half-IgG antibody could be then immobilized onto a gold surface through the generated thiol groups.

The immobilization of half-IgG on gold should provide a higher epitope density than those prepared by the direct immobilization of intact-IgG [3]. Guzman has fabricated a simple solid-phase microextraction device for on-line immunoaffinity capillary electrophoresis [4]. The device employed IgG fragments covalently bound to controlled-porosity glass, which allowed quantitation levels of analytes at approximately 5 ng mL^{-1} or lower using UV detection. However, it should be noted that sufficient specific surface area of support is also required for binding biomolecules [5–7]. Magnetic nanoparticles (MNPs) which provide sufficient specific surface area can be employed for binding biomolecules.

The synthesis and applications of MNPs have attracted much attention in recent years due to their usefulness and versatility in various scientific fields [8–13]. Various studies are focused on the fabrication of MNPs with core-shell nanostructure, such as Fe_3O_4@Al_2O_3 [14], Fe_3O_4@SiO_2 [15–17], Fe_3O_4@Ag [18,19], Fe_2O_3@Au [20,21], and Fe_3O_4@Au [22–26]. Among them, the Fe_3O_4@Au nanoparticles (not commercially available) are the most commonly studied. Gold has been found to be a splendid material for modifying Fe_3O_4 particles because of its chemical inertness and biocompatibility. The gold-coated MNPs can be applied to antibody immobilization with following advantages: (1) these MNPs are environmentally friendly and reusable; (2) the specific surface area is sufficient; (3) the process of half-IgG antibodies attachment is easy and safe; (4) a pseudo-homogeneous immunoextraction is carried out in the sample solution.

Herein, the half-IgG antibodies coated Fe_3O_4@Au nanoparticles were successfully applied to the analysis of epitestosterone (ET) in spiked human urine as a model example. Epitestosterone is the inactive 17α-epimer of testosterone (T) [27].

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Testosterone is one kind of endogenous anabolic androgenic steroids which is able to improve athletic performance in sports when administered exogenously. The abuse of T has been forbidden by the International Olympic Committee (IOC) since 1983 and the World Anti-Doping Agency (WADA) [28]. The concentration ratio of T to ET in human urine samples after enzymatic hydrolysis remains constant. If the T/ET ratio of a sample is above 4, testosterone abuse may have happened and further investigations are needed. Any urine samples with ET concentration higher than 200 ng mL^{-1} is indicative of drug abuse because the ratio of T/ET can be modified by the administration of ET as a masking agent. In human urine, T and ET mainly appear in the form of glucuronide and sulfate conjugates [29,30]. The determination of T and ET in urine samples after enzymatic hydrolysis is normally performed by gas chromatography–mass spectrometry (GC–MS) [31], high-performance liquid chromatography–mass spectrometry (HPLC–MS) [32], and capillary electrophoresis (CE) [33]. Compared with CE and GC–MS, HPLC–MS is an appropriate method for analytes detection because it allows for better reproducibility and does not require derivatization. Different pretreatment methods have been described including liquid–liquid extraction [34], solid-phase extraction [35], and immunoextraction [33]. Immunoextraction is a popular technique for sample pretreatment due to its high specificity.

In this paper, the Fe_3O_4@Au nanoparticles were synthesized on which half-IgG antibodies (anti-ET) were immobilized. A pseudo-homogeneous immunoextraction was carried out using Fe_3O_4@Au nanoparticles to specific extract ET from solution. Thus, a rapid, simple and highly specific approach was developed for the detection of ET in spiked human urine samples. To the best of our knowledge, there are few reports describing the application of coating half-IgG antibodies onto Fe_3O_4@Au nanomaterials that allowing the preconcentration of analyte from sample solution.

