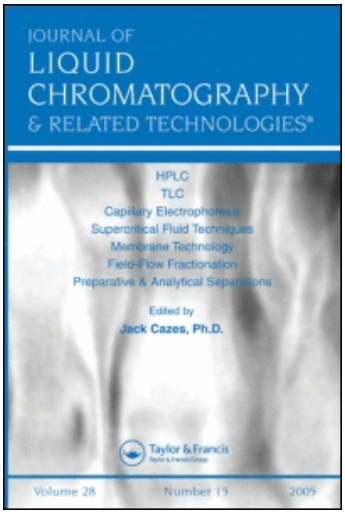

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Shimada, Kazutake , Masue, Toshiko , Toyoda, Kazuo , Takani, Masako and Nambara, Toshio(1988) 'The Utility of Cyclodextrin in Mobile Phase for High-Performance Liquid Chromatographic Separation of Isomeric Estrogens', Journal of Liquid Chromatography & Related Technologies, 11: 7, 1475 — 1484
To link to this Article: DOI: 10.1080/01483918808067187
URL: http://dx.doi.org/10.1080/01483918808067187

JOURNAL OF LIQUID CHROMATOGRAPHY, 11(7), 1475-1484 (1988)

THE UTILITY OF CYCLODEXTRIN IN MOBILE PHASE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF ISOMERIC ESTROGENS

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ABSTRACT

Cyclodextrin was used as a component of mobile phase for the separation of isomeric estrogens by reversed-phase high-performance liquid chromatography. The positional isomers of catechol and guaiacol estrogens were distinctly resolved by the addition of β-cyclodextrin to the mobile phase. The separation of estriol 16- and 17-glucuronides requires usually a prolonged time. The use of β-cyclodextrin in the mobile phase, however, reduced the retention time considerably.

The effect of β-cyclodextrin concentration in the mobile phase on the detector response was also investigated. The response of a fluorescence detector was raised with an increasing concentration of β-cyclodextrin, while that of an electrochemical detector was significantly depressed.

INTRODUCTION

Cyclodextrins (CDs) are toridal-shaped cyclic oligosaccharides consisted of α-1,4-linked D-glucopyranose units. They exhibit a highly stereoselective ability to form inclusion complexes with a variety of molecules and ions. Some attempts to utilize this phenomenon have been made in gas and liquid chromatography [1,2]. The CD-bonded column is often preferable to conventional reversed-phase one for the separation of optical, geometrical, and structural isomers [3]. Armstrong et al. first reported the separation of steroid epimers on CD-bonded stationary phase [4]. One of major limitations with this column is that the retention time is less reproducible as compared with the conventional column [5].

This paper deals with the separation of several estrogen isomers by high-performance liquid chromatography (HPLC) on the reversed-phase column with the mobile phase containing CD. The effect of CD concentration on the detector response has also been investigated.

MATERIALS AND METHODS

Materials

CDs were kindly supplied by Nihon Shokuhin Kako Co., Ltd. (Tokyo, Japan). Heptakis-(2,6-di-O-methyl)-β-CD (Me-β-CD) was prepared and donated by Kao Co. (Tokyo). Estrone and estriol were also generous gifts from Teikoku Hormone Mfg. Co. (Tokyo, Japan). Isomeric estrogens were synthesized in these laboratories [6]. Other reagents were purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). Solvents were purified by distillation prior to use.

Apparatus

HPLC was carried out on a JASCO TRI ROTAR chromatograph equipped with a JASCO UVIDEX-100-II ultraviolet detector (UV)

(Japan Spectroscopic Co., Ltd., Tokyo), Yanagimoto VMD-101 electrochemical detector (ECD) (Yanagimoto Co., Kyoto), or Hitachi F-1000 fluorescence detector (FL) (Hitachi Ltd., Tokyo) at a flow rate of 1 ml/min. The applied potential of the ECD was set vs. an Ag/AgCl reference electrode. A Develosil ODS-5 (5 μm) column (15 cm x 0.4 cm i.d.) (Nomura Chemical Co., Seto, Japan) was used at ambient temperature. The pH of the mobile phase was adjusted with H_3PO_4. The concentration of CD added to the mobile phase was limited to less than 9×10^{-3} M, because of its sparing solubility. The dead time was determined by the use of $NaNO_3$ (UV) [7].

RESULTS AND DISCUSSION

Effect of CD in the Mobile Phase on the Retention

The effects of α-, β-, γ- and Me-β-CD contents in the mobile phase on capacity factor (k') of 16-epiestriol (16-epiE_3) [8] were examined (Figure 1). Among the CDs examined, β-CD was most effective in decreasing the k' value. On the basis of these data, further study was carried out with β-CD. The k' values of estrogens and their conjugates decreased with an increasing concentration of β-CD in the mobile phase. As illustrated in Figure 2, the elution order was reversed by the addition of β-CD to the mobile phase. It is of interest that the formation of the inclusion complex from the solute and β-CD exhibits such a characteristic pattern. It would be helpful to identify unambiguously the peak on the chromatogram obtained with biological samples. The interpretation of these phenomena has previously been discussed by Hinze [1].

