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STUDIES ON STEROIDS

CCXXIX*. SEPARATION AND CHARACTERIZATION OF CATECHOL OESTROGEN GLUCURONIDES IN URINE OF PREGNANT WOMEN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation and characterization of catechol oestrogen glucuronides in the urine of pregnant women by high-performance liquid chromatography with electrochemical detection is described. The urine samples from pregnant women were passed through Amberlite XAD-2 and Sep-Pak C_{18} columns and subsequented to ion-exchange chromatography on piperidinohydroxypropyl Sephadex LH-20 to isolate the oestrogen glucuronide fraction. The subsequent resolution into individual oestrogen glucuronides was achieved by high-performance liquid chromatography on a reversed-phase column. 2-Hydroxyoestrone 2-glucuronide and 4-hydroxyoestrone 4-glucuronide were identified on the basis of their behaviour in high-performance liquid chromatography with three different mobile phases. Derivatization of two glucuronides with 2-ferrocenylethylamine, followed by chromatographic separation and measurement of hydrodynamic voltammograms with an electrochemical detector was carried out for unequivocal identification. Enzymatic cleavage of the glucuronoside linkage followed by the identification of deconjugated catechol oestrogens by high-performance liquid chromatography with electrochemical detection further supported the structural assignment of these metabolites. The ratio between the amounts of 2-hydroxyoestrone 2-glucuronide and 4-hydroxyoestrone 4-glucuronide excreted in the urine of pregnant women was found to be *ca.* 5:1.

INTRODUCTION

Since the first reports by three groups[1–3] on the occurrence of 4-hydroxyoestrogens and 2-hydroxyoestrogens in the urine of pregnant women, considerable attention has been paid to the metabolism of catechol oestrogens in connection with

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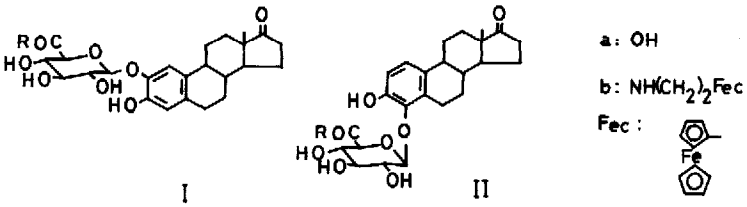

Fig. 1. Structures of catechol oestrogens and their derivatives obtained after reaction with 2-ferrocenylethylamine.

their potent physiological activities. Fotsis *et al.*[4] measured catechol oestrogen conjugates in the urine of pregnant women by gas chromatography–mass spectrometry after hydrolysis of the conjugates. The amount of catechol oestrogen glucuronide was determined to be one-fiftieth of that of the classical oestrogen conjugates (mainly oestriol 16-glucuronide). However, the conjugation positions have been remained unclear. Yoshizawa *et al.*[5] reported that glucuronidation of 2-hydroxyoestrone occurred selectively at the 3-position when radioactive oestradiol was administered to human subjects. In contrast, we demonstrated that only 2-hydroxyoestrone 2-glucuronide (Ia) and 4-hydroxyoestrone 4-glucuronide (IIa) were formed when 2- and 4-hydroxyoestrones, respectively, were incubated with human liver homogenate[6] (Fig. 1). These results prompted us to study the presence of catechol oestrogen glucuronides in the urine of pregnant women by means of high-performance liquid chromatography (HPLC) with electrochemical detection (ED).

EXPERIMENTAL

Instruments

The apparatus used for HPLC was a Waters Model ALC/GPC 202 chromatograph (Waters, Milford, MA, U.S.A.), equipped with a Yanagimoto Model VMD 101 electrochemical detector (Yanagimoto, Kyoto, Japan) or an ultraviolet (UV) detector (Waters) monitoring the absorbance at 280 nm. The applied potential of the electrochemical detector was established against an Ag–AgCl reference electrode. A test sample was introduced using a Waters Model U6K injector with an effective volume of 2 ml. HPLC was carried out on a Develosil ODS-5 column (5 μm; 15 cm × 0.4 cm I.D.) (Nomura, Seto, Japan) at ambient temperature. The pH of the mobile phase was adjusted to 3.0 with phosphoric acid and the flow-rate was set at 1 ml/min. Field-desorption mass spectra were recorded on a JEOL JMS-01SG-2 spectrometer (JEOL, Tokyo, Japan).

