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STUDIES ON STEROIDS

CCXIX*. SEPARATION AND DETERMINATION OF 4-HYDROXYOESTRIOL MONOGLUCURONIDES AND MONOSULPHATES IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

The separation and determination of 4-hydroxyoestriol monoglucuronides and monosulphates by high-performance liquid chromatography with electrochemical detection on a reversed-phase column has been carried out. The effects of the salt, composition and pH of the mobile phase on the resolution were investigated with a Develosil ODS-5 column. Each group of isomeric monoglucuronides and monosulphates of 4-hydroxyoestriol was efficiently resolved on this column when 0.5% sodium acetate—acetonitrile and 0.5% sodium acetate—tetrahydrofuran—acetonitrile were used as mobile phases, respectively. The use of the present method revealed that 4-hydroxyoestriol orally administered to the rat was excreted as 4-, 3-, 16-glucuronides and 4-sulphate in bile.

INTRODUCTION

Since the occurrence of 4-hydroxyoestrogens as well as 2-hydroxyoestrogens in pregnancy urine was disclosed by three groups [1—3], considerable attentions have been focused on the metabolic fate of catechol oestrogens in connection with their potent physiological activities. The catechol oestrogen conjugates were determined by gas chromatography—mass spectrometry [2—4], radioimmunoassay [5] and high-performance liquid chromatography

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(HPLC) [3] involving prior hydrolysis and/or solvolysis of the conjugates. Deconjugation, however, has inevitable disadvantages, such as the lack of reliability in the results and the loss of information on the conjugated form. It appears to be attractive to develop a method for the direct determination of catechol oestrogen conjugates without prior deconjugation. In the previous works, we investigated the in vitro bioconversion of 4-hydroxyoestrone and observed the species difference and pH-dependency of enzymic sulphation of this substrate [6–8]. These results prompted us to clarify the metabolic fate of 4-hydroxyoestriol, which is one of the principal catechol oestrogens in human urine [2]. The present paper deals with the separation of isomeric monoglucuronides and monosulphates of 4-hydroxyoestriol by HPLC with electrochemical detection (ED). The application of this method for the determination of 4-hydroxyoestriol conjugates in rat bile is also described.

EXPERIMENTAL

High-performance liquid chromatography

The apparatus used for this work was a Waters ALC/GPC 202 high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Yanagimoto VMD 101 electrochemical detector (Yanagimoto, Kyoto, Japan). The applied potential was set at +0.9 V vs. an Ag/AgCl reference electrode. A Develosil ODS-5 (5 μm) column (25 cm × 0.4 cm I.D.) (Nomura Chemical, Seto, Japan) was used at a flow-rate of 1.0 ml/min under ambient conditions. The pH of the mobile phase was adjusted with phosphoric acid.

Materials

The catechol oestrogen conjugates were synthesized in these laboratories by the method previously reported [9]. All reagents used were of analytical-reagent grade. Solvents were purified by distillation prior to use and degassed by sonication. The β-glucuronidase preparation derived from *Escherichia coli* (type II) was supplied by Sigma (St. Louis, MO, U.S.A.).

Collection of rat bile

Male Wistar rats weighing ca. 200 g were anaesthetized with diethyl ether, cannulated to the bile duct with polyethylene tube (PE 10) (Clay Adams, Parsippany, NJ, U.S.A.) by surgical operation and housed in Bollman cages for collection of bile. All animals were starved overnight prior to administration of 4-hydroxyoestriol. A suspension of 4-hydroxyoestriol (50 mg per head) in dimethyl sulphoxide (0.1 ml) with saline (0.7 ml) and Tween 80 (0.2 ml) was given orally to the rat, and bile was collected in a test tube containing 0.005 *M* ascorbic acid solution (0.5 ml) over a period of 24 h following administration of 4-hydroxyoestriol [4, 10].

Determination of conjugated metabolites in biological fluids

To an aliquot of bile was added 6β-hydroxyoestradiol 17-glucuronide or oestriol 16-glucuronide (each 5 μg) as an internal standard for the determination of glucuronides or sulphates. The solution was percolated through a column (5 cm × 0.6 cm I.D.) of Amberlite XAD-4 resin (Rohm and Haas,

Philadelphia, PA, U.S.A.). After thorough washing with distilled water (5 ml), the conjugate fraction was eluted with methanol (10 ml). After addition of 0.005 *M* ascorbic acid in methanol (0.5 ml), the effluent was evaporated down under reduced pressure and below 40°C. The residue obtained was dissolved in methanol, an aliquot of which was subjected to HPLC.

