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Journal of Chromatography B, 816 (2005) 7–14
JOURNAL OF CHROMATOGRAPHY B
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Development and application of a multi-target immunoaffinity column for the selective extraction of natural estrogens from pregnant women's urine samples by capillary electrophoresis

Ping Su, Xin-Xiang Zhang*, Wen-Bao Chang

College of Chemistry, Peking University, Beijing 100871, China

Received 6 April 2004; accepted 12 July 2004
Available online 22 December 2004

Abstract

In this paper, a methodology for the determination of three naturally occurring estrogens (estradiol, estrone and estriol) in pregnant women's urine has been described. The procedure included immunoaffinity column (IAC) extraction of 4 mL of urine sample and subsequent analysis of the extraction by micellar electrokinetic chromatography (MEKC). A multi-target polyclonal antibody that has high affinity to three estrogens was produced. Then the IAC was developed by coupling polyclonal antibody to CNBr-activated Sepharose 4B. The IAC showed high affinity for these estrogens. Recoveries of three estrogens from human serum matrix were greater than 92% with R.S.D. less than 4.5%. The final elute of urine sample was diluted with running buffer and then quantitated with MEKC. The experimental results demonstrated that IAC was a useful technique for extraction and concentration of estrogens from biological samples. Three estrogens levels in six pregnant women's urine were measured by both the present method and enzyme-linked immunoadsorbent assay (ELISA). The results of this method have been found to correlate well with those of ELISA.

Keywords: Estrogens; Polyclonal antibody; Immunoaffinity column

1. Introduction

Estrogens play important roles at different stages of mammalian development including prenatal development, growth, reproduction and sexual behaviors. The most potent naturally occurring estrogen is estradiol (E_2), which is interconvertable with the less potent compound, estrone (E_1). Both of these estrogens can be metabolized to estriol (E_3), which also has limited estrogenic activity. During pregnancy, estrogens are synthesized in large amounts by placenta instead of ovaries. The E_3 concentration secreted by placenta is known to gradually increase to a maximum concentration of approximately 1 mg/day as normal pregnancy progresses [1]. Urinary E_2 and E_1 concentrations are typically low relative to E_3 and fluctuate during gestation. Interest in the measurement of estrogens in serum, saliva, urine and other biological fluids has intensified as a result of the numerous potential clinical applications.

Various immunological techniques such as radioimmunoassay (RIA) [2], enzyme immunoassay (EIA) [3], fluorescence immunoassay (FIA) [4] and chemiluminescent immunoassay (CLIA) [5] have been widely used in estrogen screening and determination. In these immunology-related methods, only one target estrogen can be determined with high-specificity antibodies. On the other hand, different chromatographic methods, such as gas chromatography (GC), HPLC and capillary electrophoresis (CE), with different detectors have been reported for simultaneous determination of various estrogens [6–8]. Pretreatment procedure based on solid-phase extraction (SPE) or liquid–liquid extraction (LLE) is often required when the targeted estrogens are at trace levels and interferences at higher concentrations. Because the selectivity of valid immunoassay is generally better than that of chromatographic procedures, recently some

* Corresponding author. Tel.: +86 10 6275 4174; fax: +86 10 6275 1708.
E-mail address: zxx@pku.edu.cn (X.-X. Zhang).

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doi:10.1016/j.jchromb.2004.07.009

immunology-based pretreatment techniques for biological samples have been introduced [9–11].

The aim of this study is to develop a new simple pretreatment method based on an immunoaffinity column (IAC). Due to the high selectivity of the immunoaffinity reaction, the IAC showed specific affinity for the three naturally occurring estrogens. The final elute of urine sample is diluted with running buffer and then quantitated with MEKC. The IAC proved to be a simple, practical and reliable sample pretreatment method for biological samples.

2. Experimental

2.1. Instruments

Capillary electrophoresis system of the P/ACE 5000 series, GS-15R multi-purpose refrigerate centrifuge and DU-600 spectrophotometer were obtained from Beckman Instruments (Fullerton, CA, USA). The experiment of ELISA was carried out on GENios Microplate Reader (TECAN Co., Austria). HZQ-F incubator shaker was supplied by Harbin Donglian Electronic Technology Development (China).

