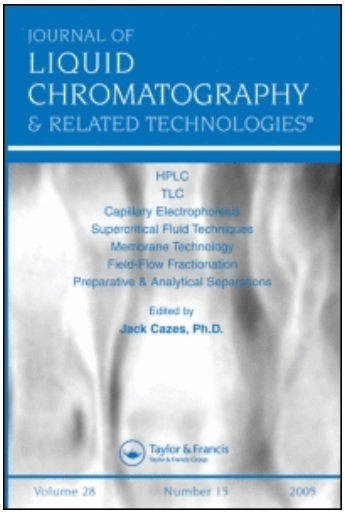

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STUDIES ON STEROIDS CLXXXIV. SEPARATION OF CATECHOL ESTROGEN MONOGLUCURONIDES AND MONOSULFATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION[1]

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ABSTRACT

Separation of catechol estrogen monoglucuronides and monosulfates by high-performance liquid chromatography with electrochemical detection on a reversed-phase column has been carried out. The effects of composition and pH of mobile phases on the capacity factor were investigated with a TSKgel ODS-120T column. Each group of isomeric monoglucuronides and monosulfates of 2- and 4-hydroxyestrogens was efficiently resolved on this column when the 0.5% ammonium dihydrogen phosphate-tetrahydrofuran-acetonitrile system was used as a mobile phase.

INTRODUCTION

Since the reports on the occurrence of 4-hydroxyestrogens as well as the well-known 2-hydroxyestrogens in pregnancy urine by three groups (2-4), considerable attentions have been drawn to the metabolic fate of catechol estrogens in connection with their potent physiological activities. The catechol estrogen conjugates were determined by gas chromatography-mass spectrometry (5), radioimmunoassay (6), and high-performance liquid chromato-

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graphy (HPLC) (4) involving prior hydrolysis and/or solvolysis of the conjugates. These procedures, however, have inevitable disadvantages: the lack of reliability in the results and the loss of informations on the conjugated forms. It appears to be attractive to develop a method for the direct determination of catechol estrogen conjugates without deconjugation. The present paper deals with the separation of isomeric monoglucuronides and monosulfates of 2- and 4-hydroxyestrogens by HPLC with electrochemical detection (ECD).

EXPERIMENTAL

Instruments

The apparatus used for this work was a Yanagimoto L-4000W high-performance liquid chromatograph equipped with a Yanagimoto VMD 101A electrochemical detector (Yanagimoto Co., Kyoto, Japan). The potential of the detector was set at +0.9V vs a Ag/AgCl reference electrode. A TSKgel ODS-120T (5 μm) column (25 cm x 0.4 cm i.d.) (Toyo Soda Co., Tokyo, Japan) was used under ambient conditions. The pH of the mobile phase was adjusted with phosphoric acid or ammonium hydroxide. All solvents were degassed by sonication. The mobile phase was used at a flow rate of 1 ml/min.

Chemicals and Reagents

The catechol estrogen conjugates were synthesized in these laboratories by the methods previously reported (7,8). All the reagents used were of analytical reagent grade. Solvents were purified by distillation prior to use.

RESULTS AND DISCUSSION

In reversed-phase HPLC, the methanol-, acetonitrile-, and tetrahydrofuran (THF)-buffer systems are usually employed as

mobile phases. Initially, our effort was directed to the separation of the isomeric monoglucuronides and monosulfates of 2- and 4-hydroxyestrogens with these solvent systems. Among several commercially available columns tested a TSKgel ODS-120T column provided the most promising result. Therefore, suitable conditions for the separation were examined in detail with this column.

First, acetonitrile, methanol, or THF combined with 0.5% ammonium dihydrogen phosphate (pH 3.0) at various ratios was employed as a mobile phase. The capacity factors (k') of all the substrates increased with a decreasing ratio of organic solvent. Satisfactory separation, however, was not attained by any binary solvent systems. For instance, when acetonitrile-buffer (5:16) was used, 2-OHE_1 3-S and 2-OHE_2 2-S were not resolved, and 2-OHE_1 2-S was eluted with the retention of more than 1 hr. The similar chromatographic behaviors were also observed for other groups of catechol estrogen conjugates.