Materials

Catechol oestrogen glucuronides and oestrogen glucuronides were synthesized in our laboratories by the methods previously reported[7,8]. The β-glucuronidase preparation derived from *Escherichia coli* (Type II) was supplied by Sigma (St. Louis, MO, U.S.A.). Amberlite XAD-2 resin was purchased from Rohm and Haas (Philadelphia, PA, U.S.A.), Sep-Pak C_{18} cartridges with packing materials were from Waters. Silica gel 60 HF_{254} (Merck, Darmstadt, F.R.G.) was used for preparative thin-layer chromatography (TLC). Piperidinohydroxypropyl Sephadex LH-20

(PHP-LH-20 in the acetate form)[9] and 2-ferrocenylethylamine[10] were prepared as previously reported. Other reagents used were of analytical-reagent grade, and solvents were purified by distillation prior to use.

Separation and characterization of catechol oestrogen glucuronides in the urine of pregnant women

Pregnancy urine (32–38 weeks of gestation; 3.5 l) was diluted with water (10 l) and percolated through an Amberlite XAD-2 column (40 cm × 2.5 cm I.D.). After washing the column with water (5 l), the conjugate fraction was eluted with methanol (1 l). The solvent was evaporated *in vacuo* at a temperature below 40°C, and the residue was redissolved in water (20 ml) and passed through a Sep-Pak C_{18} column (24 cm × 1 cm I.D.). After successive washing with water (100 ml), 10% methanol (100 ml) and 20% methanol (100 ml), the conjugates were eluted with 50% methanol (100 ml). The eluate was evaporated *in vacuo* at a temperature below 40°C, and the residue was redissolved in 90% methanol and transferred to the PHP-LH-20 column (30 cm × 1 cm I.D.). After washing the column with 90% methanol (80 ml) and 0.1 *M* acetic acid in 90% methanol (80 ml), the glucuronide fraction was eluted with 0.3 *M* acetic acid–potassium acetate (pH 7.0) in 90% methanol (80 ml) and the solvent was evaporated *in vacuo*. The residue was applied to a Sep-Pak C_{18} column, which was washed with water (100 ml) to remove inorganic salts, and then eluted with 50% methanol (50 ml). Further purification was performed by HPLC, using 0.5% sodium acetate (pH 3.0)–tetrahydrofuran–acetonitrile (40:9:3, v/v/v) as the mobile phase. The fractions with capacity ratios (k') between 8.6 and 10.3 (Ia) and between 12.7 and 14.4 (IIa) were collected and subjected to rechromatography, using 0.5% sodium acetate (pH 3.0)–acetonitrile (16:5) and 0.5% sodium acetate (pH 3.0)–methanol (6:5) as mobile phases, respectively. Compounds Ia (*ca.* 10 μg) and IIa (*ca.* 2 μg) were obtained as colourless oily residues with a single peak in the chromatogram by HPLC–ED (applied potential +0.8 V).

Hydrolysis of catechol oestrogen glucuronides with β-glucuronidase

The dried eluate (< 1 μg) fraction of each peak in the chromatogram was incubated with the β-glucuronidase preparation (*ca.* 80 units) in 0.1 *M* acetate buffer (pH 5.0; 1 ml) at 37°C overnight. The incubation mixture was adjusted to a pH value between 1 and 2 with 5% hydrochloric acid and extracted with ethyl acetate (3 ml) and washed with water. The organic layer was evaporated under a nitrogen stream and the residue was subjected to HPLC–ED (applied potential +0.8 V), using 0.5% sodium acetate (pH 3.0)–acetonitrile (8:5) as the mobile phase. The retention time for 2-hydroxyoestrone was 7.0 min, that for 4-hydroxyoestrone 8.0 min.