2.2. Material and reagents

E_2, E_1, E_3, E_2-17-HS, 3-3′-5-5′-tetramethylbenzydine (TMB), *N*-hydroxysuccinimide (NHS), progesterone, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide·HCl (EDC), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIC), sodium cholate, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA) and ovalbumin (OVA) were obtained from Sigma (St. Louis, MO, USA). CNBr-activated Sepharose 4B was supplied by Pharmacia Biotech (Uppsala, Sweden). Horseradish peroxidase (HRP)-conjugated goat anti-rabbits IgG and HRP-conjugated goat anti-mouse IgG were supplied by Huamei Biochemicals (Beijing, China). Steroid-free serum was obtained from Fureite Biochemicals (Beijing, China). Ultrafiltration centrifuge tube was purchased from Millipore (Bedford, MA, USA). The microtiter plates (Nunc Co., Denmark) with all total volumes of 0.36 mL were used in ELISA. Urine samples were collected from Beijing Maternity Hospital (Beijing, China). The antigens (E_2-BSA and E_2-OVA) were synthesized by coupling E_2 with BSA and OVA, respectively. The polyclonal antibodies were prepared and purified from serum collected from the immunized rabbits (Monoclonal Laboratories, Department of Biology, Peking University, China).

Stock solutions of E_2, E_1 and E_3 (2.0 mg/mL) for separation study were prepared by dissolving 4.0 mg of the compounds in 2.0 mL of methanol. The working solutions were made freshly before use by diluting the stock solutions with 0.01 mol/L phosphate-buffered saline (PBS).

Phosphate-buffered saline, PBS (136 mmol/L NaCl, 2.7 mmol/L KCl, 6.5 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4, pH 7.4), 0.1 mol/L HAc–NaAc buffer (containing 0.5 mol/L NaCl, pH 4.0), 0.1 mol/L Tris–HCl, 0.1 mol/L Tris–HCl (containing 0.5 mol/L NaCl, pH 8.0), 0.1 mol/L sodium carbonate solution (containing 0.5 mol/L NaCl, pH 8.3, coupling buffer) 0.1 mol/L phosphate-buffered saline–Tween 20, PBST (0.1% Tween, washing solution, pH 7.4), 0.05 mol/L sodium carbonate solution (pH 9.6) and other solutions were prepared by dissolving the reagents with distilled water.

2.3. Separation conditions for MEKC

A 47 cm × 50 μm i.d. fused-silica capillary (Yongnian Optical Fiber Factory, Hebei, China) was utilized with an effective length of 40 cm, and the temperature was maintained at 20 °C. The applied voltage was 17 kV and sample injection was at 50 mbar for 10 s. Peaks were detected by UV absorption at 200 nm.

The running buffer solution: 10 mmol/L sodium borate (pH 9.2) containing 100 mmol/L sodium cholate and 10 mmol/L sodium phosphate (pH 7.0) containing 50 mmol/L SDS and 20% methanol were filtered through a 0.22 μm membrane and degassed by ultrasonication for approximately 10 min before use. The capillary was conditioned daily by washing first with 0.2 mol/L NaOH (10 min), then with water (5 min) and finally with the running buffer (5 min). Between consecutive analysis, the capillary was flushed with 0.2 mol/L NaOH (2 min), then with water (1 min) and finally with the running buffer (2 min) in order to improve the migration time and peak-shape reproducibility.

2.4. Preparation of the complete antigen

NHS and EDC were used to activate the E_2-17-HS. The molar ratios of E_2-17-HS, NHS and EDC were 1:1.25:1.25. Typically, 6.6 mg of E_2-17-HS, 3.5 mg NHS and 5.9 mg EDC. The conjugate of E_2-17-HS and BSA was prepared according to a procedure reported previously by Zhao's work in our group [12]. The molar ratio of E_2-BSA conjugate was measured using a Coomassie Brilliant Blue spectrophotometric method [13]. The E_2-OVA conjugate was prepared in the same way as the E_2-BSA. The synthesized antigens were stored at −20 °C.

2.5. Production and purification of the polyclonal antibody

Three rabbits were immunized by subcutaneous injections according to a standard protocol consisting of a first injection of 1.0 mL containing approximately 1.0 mg E_2-BSA complex emulsified in complete Freund adjuvant followed by injection at 12-day intervals of the half quantity of immunogen emulsified in incomplete Freund adjuvant. Blood samples were taken 7 days after the final injection and tested by ELISA.

The obtained antibody was purified according to a modified caprylic acid-saturated ammonium sulfate method

[12,14]. The immunoglobulin (IgG) fraction concentration in the antibody solution was measured with UV–vis spectrophotometer and calculated based on the UV absorption difference between 260 and 280 nm [13]. The purified antibody was stored at −20 °C.

