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Short communication

Determination of urinary 15α-hydroxyestrogen levels via immunoaffinity extraction

Madoka Nakagomi*, Emako Suzuki

Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 257-8523, Japan

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ABSTRACT

15α-Hydroxyestrogens (15α-OHEs) are metabolites of the female hormone estradiol. In this study, to discover physiological markers that can be utilized for monitoring fetal conditions and estrogen-induced cancers, we established a method for quantifying 15α-OHEs in rat urine via immunoaffinity column extraction and HPLC-electrochemical detection, and detected 15α-OHEs in urine obtained male rats treated with estradiol. Notably, the standard curves for quantification obtained using the column were linear over a range of 0.5–50 ng 15α-OHEs. The accuracy of the analytical method with cleanup was 97–109% for the three kinds of 15α-OHEs examined, and the intra-assay precision of the measured values had a coefficient of variation of ≤20.6%. Therefore, the theoretical limit of quantification was 0.5 ng. However, the actual measured values obtained from the urine of male rats indicated that the detection limits were 0.425, 0.103, and 0.047 ng for estetrol, 15α-hydroxyestradiol, and 15α-hydroxyestrone, respectively. Our newly established method for measuring 15α-OHE concentrations in urine could facilitate characterization of the *in vivo* metabolic profile of 15α-OHEs in mammals under various physiological conditions, which could comprise clinical markers for monitoring human fetal health conditions in mammals.

1. Introduction

15α-Hydroxyestrogens (15α-OHEs) (Fig. 1) are metabolites of the female hormone estradiol (estra-1,3,5(10)-triene-3,17β-diol: E_2). In a previous study, Heikkilä et al. [1] detected estriol (estra-1,3,5(10)-triene-3,16α,17β-triol: E_3), 15α-hydroxyestriol (estetrol, estra-1,3,5(10)-triene-3,15α,16α,17β-tetraol: E_4), and conjugates of these two compounds in the urine of pregnant women via gas chromatography (GC) analysis. Furthermore, this group showed that, in certain cases of toxemia, evaluation of E_4 levels was a more reliable method for monitoring intrauterine fetal physiology than evaluation of E_3 levels [1]. E_4 is considered a specific product of fetal metabolism and is generated from E_2, not E_3, in the fetal liver [2]. As such, E_4 is better physiological marker for fetal health than E_3. Notably, in Wistar rats, Maggs et al. [3] found that 15α-hydroxylation of exogenously administered E_2 was unique to males. Likewise, 15α-hydroxyestradiol (estra-1,3,5(10)-triene-3,15α,17β-triol: 15α-OHE_2) was detected in kidney microsomal preparations generated from estrogen-treated male golden Syrian hamsters, which are a well-known experimental model for estrogen-induced and estrogen-dependent cancers, but not in those generated from untreated male hamsters [4]. Moreover, this group suggested that 15α-OHE_2 could potentially serve as a biomarker for changes occurring in hamster kidney cells under continuous estrogen exposure [4]. However, there is little information regarding 15α-OHEs as E_2 metabolites, and the physiological functions of these compounds are unclear, as the primary metabolites of E_2 are 16α-, 2-, and 4-hydroxylate. Hence, it is important to evaluate the urinary levels of 15α-OHEs in mammals.

In previous work, Fotsis et al. [5,6] established a GC/mass spectrometry method for quantifying estrogen metabolites in the urine of women during the third trimester of pregnancy, and successfully utilized this approach to measure urine levels of 15α-OHE. Meanwhile, we previously prepared antisera specific to 15α-hydroxyestrone (estra-1,3,5(10)-triene-3,15α-diol-17-one: 15α-OHE_1), 15α-OHE_2, and E_4, respectively, and demonstrated the specificity of these antibodies using a novel enzyme immunoassay [7]. Additionally, we established an analytical method using an immunoaffinity column and HPLC-connected electrochemical detection (ECD) for quantifying the amounts of *N*-acetylcysteine conjugates of catechol estrogens in rat urine. Using this approach, we detected 4-hydroxyestrone 2-*N*-acetylcysteine thioether (*N*-Acetyl-*S*-[3,4-dihydroxy-17-oxo-1,3,5(10)-estratrien-2-yl]-L-cysteine) in the urine of male Syrian hamsters [8]. Additionally, we