Preparation of 2-ferrocenylethylamine derivatives of oestrogen glucuronides

2-Ferrocenylethylamine (500 μg) in dichloromethane (0.1 ml), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (400 μg) in dichloromethane (0.4 ml) and 1-hydroxybenzotriazole (20 μg) in pyridine (20 μl) were added to a solution of oestrogen glucuronide (100 μg) in pyridine (50 μg)–chloroform (0.4 ml), and the mixture was kept at 37°C overnight. The reaction mixture was extracted with ethyl acetate, which was washed successively with 5% hydrochloric acid, water, 5% sodium bicarbonate, and water. After evaporation of the solvent, the residue was subjected to preparative

TLC, using chloroform–methanol (19:1) as the developing solvent. The adsorbent was collected from the spots of four solutes: 2-hydroxyoestrone 3-glucuronide derivative (der.), R_F 0.30; 2-hydroxyoestrone 2-glucuronide der., R_F 0.31; 4-hydroxyoestrone 3-glucuronide der., R_F 0.13; 4-hydroxyoestrone 4-glucuronide der., R_F 0.33. These compounds were eluted from the adsorbent with chloroform–methanol (9:1) to give 2-ferrocenylethylamine derivatives of oestrogen glucuronides as yellow oily residues. Field desorption mass spectra gave molecular ion peaks for each compound: 2-hydroxyoestrone 3-glucuronide der. m/z 674 $[M+H]^+$, 2-hydroxyoestrone 2-glucuronide der. m/z 674 $[M+H]^+$, 4-hydroxyoestrone 3-glucuronide der. m/z 674 $[M+H]^+$ and 4-hydroxyoestrone 4-glucuronide der. m/z 673 $[M]^+$. Other derivatives of oestrogen glucuronides were prepared as previously reported[10].

Derivatization of catechol oestrogen glucuronides from the urine of pregnant women with 2-ferrocenylethylamine

The catechol oestrogen glucuronide fraction (*ca.* 1 μg) from the urine of pregnant women was mixed with oestradiol 17-glucuronide (internal standard; 1 μg) in pyridine (50 μl), and the mixture was derivatized with 2-ferrocenylethylamine as described above. The amido derivatives obtained were subjected to HPLC–ED, using two solvent systems.

RESULTS AND DISCUSSION

Since only small amounts of catechol oestrogen glucuronides are excreted in the urine of pregnant women[4], the separation of these conjugates may be markedly influenced by the clean-up procedure employed. In a preliminary experiment we found that each catechol oestrogen glucuronide (2- or 4-hydroxyoestrone, 2- or 4-hydroxyoestradiol and 4-hydroxyoestriol A-ring glucuronide; 1 μg) was recovered for more than 90% from the Sep-Pak C_{18} cartridge and the PHP-LH-20 column (5 cm × 0.6 cm I.D.) with 50% methanol (10 ml) and 0.3 *M* acetic acid–potassium acetate (pH 7.0) in 90% methanol (10 ml), respectively. From these data, it was concluded, that the separation and characterization of catechol oestrogen glucuronides in the urine of pregnant women could be carried out. The urine sample was subjected successively to chromatography on Amberlite XAD-2, Sep-Pak C_{18}, and PHP-LH-20 columns. After removal of the neutral compounds and unconjugated oestrogens, the desired catechol oestrogen glucuronide fraction was eluted with the appropriate solvent[11]. Inspection of the eluate by HPLC–ED showed that the fraction also contained classical oestrogen glucuronides, necessitating further purification (Fig. 2).

In our previous studies[12,13] the chromatographic behaviour of oestrogens, their sulphates, and glucuronides on a reversed-phase column was investigated. Using these data, the glucuronide fraction could be purified successfully by HPLC on a reversed-phase column, using three solvent systems (Table I). The fractions containing Ia and IIa have been obtained as single peaks in an HPLC chromatogram. The chromatographic behaviour of these compounds, which depends upon the pH and the composition of the mobile phase, was identical with that of authentic glucuronides[12,13]. No peak corresponding to 2-hydroxyoestrone 3-glucuronide has been obtained, despite much effort.