2.6. Titration-level assessment of antiserum and purified antibodies

The optimum concentration of E_2-OVA and the titers of antiserum and purified antibodies were determined by a chequerboard method [12,15]. In brief, a 96-well microtiter plate was coated with 100 μL of E_2-OVA at different concentrations in 50 mmol/L carbonate buffer (pH 9.6) overnight at 4 °C. Then the wells were washed three times with 320 μL of PBST and blocked using 200 μL of 0.8% gelatin in 0.01 mol/L PBS for 2 h at 37 °C. After washing three times with PBST, the wells were incubated with 100 μL of 10-fold serially diluted antiserum or purified antibodies for 1 h at 37 °C. To the washed wells 100 μL of 1:1000 diluted HRP-labeled goat anti-rabbit IgG was added and incubated for 1 h at 37 °C. After washing three times with PBST and then twice with water, the wells were filled with 100 μL of phosphate buffer containing 1 μL of 60 mg/L TMB and 0.15 μL of H_2O_2. After reaction for 10–15 min at room temperature in dark, 50 μL of 2 mol/L H_2SO_4 was added to stop the enzyme reaction and the absorbance of each well was measured by the Microplate Reader at 450 nm.

2.7. Competitive ELISA for specificity study of estrogens

The specificity of the polyclonal antibody was investigated by testing E_2, E_1, E_3 and progesterone. The microtiter plate was coated with 100 μL of 8.0 μg/mL E_2-OVA and blocked in the same way as described above. Standard solutions of known concentration (0–10 mg/mL, 50 μL per well) of one of the competitive compounds were added together with 50 μL of the $1{:}10^4$ diluted purified antibody solution and incubated for 1 h at 37 °C. The following steps were similar to the procedure described in Section 2.6.

2.8. Preparation of the immunoaffinity column

The IAC was prepared as follows [16,17]: 1 g of CNBr-activated Sepharose 4B was swelled and washed with 1 mmol/L HCl and then added to 5 mL of coupling buffer (0.1 mol/L $NaHCO_3$, 0.5 mol/L NaCl, pH 8.3) containing 28.8 mg of the polyclonal antibodies. The coupling reaction was performed in a stopped vessel and rotated end over end for 3 h at 20 °C. The slurry was poured into a column of 1 cm diameter and the excess ligand was washed out with 30 mL of coupling buffer. The remaining active groups were blocked with 0.1 mol/L Tris–HCl buffer (pH 8.0) for 2 h and then the gel was washed by repeating three times the cycles of 20 mL of 0.1 mol/L NaAc–HAc buffer (pH 4.0) containing 0.5 mol/L NaCl followed by 20 mL 0.1 mol/L Tris–HCl buffer (pH 8.0) containing 0.5 mol/L NaCl. Finally, the column was equilibrated with 0.01 mol/L PBS containing 0.02 % NaN_3 at 4 °C until use. The coupling efficiency was estimated by comparing the protein content of the solution collected after the coupling reaction and the original antibody solution.

2.9. Elution study

A preliminary study which we called the elution study was used to determine the volumes as well as the percentages of the methanol concentration needed in the elution step for the subsequent IAC procedure. An aliquot of 4 mL of estrogens sample solution, prepared in PBS, was loaded onto the IAC. The analytes were eluted from the IAC with methanol–water mixtures increasing methanol concentration (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%). Elutions were performed in 200 and 400 μL portions. All wash and elution eluates were collected separately and brought to a final volume of 400 μL with running buffer. These final solutions were then analyzed under the MEKC conditions as described in Section 2.3.

2.10. IAC procedure

Aliquots of human steroid-free serum were spiked with estrogen solutions to produce the desired concentrations. Two levels of sample concentrations were prepared. At the high level, the concentrations for E_1, E_2 and E_3 were 4.92, 5.08, 4.61 μg/mL, respectively. At the low level, the respective concentrations were 0.98, 1.02, 0.92 μg/mL. After loading 4.0 mL of serum sample solution onto the IAC, it was washed with 1.0 mL of 0.01 mol/L PBS and then the captured estrogens were eluted with 300 μL of 80% methanol. The final elutes were diluted with running buffer in a ratio of 3:1 (adding 100 μL of buffer to 300 μL of final eluate). The final diluted sample solutions were then analyzed by MEKC as described above.

2.11. Application to the urine samples

Urine samples obtained from normal pregnant women were frozen and stored until analysis. Before MEKC analysis, the urinary estrogen treatment was accomplished as follows: 4 mL of urine sample was centrifuged in ultrafiltration centrifuge tube at 4 °C with 10,000 rpm for 30 min. Then, the urine solution was loaded onto the IAC. The subsequent steps were similar to the procedure described in Section 2.10. The final diluted sample solutions were then analyzed by MEKC following the same procedure as described in Section 2.10.