2. Experimental

2.1. Instrumentations

HPLC analysis was carried out on a Shimadzu Prominence LC-20A HPLC system (Kyoto, Japan) consisting of a LC-20AT quaternary solvent delivery unit, a SPD-M20A diode array detector, a SIL-20A auto sampler, and a DGU-$20A_5$ degasser. Data acquisition and processing was accomplished with Shimadzu LC solution software (Version 1.23). HPLC separations were performed at room temperature using a Gemini C_{18} reversed-phase column (250 mm × 4.6 mm i.d., 5 μm particle size, 110 Å pore diameter) from Phenomenex (Torrance, CA) with a C_{18} guard column (4 mm × 3.0 mm). Ultraviolet-visible study was recorded on a CARY 1E UV–vis spectrophotometer (Varian, Australia). Centrifugation was performed on an Avanti J-25 centrifuge (Beckman Coulter, Fullerton, CA, USA). Microcon YM-100 centrifugal filter units were purchased from Millipore (Billerica, MA). Dialysis bag was provided by Huamei Biochemicals (Beijing, China). Nd–Fe–B strong magnet was 29 mm in diameter and 6 mm thickness.

2.2. Reagents and materials

Epitestosterone, ovalbumin (OVA) and Amberlite XAD-2 resin were purchased from Sigma–Aldrich. Ferric ammonium sulfate dodecahydrate ($FeNH_4(SO_4)_2{\cdot}12H_2O$), ferrous ammonium sulfate hexahydrate ($Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$), and chloroauric acid tetrahydrate ($HAuCl_4{\cdot}4H_2O$) were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Triton X-100 was the product of Beijing Chemical Reagents Company (Beijing, China). 2-Aminoethanthiol hydrochloride (98%) was obtained from Acros Organics. Mouse monoclonal antibody (anti-ET) was prepared and purified from ascites collected from immunized mice (Monoclonal Laboratories, College of Life Science, Peking University, China). Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG, skimmed milk powder, and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Zhongshan Goldenbridge Biotechnology Co., Ltd. (Beijing, China), Inner Mongolia Yili Industrial Group Co., Ltd. (Hohhot, China), and Huamei Biochemicals (Beijing, China), respectively. HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Pure water obtained from a Milli-Q water purification system (Millipore, Molsheim, France) was used throughout the study (unless otherwise stated). Other reagents were of analytical grade and were used without further purification. All solutions used in HPLC were filtered through a 0.22 μm filter.

2.3. Buffers and stock solutions

The 0.01 M PBS buffer (pH 7.4) was prepared by dissolving 8.0 g of NaCl, 2.9 g of $Na_2HPO_4{\cdot}12H_2O$, 0.2 g of KH_2PO_4, and 0.2 g of KCl to 1 L of deionized water. Stock solutions of ET were prepared in methanol and stored at −20 °C.

Samples of 80 mL human urine were collected from a healthy male volunteer (27 years old) and treated with 20 g of Amberlite XAD-2 resin to remove steroid to obtain blank urine sample [28,30].

Stock solution of Fe^{2+} and Fe^{3+} was prepared by dissolving $FeNH_4(SO_4)_2{\cdot}12H_2O$ and $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ in 100 mL of 0.4 M aqueous sulfuric acid solution at a molar ratio of 2:1.

2.4. Fe_3O_4@Au magnetic nanoparticles

2.4.1. Synthesis of Fe_3O_4 nanoparticles

The Fe_3O_4 nanoparticles were synthesized by chemical coprecipitation method according to previous reported method with some modifications [36]. In a three-neck round bottom flask, 250 mL of 1.0 M NaOH solution was mixed with Triton X-100 solution (0.1 M) to the final concentration of 0.01 M. Then, the solution was heated to 85 °C in a water bath. At that temperature, 25 mL of stock solution of Fe^{2+} and Fe^{3+} was added dropwise with vigorous stirring using a non-magnetic stirrer under nitrogen protection. The mixture was stirred for 30 min after the addition of iron stock solution. The obtained black Fe_3O_4 nanoparticles were rinsed several times with deionized water until a neutral pH was reached. Finally, the Fe_3O_4 nanoparticles were rinsed three times with ethanol and dried in a vacuum oven at 70 °C for 3 h. The size of the Fe_3O_4 nanoparticles was confirmed to be about 13 nm by transmission electron microscopy (TEM).