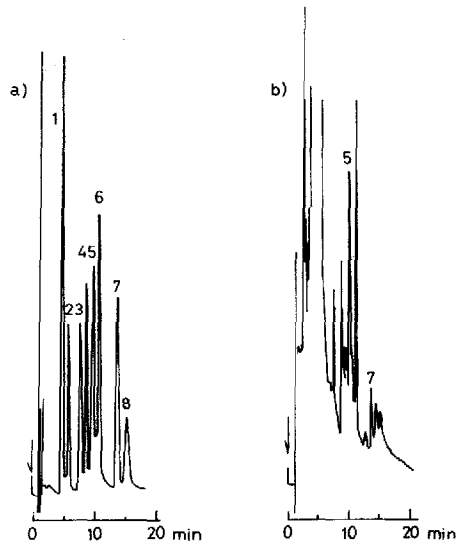

Fig. 2. Separation of oestrogen glucuronides by HPLC.Conditions: mobile phase, 0.5% sodium acetate (pH 3.0)–tetrahydrofuran–acetonitrile (40:9:3); applied potential +0.8 V. Abbreviations used are the same as in Table I. (a) authentic samples, (b) from urine of a pregnant woman. 1 = E_3 16-G; 2 = 2-OHE_2 3-G; 3 = 2-OHE_1 3-G/4-OHE_2 3-G; 4 = 4-OHE_2 4-G; 5 = 2-GHE_1 2-G; 6 = 4-OHE_1 3-G/2-OHE_2 2-G; 7 = 4-OHE_1 4-G; 8 = E_2 17-G.

A method for the determination of classical oestrogen glucuronides in urine, employing HPLC with pre-column labelling of the carboxyl groups with 2-ferrocenylethylamine has previously been developed[10]. In similar fashion, catechol oestrogens were converted to the N-(2-ferrocenylethyl)amides (Ib, IIb; see Fig. 1), which were effectively separated on the reversed-phase column with a detection limit of 0.5 pmol (S/N = 5; 4 nA full scale; see Table II). The hydrodynamic voltammograms of authentic oestrogen glucuronide derivatives are shown in Fig. 3. The oestrone 3-glucuronide derivative showed a constant value above +0.45 V, due to oxidation of the ferrocenyl moiety. The voltammogram of the oestradiol 17-glucuronide derivative exhibited a two-step oxidation, due to the ferrocenyl (+0.45 V) and the phenolic ($\geqslant$ +0.7 V) residues. In contrast, the catechol oestrogen glucuronide derivative showed unique voltammogram, involving oxidation of the phenolic group from +0.55 V. These characteristic patterns are helpful for differentiating the structural features of oestrogen glucuronides.

The obtained catechol oestrogen glucuronides were treated with 2-ferrocenylethylamine, after the addition. The resulting derivatives showed chromatographic behaviour and hydrodynamic voltammograms which were virtually identical to those of the authentic samples. The ratio of the amounts of Ia and IIa from the urine of pregnant women was estimated to be *ca.* 5:1. Enzymatic hydrolysis of Ia and IIa with β-glucuronidase gave 2-hydroxyoestrone and 4-hydroxyoestrone, respectively. This was established unambiguously by HPLC–ED.

It is evident from these results that 2-hydroxyoestrone 2-glucuronide (Ia) and 4-hydroxyoestrone 4-glucuronide (IIa) are excreted in the urine of pregnant women.

TABLE I

k' VALUES OF OESTROGEN CONJUGATES RELATIVE TO OESTRIOL 16-GLUCURONIDE

Oestrogen conjugates	*Mobile phase**		
	A	*B*	*C*
E_1 3-G**	2.21	5.34	2.95
E_2 3-G	4.11	4.80	4.75
E_3 3-G	0.20	0.56	0.22
E_2 17-G	4.22	4.80	4.75
E_3 16-G	1.00 (4.75 min)	1.00 (3.00 min)	1.00 (4.75 min)
E_2 3-S 17-G	0.00	0.67	0.76
E_3 3-S 16-G	0.00	0.00	0.00
4-OHE_1 3-G	2.72	4.91	3.58
4-OHE_2 3-G	1.92	2.41	3.19
4-OHE_1 4-G	3.59	5.56	4.36
From urine	3.59	5.56	4.36
4-OHE_2 4-G	2.15	2.41	3.19
4-OHE_3 3-G	0.00	0.00	0.00
4-OHE_3 4-G	0.00	0.00	0.00
2-OHE_1 3-G	1.75	3.39	2.41
2-OHE_2 3-G	1.17	1.65	2.02
2-OHE_1 2-G	2.49	6.21	4.75
From urine	2.49	6.21	4.75
2-OHE_2 2-G	2.96	4.91	7.41

* (A) 0.5% sodium acetate (pH 3.0)–tetrahydrofuran–acetonitrile (40:9:3), t_0 1.15 min, (B) 0.5% sodium acetate (pH 3.0)–acetonitrile (3.2:1), t_0 1.25 min, (C) 0.5% sodium acetate (pH 3.0)–methanol (1.2:1), t_0 1.31 min.