3. Results and discussion

3.1. Preparation of the complete antigen

E_2 is a small hapten with a molecular weight of 272 Da. To increase its immunogenicity, E_2 was covalently attached

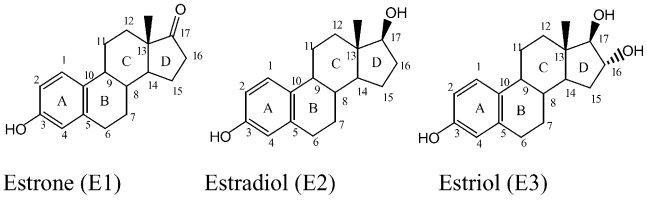

Fig. 1. Structure of estrone, estradiol and estriol.

to an antigenic carrier molecule such as BSA or OVA. Since E_2 does not have any usual conjugation groups such as carboxyl or amino groups, the E_2-17-HS was used to conjugate with BSA to form the complete antigen. In the preliminary study [18], the antigens was synthesized by activating the hydroxyl group on the A ring of E_2 and then coupling with the carrier protein. In such a conjugate, the characteristic D ring of the estrogen structure was exposed to the largest extent, resulting in a low selectivity for E_1 and E_3 (cross-reactivity lower than 5%). In our case, the E_2-17-HS was used to prepare the complete antigen due to its relatively long carbon chain with a reactive carboxyl group on the end. The conjugation took place between the amino group of BSA and the carboxyl group of E_2-17-HS, allowing the structure of interest to be favorably exposed (Fig. 1). The purpose of our study was to produce a kind of polyclonal antibody with specific selectivity towards three naturally occurring estrogens. The molar ratio of E_2-17-HS to BSA was found to be 25:1.

3.2. Specific selectivity of the polyclonal antibody

Fig. 2 shows the titration results for the antiserum. Titer is defined as the antiserum dilution required to bind 50% of a given amount of antigen. The titers of the antiserum and the purified antibody were found to be $1{:}10^6$ and $1{:}10^5$, respectively, and the optical concentration for E_2-OVA as the coating antigen was 8.0 μg/mL.

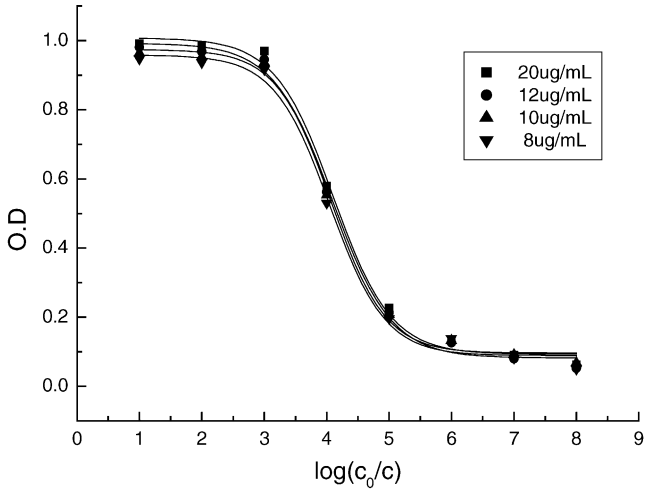

Fig. 2. Chequerboard titration results of the antiserum. The microtiter plate was coated with 100 μL of E_2-OVA at different concentrations (20.0, 12.0, 10.0 and 8.0 μg/mL) in 50 mmol/L (pH 9.5) caronate buffer.

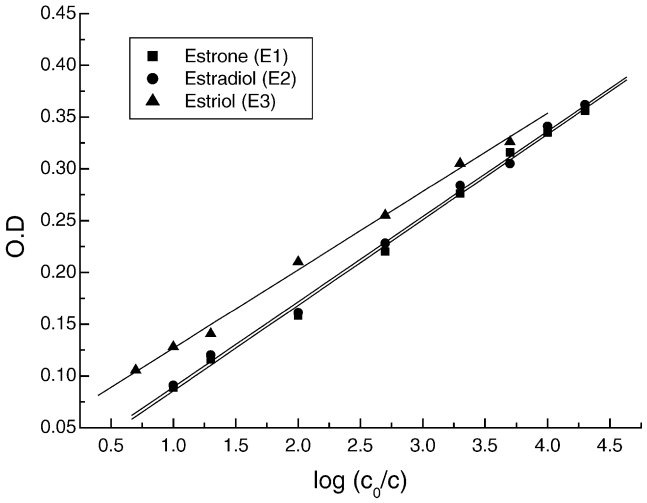

Fig. 3. The independent calibration curve of estrone, estradiol and estriol.