Recovery test for 4-hydroxyoestriol conjugates

Control rat bile (0.1 ml) was spiked with known amounts of 4-hydroxyoestriol conjugates. The assay was then carried out according to the procedure described above.

Enzymic hydrolysis of 4-hydroxyoestriol monoglucuronides

The dried eluate corresponding to each peak on the chromatogram was incubated with the β-glucuronidase preparation in 0.1 *M* acetate buffer (pH 5.0) at 37°C overnight.

Solvolysis of 4-hydroxyoestriol 4-sulphate

The dried eluate corresponding to each peak on the chromatogram was subjected to usual solvolysis [11].

RESULTS AND DISCUSSION

Initially, our effort was directed towards developing an efficient chromatographic system for the separation of isomeric monoglucuronides and monosulphates of 4-hydroxyoestriol. Among the several commercially available columns tested, a Develosil ODS-5 column provided the most promising result. Therefore, suitable conditions for the separation were examined in detail with this column.

First, the effect of the salt in the mobile phase on the resolution of closely related compounds was investigated with disodium hydrogen phosphate, ammonium dihydrogen phosphate and sodium acetate (Table I). When the first two were used, the resolution of these three pairs, especially glucuronides, was insufficient. However, the satisfactory separation ($R > 1.3$) was attained for all the pairs by using sodium acetate in the mobile phase. The data suggested

TABLE I

EFFECT OF SALTS IN MOBILE PHASE ON RESOLUTION OF 4-HYDROXYOESTRIOL MONOGLUCURONIDES AND MONOSULPHATES

Salt	Resolution (R)		
	17-Glucuronide* 16-Glucuronide	16-Glucuronide* 4-Glucuronide	17-Sulphate** 16-Sulphate
Na_2HPO_4	0.78	0.71	1.08
$NH_4H_2PO_4$	0.73	0.80	0.89
NaOAc	1.35	1.31	1.33

*0.5% Salt (pH 3.2)—acetonitrile (6:1).
**0.5% Salt (pH 3.2)—THF—acetonitrile (10:1:1).

that the salt in the mobile phase exerted much influence on the resolution of these compounds. Otto and Wegscheider [12] investigated the effect of buffer in the mobile phase on the retention of organic acids, amino acids and dipeptides, and assumed that the modification of accessible silanol groups of the stationary phase by buffer anion would be responsible for this effect. Papp and Vigh [13] observed that the separation of aromatic amines was influenced by buffer cation. They interpreted this phenomenon in terms of the change in ionization of the surface silanols and the pK-dependent protonation of the amine solutes. Although no plausible explanation is available at present, our results may be ascribable to the different effects of buffer anion in the mobile phase on the modification of silanol groups of the stationary phase and the pK-dependent protonation of the acid solutes.

The effect of pH of the mobile phase on the k' value was also examined. The k' values of these substrates were plotted against pH of the buffer in the mobile phase. It is of particular interest that the effect of pH on the k' value was quite different between the two groups. 4-Hydroxyoestriol glucuronides exhibited remarkably increased k' values with a decreasing pH value in the range 5.0–3.0 (Fig. 1a), while 4-hydroxyoestriol sulphates showed no significant change in the k' value in the pH range 5.0–3.0 (Fig. 1b). This phenomenon can be explained in terms of dissociation of these compounds. With a decreasing pH of the mobile phase, dissociation of the compounds having a glucuronic acid moiety (pK_a 3.2) decreased, exerting the increased k' values and the improved resolution. However, 4-hydroxyoestriol sulphates possessing a sulphuric acid moiety (pK_a 1.92) were ionized in the pH range 5.0–3.0 and no marked effect of pH was observed. These results were compatible with our previous reports [6, 14]. The most satisfactory separation of 4-hydroxyoestriol glucuronides was attained in the pH range 3.0–3.2 (Fig. 1a).

