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STUDIES ON STEROIDS

CLXV. DETERMINATION OF ISOMERIC CATECHOL ESTROGENS IN PREGNANCY URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A method for the determination of 2- and 4-hydroxylated estrone and estradiol in pregnancy urine by high-performance liquid chromatography with electrochemical detection (HPLC—ECD) is described. The urine catechol estrogens were deconjugated, purified by adsorption on alumina, and subjected to HPLC—ECD. Two pairs of isomeric catechol estrogens were distinctly separated on a μBondapak C_{18} column with acetonitrile—0.5% ammonium dihydrogen phosphate (pH 3.0). The amounts of these four compounds were satisfactorily determined with a quantitation limit of 1 ng using 4-hydroxy-16-oxoestradiol 17-acetate as an internal standard. The validity of the present method for the determination of urine catechol estrogens was verified by the recovery test.

INTRODUCTION

Recently considerable attention has been directed to the biological function of catechol estrogens in living animals [1—3]. In a previous paper of this series we reported the separation of catechol estrogens and their related compounds by means of high-performance liquid chromatography (HPLC) [4]. The present paper deals with the separation and determination of 2- and 4-hydroxylated estrone and estradiol in pregnancy urine by HPLC with electrochemical detection (ECD).

MATERIALS AND METHODS

Reagents

2-Hydroxyestrone, 4-hydroxyestrone, 2-hydroxyestradiol and 4-hydroxy-

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estradiol were prepared by the known methods. 4-Hydroxy-16-oxoestradiol 17-acetate (internal standard; m.p. 292°C) was synthesized from 16-oxoestradiol 17-acetate by oxidation with Fremy's salt. Acid-washed alumina 150 (Type T; E. Merck, Darmstadt, G.F.R.) was used as an adsorbent for clean-up of catechol estrogens. All reagents and chemicals were of analytical-reagent grade. Solvents were purified by distillation prior to use.

Sample collection

The pregnancy (16—39 weeks) urine specimens were kindly supplied by Dr. K. Muraguchi, School of Medicine, Tohoku University.

High-performance liquid chromatography

The apparatus used was a Waters Model ALC/GPC 202 high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.), equipped with a Yanagimoto Model VMD-101 electrochemical detector (Yanagimoto Co., Kyoto, Japan). The potential of the detector was set at +0.8 V vs. the Ag/AgCl reference electrode. The samples were introduced by means of a Model U6K sample loop injector (Waters Assoc.) with an effective volume of 2 ml. A μBondapak C_{18} (8—10 μm) column (30 cm × 3.9 mm I.D.) (Waters Assoc.) was used under ambient conditions. Acetonitrile—0.5% $NH_4H_2PO_4$ (pH 3.0) (1:2.1) was employed as a mobile phase at a flow-rate of 1 ml/min.

Assay procedure

The hot acid hydrolysis of pregnancy urine (0.2 ml) was carried out according to the procedure described by Gelbke et al. [5]. To the hydrolyzed urine sample was added a known amount of internal standard (ca. 100 ng), and the solution was extracted three times with 2 ml each of ethyl acetate. The organic layers were combined, washed successively each three times with 0.5 ml each of 0.05 *M* Tris·HCl buffer (pH 8.5) and water and then evaporated down under reduced pressure at room temperature. The residue was dissolved in 0.05 *M* Tris·HCl buffer (pH 8.5) (0.1 ml) and stirred with acid-washed alumina (500 mg) at pH 8.5 under ice-cooling for 10 min. After washing five times with 3 ml each of chilled 0.05 *M* Tris·HCl buffer (pH 8.5), the desired catechol estrogen fraction was obtained by elution with 1 *N* HCl. The effluent was extracted with ethyl acetate (10 ml), and the organic layer was washed with 0.5 ml of 0.05 *M* Tris·HCl buffer (pH 8.5), three times with 0.5 ml each of water and then evaporated down under reduced pressure. The residue was dissolved in methanol (0.1 ml), an aliquot of which was injected into the high-performance liquid chromatography.

Gas chromatography—mass spectrometry

A JEOL JGC-20K (GC)-JMS-01SG-2 (MS) combined instrument (JEOL, Tokyo, Japan) equipped with a JMA-2000 data-processing system was used. The coiled glass column (1 m × 0.3 cm I.D.) was packed with 3% OV-1 on Chromosorb W (100—120 mesh), and helium was used as a carrier gas. The temperature of the column was 230°C, and the injection port and ion source were kept at 260°C and 300°C, respectively. The accelerating voltage, ionization voltage and trap current were 8 kV, 70 eV and 200 μA, respectively.

Preparation of trimethylsilyl ether derivatives

The trimethylsilyl (TMS) derivatives were prepared by adding 0.1 ml each of pyridine, hexamethyldisilazane and trimethylchlorosilane and incubating at 60°C for 30 min. After removal of the excess reagents under a nitrogen gas stream, the residue was dissolved in hexane and subjected to gas chromatography—mass spectrometry.