To investigate the specific selectivity of the polyclonal antibody, E_1, E_2, E_3 and progesterone were determined independently by ELISA. The results (see Fig. 3) indicated that the calibration curves of E_2 and E_1 almost overlap with each other, which means the selectivity of polyclonal antibody towards E_2 and E_1 was almost the same. The calibration curve of E_3 (see Fig. 3) showed that it was feasible to recognize E_3 with the polyclonal antibody. The logit–log algorithm was used to establish the linear regression [19]. The linear range and detection limit were deduced to be 1.0 ng/mL–10.0 μg/mL and 0.42 ng/mL for E_2, 1.0 ng/mL–10.0 μg/mL and 0.36 ng/mL for E_1, and 5.0 ng/mL–20.0 μg/mL and 2.21 ng/mL for E_3, respectively. According to 50% displacement method [20], the cross reactions of E_1 and E_3 with E_2 were 98.3, and 89.2%, respectively, while the cross reaction of progesterone was lower than 5%. The results confirmed that the polyclonal antibody could be applied in the recognition of the three naturally occurring estrogens simultaneously.

3.3. Optimization of the MEKC separation

The most potent estrogen in human is E_2, followed by E_1 and E_3. Each of these molecules contains a 17-carbon nucleus with a methyl group at C-13, and an aromatic ring with a hydroxyl group at C-3 (see Fig. 1). These estrogens are negatively charged in alkaline solution due to ionization of the phenolic hydroxyl group. CZE has been used to separate E_1, E_2 and E_3 but they were not separated with good resolution [9]. Micellar electrokinetic chromatography (MEKC) is a useful method for separating estrogens [7,21]. MEKC of estrogens using bile salt as micelles was investigated. Using a 10 mmol/L borate buffer (pH 9.2) containing 100 mmol/L sodium cholate, these three estrogens were well separated (Fig. 4A). MEKC of estrogens using SDS micelles was also investigated. In contrast to the bile salt, SDS micelles strongly retained the estrogens, resulting in poor separation. Addition

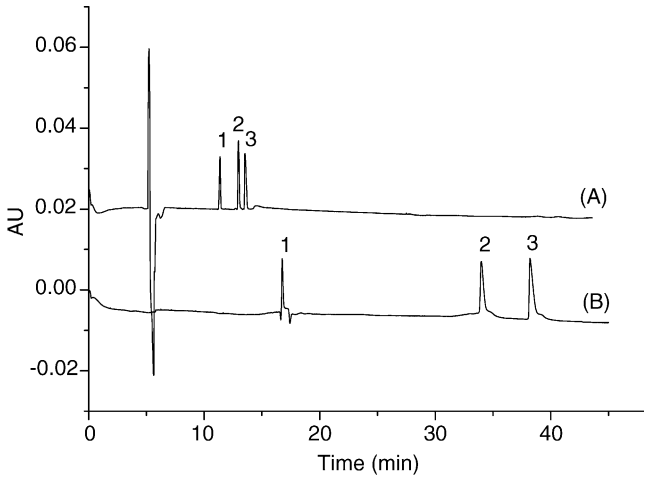

Fig. 4. Separations of three estrogens in different MEKC media: (1) estriol; (2) estradiol; (3) estrone; 10 mmol/L sodium borate (pH 9.2) containing 100 mmol/L sodium cholate; 10 mmol/L sodium phosphate (pH 7.0) containing 50 mmol/L SDS and 20% methanol. Separation conditions: applied voltage, 17 kV; column temperature, 20 °C.

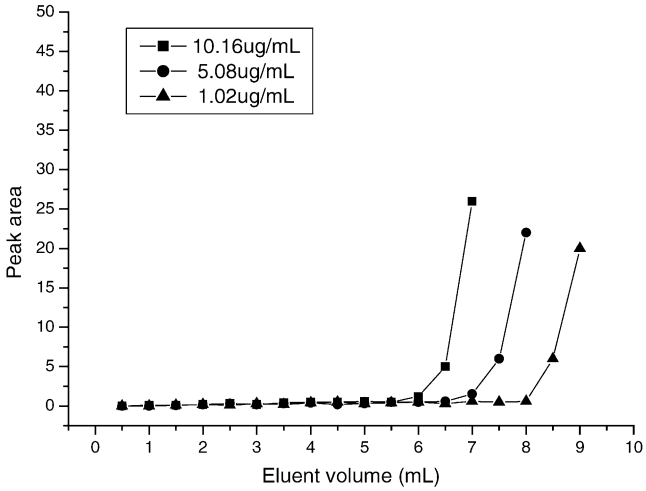

Fig. 5. Elution profiles for the continuous loading estradiol in 0.01 mol/L PBS.

of 20% methanol to the SDS buffer allowed for the separation of three estrogens (Fig. 4B). The results showed that the sodium cholate-modified buffer is more desirable because of fast analysis time (15 min versus 35 min). As a result, the 10 mmol/L borate buffer (pH 9.2) containing 100 mmol/L sodium cholate was chosen as the running buffer for the determination of estrogens.