2.4.2. Synthesis of Fe_3O_4@Au nanoparticles

The molar ratio of Fe_3O_4 to $HAuCl_4$ of 2:1 was added into a 100-mL erlenmeyer flask. The solution was heated to 85 °C in a water bath after being sonicated for 15 min. Then, 0.5 g of glucose was added drop by drop with gentle stirring. The mixture was stirred for 1 h after the addition of glucose. As the Fe_3O_4 nanoparticles were gradually coated by Au, the color of the solution was changed from black to reddish brown. The solution was cooled to room temperature and centrifuged to obtain Fe_3O_4@Au nanoparticles which were then rinsed several times with deionized water until a neutral pH was reached. Finally, the Fe_3O_4@Au nanoparticles were rinsed three times with ethanol and dried in a vacuum oven at 70 °C overnight. The size of the Fe_3O_4@Au nanoparticles was found to be about 50 nm by TEM.

2.5. Half-IgG

2.5.1. Preparation of half-IgG

Monoclonal antibodies (anti-ET) were collected from mouse ascites and were further purified based on a modified method of octanoic acid-saturated ammonium sulfate [37]. The obtained antibodies were dialyzed against 0.01 M PBS at 4 °C for 24 h using a 14 kDa cutoff dialysis bag. In the process of antibody reduction, 2 mg of EDTA·$2H_2O$ was dissolved in 0.01 M PBS buffer containing 14 mg of antibodies to reach the final concentration of 5×10^{-3} mM. Then, 11 mg of 2-aminoethanthiol was added to the antibody solution and incubated at 37 °C for 90 min. The reaction mixture was dialyzed against 0.01 M PBS overnight at 4 °C in a 14 kDa cutoff dialysis bag. Finally, unreacted intact-IgG were filtered using Microcon YM-100. The molecular weights of intact-IgG and half-IgG were determined by MALDI-TOF mass spectrometry.

2.5.2. Half-IgG immobilization on MNPs

Fe_3O_4@Au nanoparticles were added to 0.01 M PBS solution containing sufficient half-IgG antibodies. The mixture was gently stirred to disperse nanoparticles and left at 4 °C overnight. In this step, half-IgG antibodies were coated on Au surface provided by Fe_3O_4@Au nanoparticles through Au–S bonds. After the reaction was completed, a magnet was applied to separate MNPs from solution. The concentration of half-IgG in the solution was measured by a UV–vis spectrometer. The results confirmed that approximately 1 mg of half-IgG antibodies were immobilized on 10 mg of Fe_3O_4@Au nanoparticles, which was believed that Fe_3O_4@Au nanoparticles should have a better antibody immobilization efficiency than CNBr-activated Sepharose 4B [38].

2.5.3. Pseudo-homogeneous immunoextraction based on antibody-conjugated Fe_3O_4@Au MNPs

The general operation of immunoextraction method for the measurement of ET was described in Fig. 1, which was based on ET-half-IgG antibodies coated Fe_3O_4@Au nanoparticles. When these nanoparticles were incubated with sample solution at room temperature, antigen (ET) would be extracted while interferent would not be retained. The interaction between the antibody and the antigen was the principle of the immunoextraction. The antigens were captured by the MNPs if the antigen–antibody complex was formed. The process was considered to be a pseudo-homogeneous immunoextraction because the MNPs could be well dispersed in the sample solution. The MNPs were readily isolated from the sample solution by applying an external magnetic field on the side wall. Then, the supernatant liquid was entirely discarded and the collected MNPs were rinsed three times with water. After rinsing, the MNPs were eluted three times with methanol. The methanol solution containing analytes (ET) was evaporated to dryness under a gentle nitrogen stream at room temperature. Finally, analytes were redissolved in the mobile phase prior to HPLC analysis.

3. Results and discussion

3.1. Characterization of Fe_3O_4 and Fe_3O_4@Au nanoparticles

The particle size of Fe_3O_4 magnetic particles was 13 ± 1 nm measured by TEM (see Fig. S1A in the Supplementary Data for more details). These magnetic particles were well dispersed due to the addition of Triton X-100 in the synthesis of Fe_3O_4. The particle size of Fe_3O_4@Au nanoparticles was 50 ± 5 nm (Fig. S1B). The formation of large Fe_3O_4@Au nanoparticles might result from the aggregation of small Fe_3O_4@Au nanoparticles. The obtained Fe_3O_4@Au