These results prompted us to use a ternary solvent system containing an additional organic modifier, THF, which has proved to be effective for the separation of the ethereal compounds (9) and amino acid derivatives (10,11). As for the 0.5% ammonium dihydrogen phosphate-THF-acetonitrile system, the effect of the ratio of THF to acetonitrile on the retention value was investigated. The k' values of catechol estrogen ring A conjugates relative to the 17-conjugate in each group were plotted against the ratio of THF to acetonitrile in the mobile phase. The results obtained for 4-hydroxyestrogen sulfates and glucuronides are shown in Fig. 1a and 1b, respectively. The relative k' value of each substrate was reduced remarkably with an increasing ratio of THF to acetonitrile. This phenomenon indicated that THF would be more effective than acetonitrile for the early elution of ring A conjugates relative to ring D conjugates. The relative k' values of 2-hydroxyestrogen conjugates at various ratios of THF

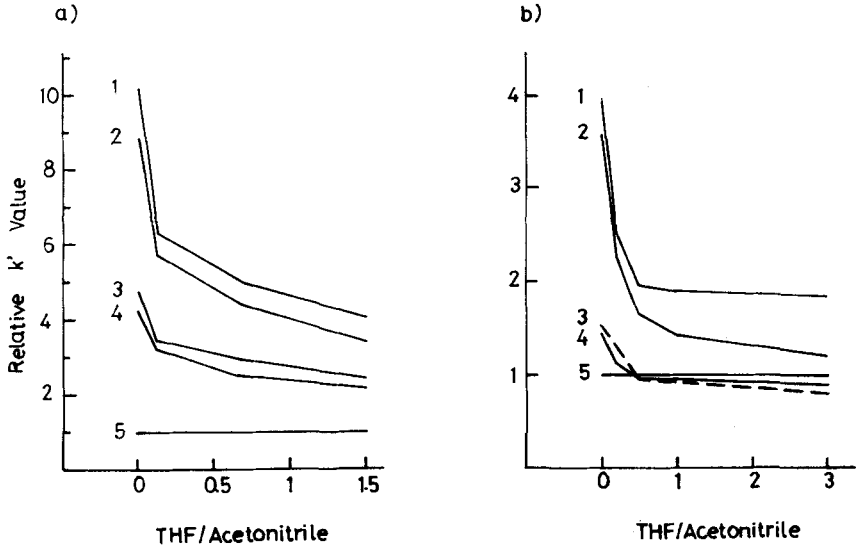

FIGURE 1. Effect of the Ratio of THF to Acetonitrile in the Mobile Phase on Relative k' Values of 4-Hydroxyestrogen Sulfates (a) and Glucuronides (b).
a) Organic solvent-buffer (5:16). 1, 4-OHE_1 4-S; 2, 4-OHE_1 3-S; 3, 4-OHE_2 3-S; 4, 4-OHE_2 4-S; 5, 4-OHE_2 17-S. b) Organic solvent-buffer (3:10). 1, 4-OHE_1 4-G; 2, 4-OHE_1 3-G; 3, 4-OHE_2 3-G; 4, 4-OHE_2 4-G; 5, 4-OHE_2 17-G.

to acetonitrile were also estimated. The results obtained were similar to those of 4-hydroxyestrogen conjugates (Table 1). The use of a suitable concentration of THF in the mobile phase improved the separation and brought about an earlier elution, providing a sharp peak of theoretical shape on the chromatogram. Based upon these data we arrived at a conclusion that THF would be an efficient modifier in the mobile phase for the separation of these conjugates. The suitable concentrations of THF in the mobile phases were found to be 14.3% for 2-hydroxyestrogen sulfates, 9.5% for 4-hydroxyestrogen sulfates, 11.5% for 2-hydroxyestrogen glucuronides, and 17.3% for 4-hydroxyestrogen glucuronides.

TABLE 1
Effect of the Ratio of THF to Acetonitrile in the Mobile Phase on Relative k' Values of 2-Hydroxyestrogen Conjugates

Compound	THF/acetonitrile				
	0	0.25	0.5	1	1.5
2-OHE_1 2-S	4.8*	3.0	2.6	2.3	2.0
2-OHE_2 2-S	3.4	2.9	2.7	2.4	2.2
2-OHE_1 3-S	3.4	2.3	2.0	1.7	1.6
2-OHE_2 3-S	1.8	1.5	1.4	1.3	1.2
2-OHE_2 17-S	1.0	1.0	1.0	1.0	1.0
2-OHE_1 2-G	1.0**	1.0	1.0	1.1	1.1
2-OHE_2 2-G	2.0	1.6	1.4	1.3	1.3
2-OHE_1 3-G	1.2	0.8	0.7	0.7	0.6
2-OHE_2 3-G	0.4	0.4	0.4	0.4	0.4
2-OHE_2 17-G	1.0	1.0	1.0	1.0	1.0