3.4. Preparations and evaluation of the IAC

The amount of bound antibody was determined by measuring the concentration of the antibody before and after coupling reaction. The coupling efficiency of the polyclonal antibody to the CNBr-Sepharose 4B was observed to be 91.1 ± 2.5% (the mean of three coupling experiments), defined as the percentage of immobilized antibody accounted for of the original amount [22]. The coupling reactions were performed three times. One was generated from 0.6 g of freeze-dried power of CNBr-Sepharose 4B with a final gel volume of about 1.8 mL. The other two were generated from 1.0 g of freeze-dried power, with a final gel volume of about 3.5 mL.

To simplify the protocol for the determination of the volume percentage of methanol in the elution step, the analytes were prepared in a PBS solution instead of in a steroid-free serum matrix. The elution effects of the concentrations and volumes of methanol were studied in detail. The elution results for E_2 are shown in Table 1. Table 1A shows five consecutive elutions of 200 μL elution volume at different percentages of methanol (from 0 to 100%) in order to obtain 100% recovery of E_2 for five consecutive elutions, >50% of methanol was needed. For the first elution (elute 1), >90% of E_2 comes out at a methanol concentration ≥80%. A similar elution pattern was observed when the elution volume increased from 200 to 400 μL. Table 1B shows three consecutive elutions of 400 μL elution volume at different percentages of methanol. Similar results were acquired for E_1 and E_3. Based on these results, 80% methanol at 300 μL was chosen as the elution solvent.

The breakthrough volumes of E_2 at 10.16, 5.08 and 1.02 μg/mL were measured and the results are shown in Fig. 5. It can be seen from Fig. 5 that, at a concentration of

Table 1
Recovery of estradiol in fractions from different concentration of methanol as elute solutions

Elution fraction (number)	Percentage concentration of methanol (%)										
	0	10	20	30	40	50	60	70	80	90	100
(A) The volume of each fraction was as 200 μL											
1	0.1	2	5	15	41	65	79	88	96	93	92
2	0	0.5	3	8	15	18	12	9	2	4	5
3	0	0.2	2	5	8	10	6	1	1	2	2
4	0	0.1	1	2	5	3	2	1	0.2	1	0.2
5	0	0	0.2	1	2	2	1	0.1	0.1	0.1	0.1
(B) The volume of each fraction was as 400 μL											
1	0.1	3	16	31	65	82	90	93	98	95	93
2	0	1	5	10	10	12	8	5	1.5	3	4
3	0	1	3	5	5	3	1	2	0.1	1	2

(A) Five consecutive fractions in 200 μL; (B) three consecutive fractions in 400 μL.

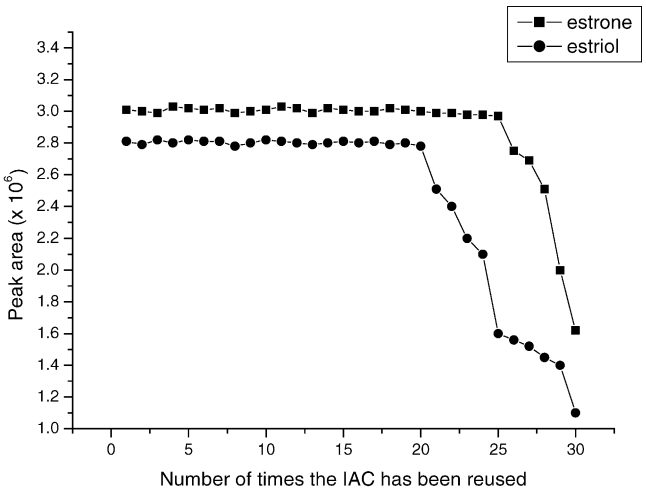

Fig. 6. Peak areas obtained for estriol and estrone from the relicate analysis (n = 30) using the same IAC of spiked steroid-free serum (the concentrations of estriol and estrone were 4.92 and 4.61 μg/mL).

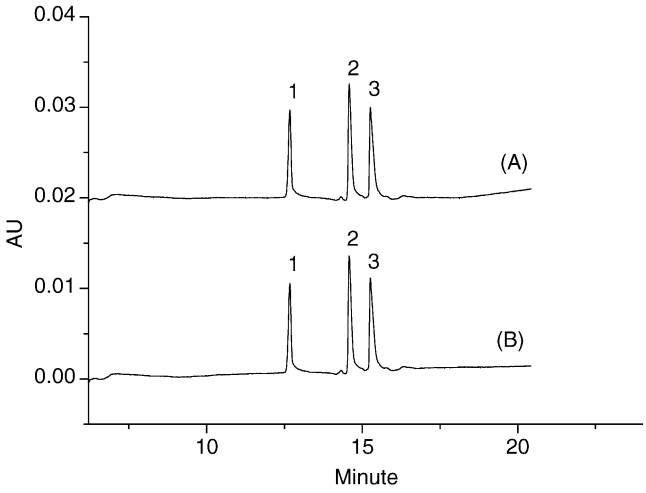

Fig. 7. Representative electropherograms of spiked estrogens from two different matrices. (1) Estriol; (2) estradiol; (3) estrone. (A) Human steroid-free serum; (B) PBS. Separation conditions: running buffer, 10 mmol/L sodium borate containing 100 mmol/L sodium cholate at pH 9.2; others as in Fig. 3.