Although 4-hydroxyoestriol glucuronides were satisfactorily separated by

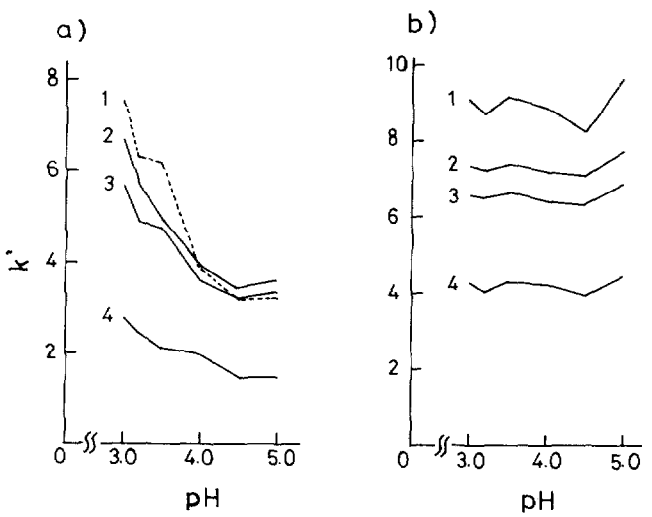

Fig. 1. Effect of pH of mobile phase on k' values of 4-hydroxyoestriol conjugates. (a) 1: 4-Glucuronide, 2: 16-glucuronide, 3: 17-glucuronide, 4: 3-glucuronide; (b) 1: 4-sulphate, 2: 16-sulphate, 3: 17-sulphate, 4: 3-sulphate.

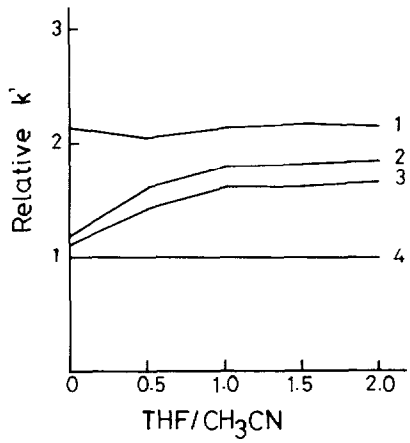

Fig. 2. Effect of the THF—acetonitrile ratio of mobile phase on relative k' values of 4-hydroxyoestriol sulphates. 1: 4-Sulphate, 2: 16-sulphate, 3: 17-sulphate, 4: 3-sulphate.

the use of a binary solvent system (0.5% sodium acetate—acetonitrile), no distinct resolution of 4-hydroxyoestriol sulphates was obtained by any binary solvent systems. According to our previous finding [6], the use of a ternary solvent system consisting of 0.5% sodium acetate—tetrahydrofuran (THF)—acetonitrile was investigated. The effect of the THF to acetonitrile ratio on the k' value relative to 4-hydroxyoestriol 3-sulphate was examined using an organic solvent—buffer (5:1) system (Fig. 2). The relative k' values of 4-hydroxyoestriol 16- and 17-sulphates were reduced remarkably with a decreasing ratio of THF to acetonitrile, while that of 4-hydroxyoestriol 4-sulphate was almost constant under these conditions. It seems likely that THF would be more effective than acetonitrile for the facile elution of ring A conjugates relative to ring D conjugates [6]. The suitable ratio of THF to acetonitrile in the mobile phase for the separation of 4-hydroxyoestriol sulphates was found in the range 1.0—1.5. On the basis of these data, 0.5% sodium acetate (pH 3.2)—acetonitrile (6:1) and 0.5% sodium acetate (pH 4.7)—THF—acetonitrile (10:1:1) were chosen as mobile phases suitable for 4-hydroxyoestriol monoglucuronides and monosulphates, respectively. It is evident from the chromatogram in Figs. 3a and 4a that each group of these conjugates was efficiently resolved within 21 min.

The present method was then applied for the determination of 4-hydroxyoestriol monoglucuronides and monosulphates in rat bile following oral administration of 4-hydroxyoestriol. A portion of rat bile was subjected to chromatography on Amberlite XAD-4 resin. As illustrated in Figs. 3b and 4b, the typical chromatograms showed the stable baselines without interfering peaks and, hence, were favourable for the determination of 4-hydroxyoestriol monoconjugates in biological specimens. The present and previous results [15] demonstrated that HPLC—ED would be particularly useful for the analysis of oestrogen conjugates in biological fluids.