* Organic solvent-buffer (5:16), ** organic solvent-buffer (3:10)

The effect of pH on the k' value was then examined with 0.5% ammonium dihydrogen phosphate-THF-acetonitrile. The k' values of these substrates were plotted against pH of the buffer in the mobile phase. It is of particular interest that the effects of pH on the k' values were quite different among the four groups. The k' values of 4- and 2-hydroxyestrogen glucuronides decreased remarkably with an increasing pH value in the range of 3.0 to 5.0 (Fig. 2a,b). The compounds having a glucuronic acid moiety (pK_a 3.2) exhibited greater k' values at pH 3.0 where undissociated species are predominant. With an increasing pH value of the mobile phase the dissociation of these conjugates increases and hence, their k' values decreased. The result was compatible with

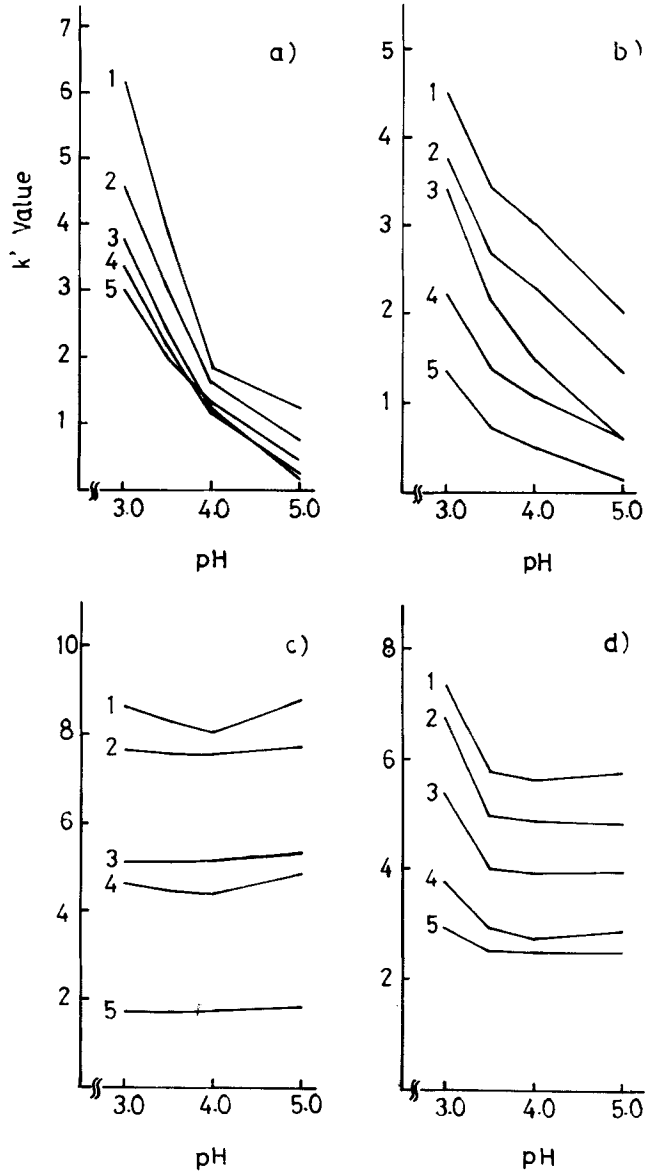

FIGURE 2. Effect of pH of Mobile Phase on k' Values of 4-Hydroxyestrogen Glucuronides (a), 2-Hydroxyestrogen Glucuronides (b), 4-Hydroxyestrogen Sulfates (c), and 2-Hydroxyestrogen Sulfates (d).
a) 1, 4-OHE_1 4-G; 2, 4-OHE_1 3-G; 3, 4-OHE_2 17-G; 4, 4-OHE_2 4-G; 5, 4-OHE_2 3-G. b) 1, 2-OHE_2 2-G; 2, 2-OHE_2 2-G; 3, 2-OHE_2 17-G; 4, 2-OHE_1 3-G; 5, 2-OHE_2 3-G. c) 1, 4-OHE_1 4-S; 2, 4-OHE_1 3-S; 3, 4-OHE_2 3-S; 4, 4-OHE_2 4-S; 5, 4-OHE_2 17-S. d) 1, 2-OHE_2 2-S; 2, 2-OHE_1 2-S; 3, 2-OHE_1 3-S; 4, 2-OHE_2 3-S; 5, 2-OHE_2 17-S.