1.02 μg/mL, the breakthrough volume was about 1.5–2.5 mL larger than that at 10.16 μg/mL. According to the above results, the sample loading volume was set at 4.0 mL.

The generated IAC was a gravity-flow column with a flow rate of about 0.2–0.3 mL/min. It could be used multiple times (≥20 times) and the performance was stable for at least three months. If frequently use the IAC was simply stored in 0.01 mol/L PBS at 4 °C. For relatively long-term storage (over a week), it was stored in 0.01 mol/L PBS containing 0.02% NaN_3 at 4 °C Fig. 6 shows the peak areas obtained for E_3 and E_1, the analytes with the shortest and longest migration times, respectively, from the replicate analysis (n = 30) of 4 mL of spiked steroid-free serum using the IAC. As can be seen, based on the peak areas obtained, the IAC_3 could be reused up to 25 times in the analysis of the most apolar compounds, such as E_1. However, in case of more polar compounds, such as E_3, only 20 times was recommended because the retention capacity exhibited by the IAC decreased with every additional use. Therefore, the IAC should not be reused more than 20 times in any case.

3.5. Recovery of three estrogens

To demonstrate the application of the IAC method developed, two different matrices spiked with E_1, E_2 and E_3 were investigated. The results are summarized in Table 2. For the PBS matrix, the recoveries were all >95% with R.S.D. values <3.5%. For the steroid-free serum matrix, the recoveries were all >92.2% with R.S.D. values <4.5%. The recoveries from PBS matrix were consistent with those from serum matrix.

The representative chromatograms from PBS and steroid-free serum are depicted in Fig. 7A and B, respectively. All three peaks were well separated, and no interfering peaks were observed. The elution sequence was E_3 (peak 1), E_2 (peak 2) and E_1 (peak 3).

3.6. Applications to urine samples

The ultimate goal of this study was to develop a method for estrogen analysis of pregnant women's urine samples. There are a number of potential pitfalls associated with estrogen analysis of urine. A major problem with some currently available methods is the interference on estrogen determination from a significant number of other steroids present in urine, which can interfere with the determination of estrogens in some methods. The purification procedure described in Section 2.8 could eliminate the majority of these interferences. The advantage of this procedure lies in that a relatively

Table 2
Recoveries of three estrogens in two different matrices

Estrogens	Concentration (μg/mL)	PBS		Steroid-free serum	
		Recovery (%, n = 5)	R.S.D. (%, n = 5)	Recovery (%, n = 5)	R.S.D. (%, n = 5)
E_1	4.92	102.1	3.4	92.3	4.2
	0.98	98.4	2.7	103.1	3.6
E_2	5.08	97.3	3.1	102.6	3.9
	1.02	101.6	2.5	93.8	3.1
E_3	4.63	102.3	2.9	100.6	3.3
	0.92	96.2	2.1	94.8	2.8

Table 3
Analytical data corresponding to the analysis of estrogens in pregnant women's urine

Numbr	Weeks of gestation	Detection level[a] (mg/day)			Detection level[b] (mg/day)			R.S.D. (n = 5, %)	
		E_3	E_2	E_1	E_1	E_2	E_3	E_1[a]	E_1[b]
1	36	0.592	0.093	0.176	0.581	0.088	0.171	2.8	3.8
2	35	0.489	0.082	0.163	0.503	0.076	0.167	3.5	4.6
3	33	0.412	0.079	0.116	0.401	0.083	0.123	4.2	5.0
4	32	0.426	0.076	0.102	0.423	0.070	0.115	3.9	3.2
5	32	0.367	0.085	0.115	0.375	0.081	0.112	4.6	5.2
6	31	0.395	0.095	0.124	0.389	0.092	0.131	3.2	2.9
7	28	0.372	0.106	0.113	0.381	0.112	0.107	3.1	3.7
8	25	0.384	0.117	0.082	0.378	0.121	0.081	2.4	2.8

[a] Data by present method.
[b] Data by ELISA.

