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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

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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

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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