RESULTS AND DISCUSSION

Separation of the four isomeric catechol estrogens has been previously established by HPLC on a μBondapak C_{18} column using acetonitrile—0.5% ammonium dihydrogen phosphate (pH 3.0) (1:2.1) as a mobile phase [4]. In the present study, the separation and determination of catechol estrogens in human pregnancy urine was undertaken employing this technique.

The urine specimen containing labile catechol estrogens was successfully collected and preserved, oxidative decomposition being prevented by the addition of ascorbic acid. The deconjugated estrogen fraction was obtained by hot acid hydrolysis followed by extraction with ethyl acetate. The clean-up procedure for urine catecholamines appeared to be similarly effective for urine catechol estrogens. The catecholic compounds were selectively adsorbed on acid-washed alumina and then recovered by eluting with hydrochloric acid. An aliquot of the eluate was subjected to HPLC—ECD according to the procedure previously established. As shown in Fig. 1, the four isomeric catechol estrogens in pregnancy urine were distinctly separated and identified on the chromatogram by comparison with the authentic samples.

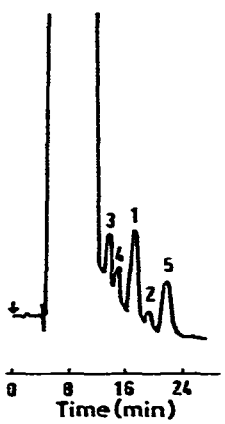

Fig.1. Separation of catechol estrogens in pregnancy urine by HPLC—ECD. 1 = 2-OHE_1 (3.4 ng); 2 = 4-OHE_1 (< 1 ng); 3 = 2-OHE_2 (1.2 ng); 4 = 4-OHE_2 (1.0 ng); 5 = 4-hydroxy-16-oxoestradiol 17-acetate (internal standard) (5.0 ng). Conditions: column, μBondapak C_{18}; mobile phase, acetonitrile—0.5% $NH_4H_2PO_4$ (pH 3.0) (1:2.1), 1 ml/min; detection, Yanagimoto Model VMD-101 electrochemical detector. Abbreviations as in Table I.

The structures of these four peaks were unequivocally characterized by means of gas chromatography—mass spectrometry. The urine catechol estrogens were transformed into the TMS ether derivatives in the usual manner and then analyzed using selected ion monitoring of parent ions at *m/z* 430

for 2- and 4-hydroxyestrone and *m/z* 504 for 2- and 4-hydroxyestradiol. A typical mass chromatogram is illustrated in Fig. 2. Two pairs of peaks obtained by single ion monitoring of each characteristic ion corresponded well with four peaks observed on reconstructed ion chromatography (RIC). In addition, the mass spectrum of each peak on RIC was identical to that of the respective authentic sample. The presence of 2- and 4-hydroxyestrogens in normal late pregnancy urine was thus definitely established. To the best of our knowledge this is the first reported occurrence of 4-hydroxyestrone and 4-hydroxyestradiol in man, although identification of 4-hydroxyestriol in pregnancy urine has recently been demonstrated by Fotsis et al. [6].

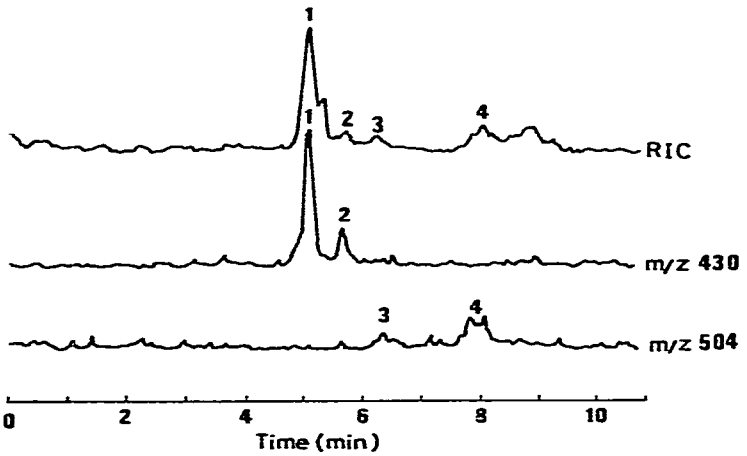

Fig. 2. Mass chromatogram of catechol estrogens in pregnancy urine. 1 = 2-OHE_1; 2 = 4-OHE_1; 3 = 2-OHE_2; 4 = 4-OHE_2. Abbreviations as in Table I.

Development of a method for the quantitation of urine catechol estrogens was then undertaken. The calibration graphs were constructed by plotting the peak height of each catechol estrogen to the internal standard (that is 4-hydroxy-16-oxoestradiol 17-acetate) against the amount of the former; satisfactory linearity was observed in the range 1—6 ng of catechol estrogens (Fig. 3).

The hydrolyzed urine was extracted with ethyl acetate and the extract was in turn subjected to the clean-up procedure used for the analysis of urine catecholamines. The use of selective adsorption on acid-washed alumina provided the catechol fraction where the desired catechol estrogens were expected to be present.