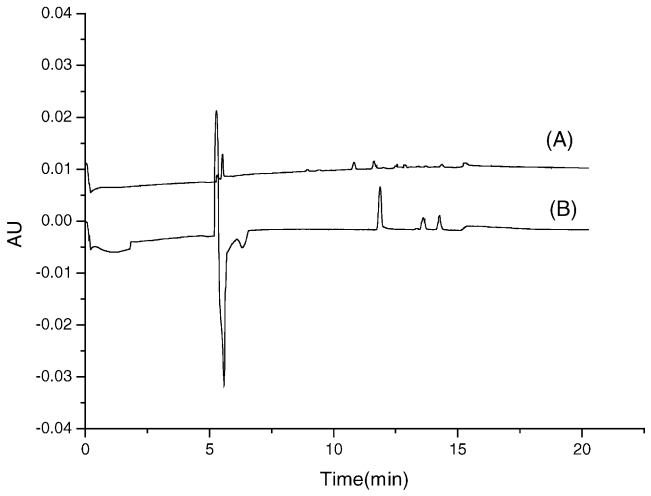

Fig. 8. Electropherograms of urine sample without pretreatment with IAC and urine sample pretreated with IAC. (1) Estriol; (2) estradiol; (3) estrone; (A) without pretreatment with IAC; (B) pretreated with IAC.

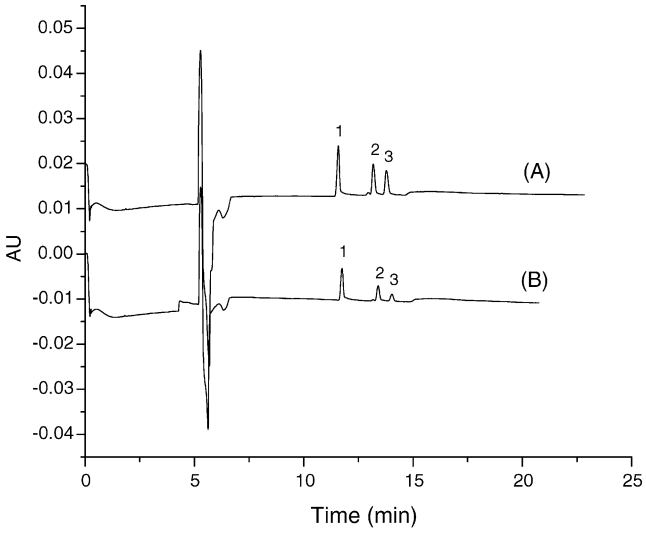

Fig. 9. Electropherograms of urinary estrogen separation. (1) Estriol; (2) estradiol; (3) estrone; (A) urine extract co-injected with the standard mixture of estrogens; (B) the same urine extract as that shown in (A) without co-injection with standards.

"clean" estrogen sample can be provided for MEKC analysis.

Since the complex matrix in urine samples would interfere with the analysis of the target compounds, different pretreatments and/or enrichment procedures were needed. Fig. 8 shows the results of separation of urine samples without and with IAC pretreatment. If the urine sample was analyzed by optimized MEKC without IAC pretreatment, no peaks could be identified and there were many unknown peaks in electropherogram (Fig. 8A). The developed IAC was specific to E_1, E_2 and E_3, which could be applied to eliminate complex matrix and enriching the targeted estrogens (Fig. 8B).

Fig. 9 shows the results of separation in 10 mmol/L borate buffer (pH 9.2) containing 100 mmol/L sodium cholate of women's urine sample and women's urine sample plus three authentic estrogens. The results show that E_3 is easily identified and clearly identifiable signals are obtained for E_1 and E_2. Therefore, our CE method can provide a rapid analysis with adequate resolution of the desired estrogens.

The optimized MEKC system was used to analyze the purified urine samples of a group of 25–36 weeks pregnant women (who were presumed to have detectable estrogens levels, especially E_3). The levels of three estrogens in urine samples could be simultaneously measured by the present method. The results of the method developed have been found to correlate well with those of ELISA in the analysis of real samples(Table 3). It is showed that the developed method is satisfactory to be applied in the clinical analysis with relative standard deviations (R.S.D.) ranging from 2.8 to 5.2% for all urine samples.

4. Conclusions

The off-line IAC-MEKC presented here allows for simultaneous analysis of the targeted naturally occurring estrogens in pregnant women's urine. Our IAC approach involves fewer operating steps and consumes much smaller volumes of organic solvents (300 μL). It presents a simple and practical method for the isolation and purification of estrogens from urine samples.

The determination of estrogens in serum or saliva samples requires either a different purification/concentration protocol

or a new detection technique more sensitive than absorption, which is currently under investigation in our laboratory.

Acknowledgement

The authors thank the financial support from the National Natural Science Foundation of China (Grant No. 20075003, 20375001).

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