TABLE 2
k' Values of Catechol Estrogen Conjugates

Compound	k'*	Compound	k'**
2-OHE_2 17-S	2.9	2-OHE_2 3-G	0.7
2-OHE_2 3-S	3.7	2-OHE_1 3-G	1.4
2-OHE_1 3-S	5.4	2-OHE_2 17-G	2.2
2-OHE_1 2-S	6.8	2-OHE_1 2-G	2.7
2-OHE_2 2-S	7.3	2-OHE_2 2-G	3.4
4-OHE_2 17-S	1.7	4-OHE_2 3-G	3.0
4-OHE_2 4-S	4.6	4-OHE_2 4-G	3.4
4-OHE_2 3-S	5.1	4-OHE_2 17-G	3.8
4-OHE_1 3-S	7.6	4-OHE_1 3-G	4.6
4-OHE_1 4-S	8.7	4-OHE_1 4-G	6.2

* t_0: 4.4 min, ** t_0: 4.8 min.

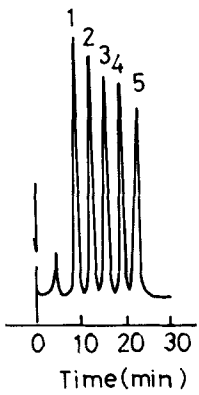

FIGURE 3. HPLC Separation of a Synthetic Mixture of 2-Hydroxyestrogen Glucuronides.
1, 2-OHE_2 3-G; 2, 2-OHE_1 3-G; 3, 2-OHE_2 17-G; 4, 2-OHE_1 2-G; 5, 2-OHE_2 2-G.
Conditions: column, TSKgel ODS-120T; mobile phase, 0.5% ammonium dihydrogen phosphate (pH 3.5)/THF/acetonitrile (20:3:3), 1 ml/min; detection, ECD at +0.9V.

the previous finding on the resolution of estriol 16-glucuronide and 17-glucuronide (12). The most satisfactory separation was obtained for 4-hydroxyestrogen glucuronides at pH 3.0 (Fig. 2a) while for 2-hydroxyestrogen glucuronides at pH 3.5 (Fig. 2b). As for 4-hydroxyestrogen sulfates no significant difference in the k' value was found in the range of pH 3.0 to 5.0 (Fig. 2c). The k' values of 2-hydroxyestrogen sulfates decreased remarkably from pH 3.0 to 3.5 and then were constant in the range of pH 3.5 to 5.0 (Fig. 2d). Although no plausible explanation is at present available, these phenomena may be ascribable to the difference in ionization of the solutes in the mobile phase (13).

On the basis of these data 0.5% ammonium dihydrogen phosphate (pH 3.0)-THF-acetonitrile (16:3:2, 16:2:3, and 40:9:3) were chosen as mobile phases suitable for 2-hydroxyestrogen sulfates, 4-hydroxyestrogen sulfates, and 4-hydroxyestrogen glucuronides, respectively. For 2-hydroxyestrogen glucuronides 0.5% ammonium dihydrogen phosphate (pH 3.5)-THF-acetonitrile (20:3:3) was found to be the most suitable mobile phase. Each group of isomeric monosulfates and monoglucuronides of 2- and 4-hydroxyestrogens was efficiently resolved by using the above solvent systems. The k' values of these catechol estrogen conjugates are listed in Table 2. A typical chromatogram of 2-hydroxyestrogen glucuronides is illustrated in Fig. 3. The detection limits of ring A sulfates and glucuronides of catechol estrogens were estimated to be 1 ng and 5 ng, respectively, while that of catechol estrogen 17-conjugates was 500 pg (S/N=2 at 2 nA full scale).

The application of the present method to the determination of catechol estrogen conjugates in biological fluids will be reported elsewhere (14).

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1) The following abbreviations are used in this paper: 2-OHE_1, 2-hydroxyestrone; 2-OHE_2, 2-hydroxyestradiol; 4-OHE_1, 4-hydroxyestrone; 4-OHE_2, 4-hydroxyestradiol; S, sulfate; G, glucuronide.

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