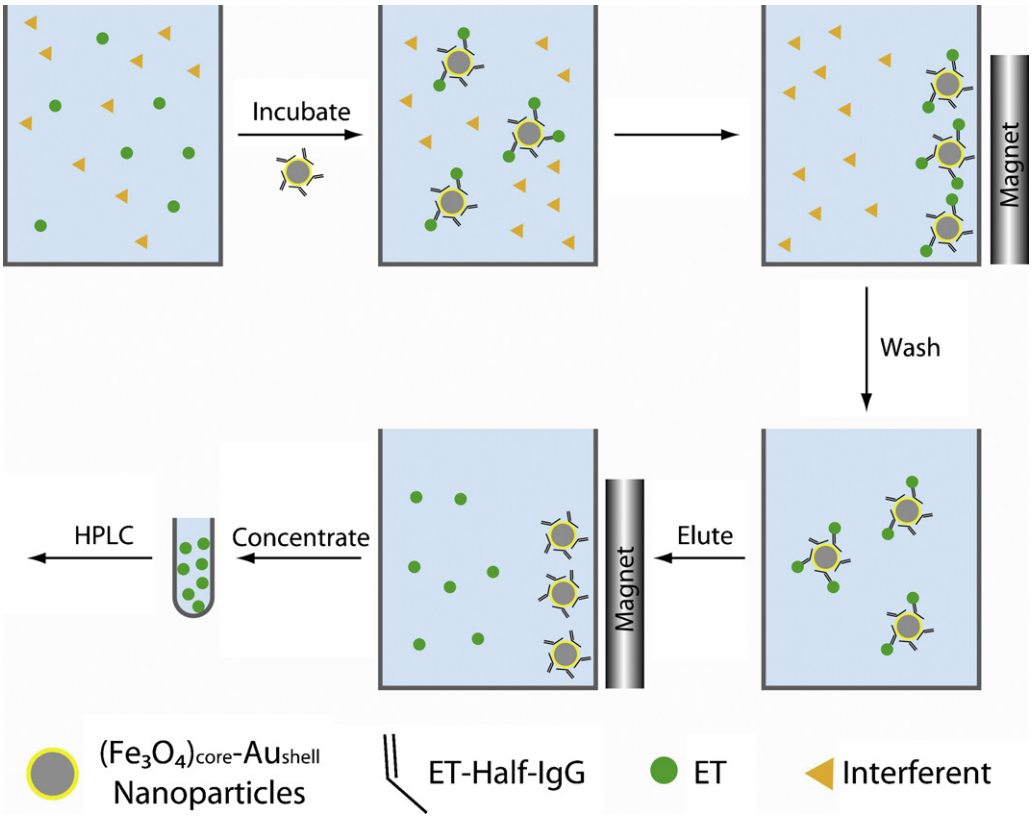

Fig. 1. Schematic description of pseudo-homogeneous immunoextraction method for ET measurement using ET-half-IgG coated Fe_3O_4@Au nanoparticles.

MNPs in this size were easily isolated from sample solution, which proved that these nanoparticles could be successfully employed as a material for rapid sample pretreatment. The unreacted Fe_3O_4 nanoparticles left in Fe_3O_4@Au nanoparticles could not affect the immobilization of half-IgG antibodies on gold because half-IgG antibodies could not attach on Fe_3O_4 nanoparticles through physical adsorption. Moreover, Fe_3O_4 and Fe_3O_4@Au nanoparticles were analyzed by Fourier transform infrared (FTIR) (see Fig. S2). The spectrum showed that the stretching frequency for the Fe–O bond at 579 cm^{-1} shifted to 586 cm^{-1} when Fe_3O_4 nanoparticles were coated by gold. The new peaks at 636 cm^{-1} and 586 cm^{-1} indicated that the surface of Fe_3O_4 nanoparticles was successfully modified with gold.