Known amounts of 4-hydroxyoestriol monoglucuronides and monosulphates were added to control rat bile specimens, and their recovery rates were then determined. As listed in Table II, ring A glucuronides and sulphates of 4-

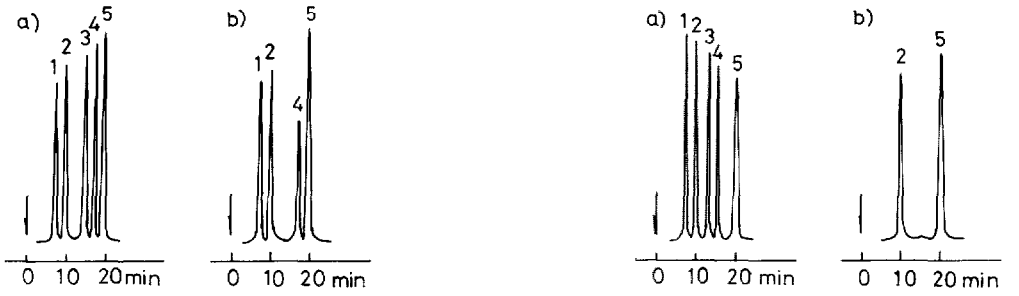

Fig. 3. Separation of 4-hydroxyoestriol monoglucuronides. (a) Authentic samples; (b) metabolites in rat bile. 1: 3-Glucuronide, 2: internal standard, 3: 17-glucuronide, 4: 16-glucuronide, 5: 4-glucuronide. Conditions: column, Develosil ODS-5; mobile phase, 0.5% sodium acetate (pH 3.2)—acetonitrile (6:1); flow-rate, 1.0 ml/min.

Fig. 4. Separation of 4-hydroxyoestriol monosulphates. (a) Authentic samples; (b) metabolites in rat bile. 1: 3-Sulphate, 2: internal standard, 3: 17-sulphate, 4: 16-sulphate, 5: 4-sulphate. Conditions: column, Develosil ODS-5; mobile phase, 0.5% sodium acetate (pH 4.7)—THF—acetonitrile (10:1:1); flow-rate, 1.0 ml/min.

TABLE II

RECOVERY OF 4-HYDROXYOESTRIOL MONOGLUCURONIDES AND MONOSULPHATES ADDED TO RAT BILE

Compound	Recovery (mean ± S.D., n = 6) (%)		
	Amount added (μg per 0.1 ml)		
	1	5	10
3-Glucuronide	91.0 ± 1.6	90.0 ± 1.4	91.2 ± 2.7
4-Glucuronide	91.2 ± 2.9	92.4 ± 2.2	90.4 ± 3.1
16-Glucuronide	82.0 ± 2.5	83.5 ± 3.0	78.2 ± 2.5
17-Glucuronide	73.6 ± 2.6	84.6 ± 3.1	79.3 ± 2.3
3-Sulphate	92.0 ± 2.9	—	91.9 ± 1.7
4-Sulphate	88.0 ± 3.3	—	92.3 ± 2.4
16-Sulphate	83.1 ± 2.4	—	81.2 ± 2.5
17-Sulphate	87.0 ± 3.0	—	85.3 ± 1.3

hydroxyoestriol spiked to rat bile were recovered at rates of more than 88%, while the ring D conjugates having the labile catechol structure were recovered at a rate of more than 73% in the presence of ascorbic acid [4]. 6β-Hydroxyoestradiol 17-glucuronide and oestriol 16-glucuronide were chosen as internal standards for the determination of 4-hydroxyoestriol monoglucuronides and monosulphates, respectively. The recovery rates of 6β-hydroxyoestradiol 17-glucuronide and oestriol 16-glucuronide were found to be 86.3 ± 3.2% and 90.9 ± 3.6% ($n \geqslant 6$), respectively. A calibration graph was constructed by plotting the ratio of the peak height of each compound to that of the internal standard dissolved in control bile against the amount of each compound, a linear response to each compound being observed in the range 0—10 μg. The detection limits of ring A sulphates and glucuronides were estimated to be 1 and 5 ng, respectively, while that of ring D conjugates was 500 pg (signal-to-noise