Abbreviations: 15α-OHEs, 15α-hydroxyestrogens; E_1, estrone; E_2, estradiol; E_3, estriol; E_4, estetrol; 15α-OHE_1, 15α-hydroxyestrone; 15α-OHE_2, 15α-hydroxyestradiol

* Corresponding author. Present address: Dept. Research, Research Foundation ITSUU Laboratory, C1232, Kanagawa Science Park R & D building, 3-2-1 Sakado, Takatsu-ku, Kawasaki, Kanagawa 213-0012, Japan.
E-mail address: mnakagomi@itsuu.or.jp (M. Nakagomi).

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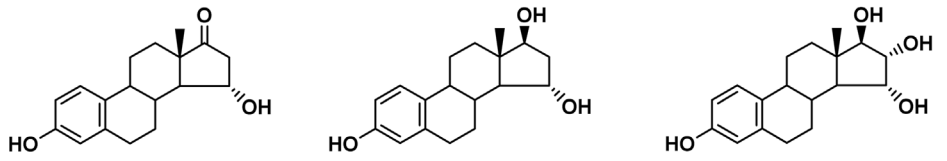

15α-hydroxyestrone (15α-OHE_1) 15α-hydroxyestradiol (15α-OHE_2) 15α-hydroxyestriol (15α-OHE_3: E_4)

Fig. 1. Chemical structures of 15α-hydroxyestrogens.

determined that the urine of male rats exhibited two- to 20-fold lower levels of 2-hydroxylate than that of females [8]. We therefore concluded that 15α-OHEs comprise the major estrogen metabolites of male rat urine and attempted to establish an analytical method for measuring the concentrations of these compounds in rat urine by applying the methodology and sensitivity of *N*-acetylcysteine conjugates of catechol estrogens.

In this study, considering the importance of quantifying the urinary levels of 15α-OHEs to discover physiological markers of fetal health conditions and estrogen-induced cancers, we continue our previous work by developing a novel 15α-OHE quantification method based on immunoaffinity and HPLC-ECD.

2. Material and methods

2.1. Chemicals

15α-Hydroxyestrone (15α-OHE_1), 15α-hydroxyestradiol (15α-OHE_2), and estetrol (E_4) were synthesized according to a previously described method [9]. Estradiol (E_2) was purchased from Sanofi (Paris, France). The anti-15α-OHE_1, anti-15α-OHE_2, and anti-E_4 antisera used herein were prepared in our previous study [7]. All other chemicals were commercial products of reagent grade.

2.2. Antibody immobilization

Antibody immobilization was performed as described in our previous paper [8]. Approximately 4.8 mL of anti-15α-OHE_2 antiserum (the cross-reactivity for 15α-OHE_1 and for E_4 were 3.4% and 2.1%, respectively) and anti-E_4 antiserum (the cross-reactivity for 15α-OHE_1 and for 15α-OHE_2 were 0.3% and 4.6%, respectively) were purified using a Protein A Sepharose® 4 Fast Flow column (GE Healthcare, Little Chalfont, UK), respectively. The resulting eluates were dialyzed against distilled water and then lyophilized, yielding 69.6 mg and 90.1 mg of IgG powder, respectively. The anti-15α-OHE_2 (50.9 mg) and anti-E_4 IgG (62.3 mg) powders were then dissolved in 10 mL of 50 mM phosphate buffer (pH 7.5), respectively, and mixed with 12 mL Affi-Gel® 10 (Bio-Rad, Hercules, CA, USA), which was pre-washed with 10 mM cold acetate buffer (pH 4.5), followed by cold deionized water. After shaking gently for 4 h at 4 °C, 1 M ethanolamine (1.25 mL, pH 8.0) was added to each mixture and agitation was continued for 1 h at ambient temperature (25 ± 2 °C). Finally, each IgG-coupled gel was washed with deionized water. The immunoaffinity column (0.3 mL) for cleanup was prepared as follows: the IgG-coupled gels were mixed at a 1:1 (v:v) ratio and added to a polypropylene column filled with 50 mM phosphate buffer (pH 7.3) containing 0.2% NaN_3, and stored at 4 °C.