** E_1, oestrone; E_2, oestradiol; E_3, oestriol; G, glucuronide; S, sulphate.

TABLE II

k' VALUES OF CATECHOL OESTROGEN GLUCURONIDE DERIVATIVES OBTAINED WITH 2-FERROCENYLETHYLAMINE

Conditions: mobile phases (A) water–acetonitrile (6:5) containing 0.05 *M* sodium perchlorate; (B) water–methanol (2:5) containing 0.05 *M* sodium perchlorate; t_0 1.25 min; applied potential, +0.5 V. Abbreviations used are the same as in Table I.

Derivative	*Mobile phase*	
	A	*B*
2-OHE_1 2-G	8.40	7.55
2-OHE_1 3-G	7.00	6.61
4-OHE_1 3-G	7.80	7.78
4-OHE_1 4-G	10.00	8.51

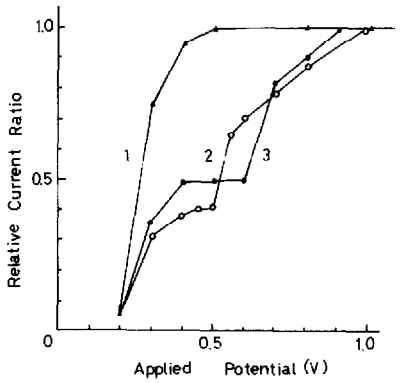

Fig. 3. Hydrodynamic voltammograms of oestrogen glucuronide derivatives. The maximum response of each derivative is arbitrarily taken as 1.0. Abbreviations used are the same as in Table I. 1 = E_1 3-G; 2 = 2-OHE_1 2-G; 3 = E_2 17-G.

This is the first definite demonstration of the occurrence of catechol oestrogen glucuronides in such urine samples. Further studies on the metabolism of catechol oestrogen conjugates during pregnancy are being conducted in our laboratories, and the results will be reported elsewhere.

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REFERENCES

1 G. Emans, H.-O. Hoppen, P. Ball and R. Knuppen, *Steroids*, 36 (1980) 73.
2 T. Fotsis, P. Järvenpää and H. Adlercreutz, *J. Clin. Endocrinol. Metab.*, 51 (1981) 148.
3 K. Shimada, T. Tanaka and T. Nambara, *J. Chromatogr.*, 223 (1981) 33.
4 T. Fotsis, H. Adlercreutz, P. Järvenpää, K. D. R. Setchell, M. Axelson and J. Sjövall, *J. Steroid Biochem.*, 14 (1981) 457.
5 I. Yoshizawa and J. Fishman, *J. Clin. Endocrinol. Metab.*, 29 (1969) 1123.
6 K. Shimada, Y. Yumura, E. Terashima and T. Nambara, *J. Steroid Biochem.*, 20 (1984) 1163.
7 K. Shimada, H. Shinkai and T. Nambara, *Chem. Pharm. Bull.*, 30 (1982) 4417.
8 K. Shimada, F. Xie and T. Nambara, *Chem. Pharm. Bull.*, 34 (1986) 179.
9 J. Goto, M. Hasegawa, H. Kato and T. Nambara, *Clin. Chim. Acta*, 87 (1978) 141.
10 K. Shimada, E. Nagashima, S. Orii and T. Nambara, *J. Pharm. Biomed. Anal.*, in press.
11 K. Shimada, F. Xie and T. Nambara, *J. Chromatogr.*, 232 (1982) 13.
12 K. Shimada, F. Xie, E. Terashima and T. Nambara, *J. Liq. Chromatogr.*, 7 (1984) 925.
13 K. Shimada, F. Xie and T. Nambara, *J. Chromatogr.*, 378 (1986) 17.