In order to confirm the validity of the present method for the determination of urine catechol estrogens, the recovery test was carried out using the authentic samples. First, catechol estrogens recovered through the whole clean-up procedure involving extraction with organic solvents and adsorption on alumina were determined. It is evident from the data in Table I that the four catechol estrogens were all recovered at satisfactory rates. Subsequently, the recovery of catechol estrogens in spiked pregnancy urine was tested. A mixture of known amounts of the four catechol estrogens was added to the urine hydrolysate, and the assay procedure involving extraction and clean-up

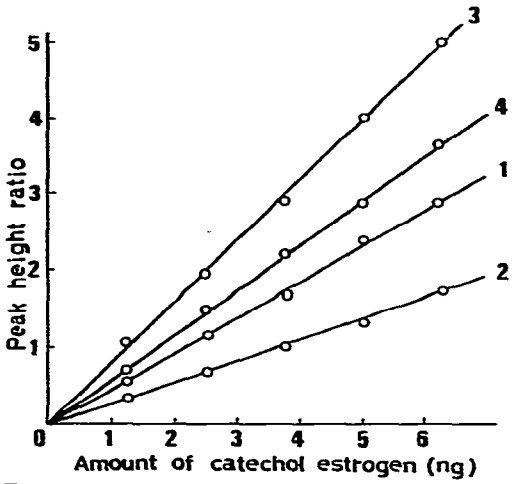

Fig. 3. Calibration graphs for catechol estrogens. 1 = 2-OHE$_1$; 2 = 4-OHE$_1$; 3 = 2-OHE$_2$; 4 = 4-OHE$_2$. Abbreviations as in Table I.

TABLE I

RECOVERIES OF CATECHOL ESTROGENS* TAKEN THROUGH THE WHOLE CLEAN-UP

An aqueous solution (0.2 ml) containing 50 ng each of the four catechol estrogens was used for the recovery test.

	Recovery (%)			
	2-OHE$_1$	2-OHE$_2$	4-OHE$_1$	4-OHE$_2$
Mean ± S.D. (n=6)	74.8 ± 3.8	73.9 ± 2.4	74.7 ± 1.2	75.1 ± 2.2

*2-OHE$_1$ = 2-hydroxyestrone = 2,3-dihydroxy-1,3,5(10)-estratrien-17-one; 4-OHE$_1$ = 4-hydroxyestrone = 3,4-dihydroxy-1,3,5(10)-estratrien-17-one; 2-OHE$_2$ = 2-hydroxyestradiol = 1,3,5(10)-estratriene-2,3,17β-triol; 4-OHE$_2$ = 4-hydroxyestradiol = 1,3,5(10)-estratriene-3,4,17β-triol.

followed by HPLC was carried out. The mean recovery values of the four catechol estrogens in spiked pregnancy urine were found in the range 95.4—106.0% (Table II).

The present method was then applied to quantitation of the four catechol estrogens excreted in pregnancy urine (Table III). In almost all cases 2-hydroxyestrone showed the highest value of these four. The amount of 4-hydroxyestrogens excreted was less than that of 2-hydroxyestrogens. It is to be noted that biologically potent catechol estrogens, in particular 4-hydroxyestrogens, can be determined with satisfactory accuracy and precision by the newly developed procedure.

The availability of a simple and sensitive method may serve to clarify the biological function and metabolic significance of catechol estrogens. Further studies on the development of a more sensitive method for determination of catechol estrogens in tissues are being conducted in these laboratories and the details will be reported in the near future.

TABLE II

DETERMINATION OF CATECHOL ESTROGENS* IN SPIKED URINE

A pregnancy urine specimen containing 2-OHE_1 (34 ng/ml), 2-OHE_2 (24 ng/ml), 4-OHE_1 (27 ng/ml) and 4-OHE_2 (21 ng/ml) was used.

Amount of each spiked (ng/ml)	Determined (ng/ml)			
	2-OHE_1	2-OHE_2	4-OHE_1	4-OHE_2
100	97	96	103	106
250	252	246	266	258
500	513	477	519	505
1000	972	966	1026	962
Relative recovery rate (%) (Mean ± S.D.; n=5)	99.1 ± 2.4	98.2 ± 3.1	103.8 ± 1.7	102.0 ± 3.6

*For abbreviations see footnote to Table I.

TABLE III

URINE LEVELS OF CATECHOL ESTROGENS* IN NORMAL PREGNANCY

Subject	Determined (ng/ml)			
	2-OHE_1	2-OHE_2	4-OHE_1	4-OHE_2
A (16 w)	125	103	51	44
B (16 w)	138	36	n.d.**	21
C (25 w)	128	138	n.d.	58
D (29 w)	276	59	n.d.	104
E (34 w)	145	305	n.d.	95
F (37 w)	382	130	36	39
G (38 w)	203	385	116	n.d.
H (38 w)	264	104	22	136
I (38 w)	749	109	73	72
J (39 w)	109	168	65	n.d.

*For abbreviations see footnote to Table I.
**n.d. = not detectable.

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