2.3. HPLC analysis

The HPLC system was connected to an ECD device (Coulochem 2 Model 5011; ESA, Inc., Chelmsford, MA, USA) set at +0.65 V for detection of 15α-OHEs. Meanwhile, an Inertsil ODS-3 column (4.6 mm inner diameter × 150 mm; GL Science Co., Inc., Tokyo, Japan) was used for HPLC analysis. HPLC was carried out at 40 °C, and the solvent system was comprised of 0.5% $NH_4H_2PO_4$ (pH 3.5)/CH_3CN (83:17 or 4:1, v/v).

2.4. Cleanup of urine samples for measuring 15α-OHE levels

Rat urine (1 mL) was filtered through a disposable disk filter (HLC-DISK ™ 25, pore length: 0.45 μm; Kanto Chemical Co., Inc., Tokyo, Japan) and diluted with 4 mL of 50 mM phosphate buffer (pH 7.3) to adjust the pH to neutral. The diluted urine was applied to the column (gel volume: 0.3 mL, see *Immobilization of antibody* section), which was pre-equilibrated with 50 mM phosphate buffer (pH 7.3). After washing the column with deionized water (2 mL), the desired fractions were eluted with 95% methanol (2.5 mL), and the collected eluates was evaporated under reduced pressure at 37 °C. The resulting residues were then re-dissolved in the mobile phase (100 μL), and 50 μL of each sample was subjected to HPLC at a flow rate of 1.0 mL/min. The standard curves for 15α-OHE_1, 15α-OHE_2, and E_4 were prepared over a range of 0.5–50 ng; known amounts of 15α-OHE_1, 15α-OHE_2, and E_4 (0.5, 1, 2, 5, 10, 25, and 50 ng each) were added to 50 mM phosphate buffer (pH 7.3), and cleanup of the mixtures was performed according to the method described above.

3. Results and discussion

3.1. Investigation of the affinity and capacity of the immunoaffinity column

The performance of the immunoaffinity column for cleanup of bodily fluids was affected by the cross-reactivity and titer of the antisera used for preparation of the immunoaffinity gel. To optimize the specificity of the cleanup column, we screened a range of immunoaffinity gel volumes and antisera titers. Initially, due to the low titer of the anti-15α-OHE_1 antiserum, this antibody was immobilized on the gel at a concentration of 30 mg IgG/1 mL gel. Notably, however, this anti-15α-OHE_1 gel bound several as yet unidentified compounds, as indicated by several non-specific peaks on the HPLC chromatogram. As such, this gel could not be used for cleanup. Hence, in this study, antibody immobilization was carried out using a few mg of IgG per 1 mL of gel. The anti-15α-OHE_2 and anti-E_4 gels were subsequently mixed together at a 1:1 ratio (0.15:0.15 mL), and the capacity of the mixed gel was investigated. Our findings indicate that the column was capable of binding up to 50 ng each of 15α-OHE_1, 15α-OHE_2, and E_4 (Fig. 2). Moreover, the standard curves for 15α-OHE_1, 15α-OHE_2, and E_4 showed good linearity ($R \geq 0.999$) at a range of 0.5–50 ng.

3.2. Accuracy, precision, and recovery efficiency of the HPLC-ECD method

The accuracy and intra-assay precision of our newly established analytical method were tested by analyzing the recovery percentages, with coefficients of variation (CV), of 15α-OHEs. Five known amounts of 15α-OHEs (0.5, 2, 5, 10 and 50 ng; five samples per concentration for each compound) were added to 50 mM phosphate buffer (pH 7.3), respectively. The amounts of 15α-OHEs in each sample were determined by HPLC analysis after the cleanup procedure, according to our established method, and were calculated using the corresponding standard