3.2. Characterization of half-IgG antibodies

As was mentioned, the immobilization of antibody fragments on gold surface was carried out through disulfide bonds. The native disulfide bonds between the two heavy chains of intact-IgG were chosen to generate thiol groups by the reduction of 2-aminoethanthiol. Thus, the half-IgG fragments were obtained by splitting the intact-IgG biomolecules. The molecular weights of intact-IgG and half-IgG were determined by MALDI-TOF mass spectrometry, which revealed that the molecular weights of intact-IgG and half-IgG antibodies were 147 798 ± 443 and 73 8160 ± 221, respectively (Fig. S3). The generated half-IgG antibodies were immobilized onto gold surface through Au-S bonds without affecting the antigen-binding sites.

3.3. Application of the pseudo-homogeneous immunoextraction method to spiked human urine samples

As shown in Fig. 2, direct injection of the spiked urine sample showed that the ET peak was submerged in high background signals resulting from the interferents in urine. The inset of Fig. 2 showed that the determination of ET in the spiked urine sample by HPLC was difficult because no sample pretreatment procedure was involved. However, with the immunoextraction method, the ET peak could be obtained and measured by subtracting the background signals. Liquid chromatography-electrospray ionization-mass spectrometry (LC–ESI-MS) was applied to identify ET in the solution of Fig. 2b (Fig. S4). The absence of the ET peak in Fig. 2c showed that without the immobilization of antibody on Fe_3O_4@Au MNPs, bare Fe_3O_4@Au MNPs had no enrichment efficiency and no non-specific adsorption of ET was observed. The results demonstrated that antibody-conjugated Fe_3O_4@Au nanoparticles exhibited high specificity for enrichment of ET in spiked urine samples.

3.4. Standard calibration curve

Antibody-coated Fe_3O_4@Au nanoparticles were incubated with 1 mL of 20, 50, 80, 100, and 200 ng mL^{-1} ET standard solutions in PBS, respectively. The process was illustrated in Fig. 1. After being evaporated with N_2 to dryness, analytes were redissolved in 1 mL of mobile phase and then analyzed by HPLC. Each sample was analyzed in triplicate injections with 20 μL per injection. The standard calibration curve was obtained by plotting peak area (Y) against the corresponding concentration of ET (X):

$$Y = 53.2X + 837$$

The standard calibration curve showed good linearity ($R^2 = 0.995$) in the range of 20–200 ng mL^{-1} in PBS solutions with acceptable accuracy and precision results. Limit of detection (LOD) (S/N ≥ 3) was proved to be 6 ng mL^{-1} for ET (data not shown). Limit of quantification (LOQ) (S/N ≥ 10) was tested to be 20 ng mL^{-1}. Concentrations of ET in each spiked urine sample were calculated by comparing their peak area to the standard calibration curve. The recoveries of 50, 100, and 200 ng mL^{-1} blank urine samples spiked with ET ranged from 92% to 109% (Table 1), which were agreed with those obtained by indirect competitive ELISAs (see Supplementary Data for more details).

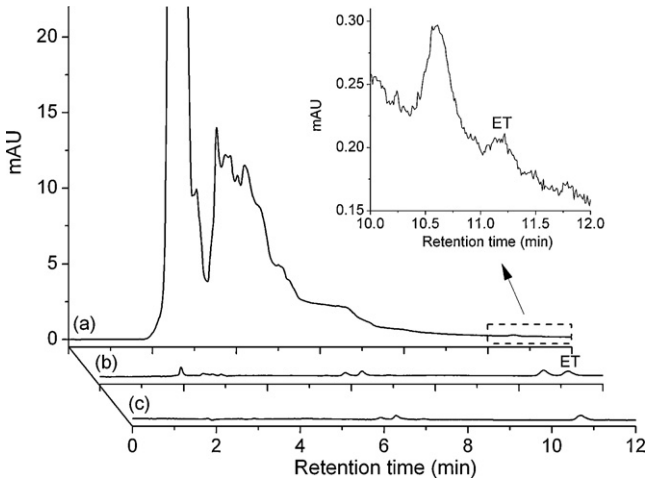

Fig. 2. HPLC separation of ET by (a) 100 ng mL^{-1} ET-spiked urine sample without the pretreatment of immunoextraction method, (b) 100 ng mL^{-1} ET-spiked urine sample with the pretreatment of immunoextraction method, and (c) 100 ng mL^{-1} ET-spiked urine sample using bare Fe_3O_4@Au MNPs. Chromatographic conditions: the mobile phase consisted of an acetonitrile/water (50/50, v/v) mixture acidified with glacial acetic acid to pH 4.1. The injection volumes were 20 μL, and the flow rate was maintained at 1.0 mL min^{-1} throughout the analysis. Retention time of ET was at approximately 11.16 min.