ratio = 3 at 2 nA full-scale). The typical chromatograms illustrated in Figs. 3b and 4b demonstrated evidently the excretion of 4-hydroxyoestriol 4-, 3-, 16-glucuronides and 4-sulphate in rat bile. The characterization of these peaks was then carried out. These metabolites showed chromatographic behaviour identical with those of the authentic samples, along with changes in pH and composition of the mobile phase. After treatment with β-glucuronidase or acid, all the peaks on the chromatogram were changed into a peak corresponding to 4-hydroxyoestriol. In addition, upon methylation of ring A conjugate fractions with diazomethane followed by treatment with alkali, then β-glucuronidase or acid, these compounds were changed into 4-hydroxyoestriol 3-methyl ether (from 4-hydroxyoestriol 4-glucuronide and 4-sulphate fractions) and 4-methyl ether (from the 4-hydroxyoestriol 3-glucuronide fraction) [9]. The peak corresponding to 4-hydroxyoestriol 16-glucuronide disappeared from the chromatogram by alkali treatment [16].

TABLE III

EXCRETED AMOUNTS OF 4-HYDROXYOESTRIOL MONOGLUCURONIDES AND MONOSULPHATES IN RAT BILE

Compound	Excreted amount		Bioconversion rate (%)
	μg per 0.1 ml	mg per 24 h	
4-Glucuronide	43.0	3.8	4.8
3-Glucuronide	10.4	0.9	1.2
16-Glucuronide	10.6	0.9	1.2
4-Sulphate	1.3	0.1	0.09

The excreted amounts of the conjugates in rat bile were determined (Table III). With regard to the glucuronide fraction, 4-hydroxyoestriol 4-glucuronide was the most predominant conjugate and the ratio of the excreted amount of 4- to 3- to 16-glucuronide was 4:1:1. As for the sulphate fraction, 4-hydroxyoestriol 4-sulphate was solely excreted in amounts much less than the glucuronides. 4-Hydroxyoestriol was transformed by biliary excretion into the 4-, 3-, 16-glucuronides and 4-sulphate (4.8, 1.2, 1.2 and 0.09%, respectively).

Previously, we found that 4-hydroxyoestrone 4-glucuronide and 4-sulphate were excreted in bile when 4-hydroxyoestrone was administered to the rat. We also clarified the excretion of oestriol 16- and 17-glucuronides in rat bile after administration of oestriol [15]. In the present study, however, it has been demonstrated that 4-hydroxyoestriol 4-, 3-, 16-glucuronides and 4-sulphate were excreted in rat bile. It is of interest that the conjugated positions are different, depending on the chemical structures of the substrates. These results are indicative of the multiplicity of UDP-glucuronyltransferase.

The application of the present method to the characterization of 4-hydroxyoestriol conjugates in pregnancy urine is being conducted in these laboratories and details will be reported elsewhere.

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REFERENCES

1 G. Emons, H.-O. Hoppen, P. Ball and R. Knuppen, Steroids, 36 (1980) 36.
2 T. Fotsis, P. Järvenpää and H. Adlercreutz, J. Clin. Endocrinol. Metab., 51 (1980) 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 G. Emons, C. Mente, R. Knuppen and P. Ball, Acta Endocrinol., 97 (1981) 251.
6 K. Shimada, F. Xie, E. Terashima and T. Nambara, J. Liq. Chromatogr., 7 (1984) 925.
7 K. Shimada, Y. Yumura, E. Terashima and T. Nambara, J. Steroid Biochem., 20 (1984) 1163.
8 K. Shimada, M. Hara and T. Nambara, Chem. Pharm. Bull., 33 (1985) 685.
9 K. Shimada, F. Xie and T. Nambara, Chem. Pharm. Bull., 34 (1986) 179.
10 T. Nambara and Y. Kawarada, Chem. Pharm. Bull., 25 (1977) 942.
11 S. Burstein and S. Lieberman, J. Biol. Chem., 233 (1958) 331.
12 M. Otto and W. Wegscheider, J. Liq. Chromatogr., 6 (1983) 685.
13 E. Papp and Gy. Vigh, J. Chromatogr., 259 (1983) 49.
14 K. Shimada, M. Kaji, F. Xie and T. Nambara, J. Liq. Chromatogr., 5 (1982) 1763.
15 K. Shimada, F. Xie and T. Nambara, J. Chromatogr., 232 (1982) 13.
16 K. Shimada, M. Kumai, H. Shinkai and T. Nambara, Anal. Biochem., 116 (1981) 287.