3.5. Analysis of ET in spiked human urine samples

The pseudo-homogeneous immunoextraction method was used to extract analytes from sample solutions at concentration level of less than 6 ng mL^{-1}. The results showed that 1, 0.5, and 0.2 ng mL^{-1} ET from 20 mL PBS solutions could be successfully extracted for HPLC analysis (Table 2). The recoveries ranged from 92% to 103% with RSD less than 6%. Applying the pseudo-homogeneous immunoextraction process, the limit of detection of ET-spiked sample solutions can reach 100 times lower than that of HPLC method (Table 3). The obtained results indicated that the pseudo-homogeneous immunoextraction method might become a novel strategy for sample pretreatment if the concentration of analyte was low.

In addition, only 1 h was required for the pretreatment process of 20 mL of sample solution when using this pseudo-homogeneous

Table 1
Comparison of pseudo-homogeneous immunoextraction-HPLC with indirect competitive ELISAs in ET determination.

Original volume (mL)	Concentration (ng mL^{-1})	Final volume (mL)	Pseudo-homogeneous immunoextraction-HPLC			Indirect competitive ELISAs		
			Found (ng mL^{-1})	Recovery (%)	RSD (%, $n = 3$)	Found (ng mL^{-1})	Recovery (%)	RSD (%, $n = 3$)
1	50	1	49 ± 3	99	6	38 ± 9	76	24
1	100	1	92 ± 4	92	4	112 ± 22	112	19
1	200	1	218 ± 6	109	3	243 ± 56	122	23

Table 2
Applying pseudo-homogeneous immunoextraction-HPLC method to ET determination at low concentrations.

Original volume (mL)	Original concentration ($ng\,mL^{-1}$)	Final volume (mL)	Final concentration ($ng\,mL^{-1}$)	Found ($ng\,mL^{-1}$)	Recovery (%)	RSD (%, $n=3$)
20	0.2	0.2	20	19 ± 1	94	4
20	0.5	0.2	50	51 ± 2	103	5
20	1	0.2	100	92 ± 4	92	4

Table 3
Comparison of pseudo-homogeneous immunoextraction-HPLC with indirect competitive ELISAs and HPLC for LOD and LOQ determination.

	Pseudo-homogeneous immunoextraction-HPLC	HPLC	Indirect competitive ELISAs
LOD ($ng\,mL^{-1}$)	0.06	6	6
LOQ ($ng\,mL^{-1}$)	0.20	20	20

immunoextraction method. However, when using conventional immunoaffinity column (CNBr-activated Sepharose 4B) technique, it might take several hours to complete the immunoextraction process. Thus, the pseudo-homogeneous immunoextraction method based on antibody-coated Fe_3O_4@Au nanoparticles was less time-consuming than the conventional immunoaffinity column technique.

4. Conclusions

A novel, rapid and highly specific approach for sample pretreatment using a pseudo-homogeneous immunoextraction method was reported. The Fe_3O_4@Au nanoparticles functionalized with half-IgG monoclonal antibodies (anti-ET) were applied to the extraction and enrichment of ET in spiked human urine samples. The pseudo-homogeneous immunoextraction method was implemented as a model enrichment strategy for quantitative analysis of ET when coupled with HPLC. Compared with liquid–liquid extraction and solid-phase extraction technique, this immunoaffinity extraction method based on antibody-conjugated Fe_3O_4@Au nanoparticles showed significant advantages such as convenience, rapid separation, high specificity, and high enrichment factor. Additionally, the pseudo-homogeneous immunoextraction method offered an enrichment factor of 100-fold to fulfill the need for measuring an analyte that had a concentration 100 times lower than the limits of quantification. However, much greater enrichment factor (for instance, 1000-fold) may be achieved for extremely dilute sample solutions in large volumes, which may have the advantage over conventional immunoaffinity column technique in the near future.

Acknowledgments

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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.talanta.2010.01.021.

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