Separation of Catechol Estrogens

In the previous paper, we reported the HPLC separation of catechol estrogens (2-OHE_1, 2-OHE_2, 4-OHE_1, 4-OHE_2) on Hitachi

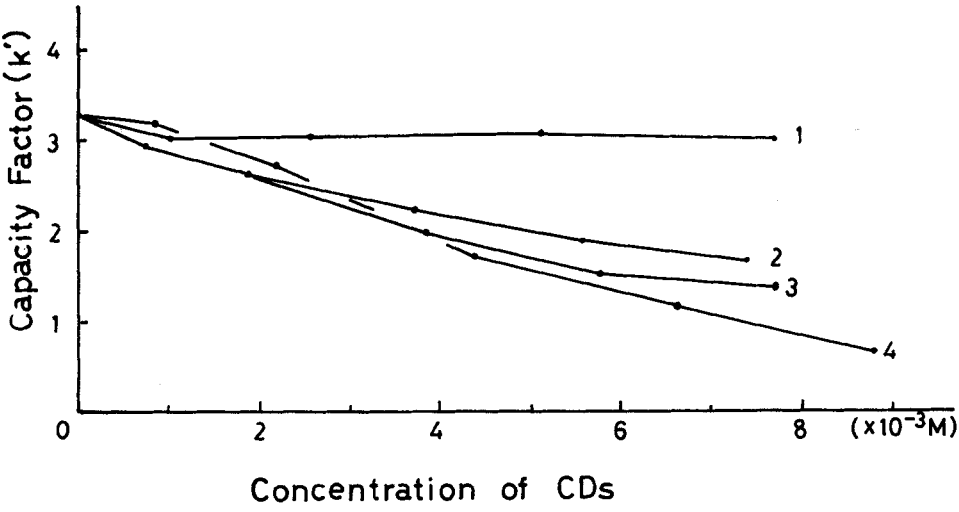

FIGURE 1. Effect of CD on the Retention of 16-epiE_3.
1: α-CD, 2: Me-β-CD, 3: γ-CD, 4: β-CD.
Conditions: solute (5 μg); mobile phase, acetonitrile/H_2O (1:2) containing each CD as indicated; detection, UV (280 nm); t_0=1.4 min.

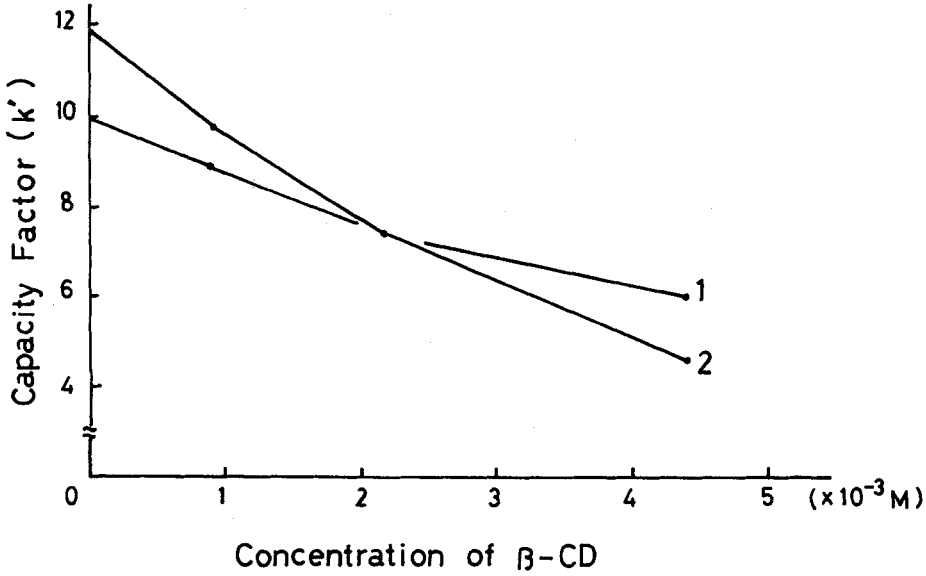

FIGURE 2. Effect of β-CD on the Retention of Catechol Estrogens.
1: 2-OHE_1, 2: 4-OHE_1.
Conditions: solute (each 5 μg); mobile phase, acetonitrile/0.06 M AcONa (pH 4.0) (1:2) containing β-CD as indicated; detection, UV (280 nm); t_0=1.4 min.

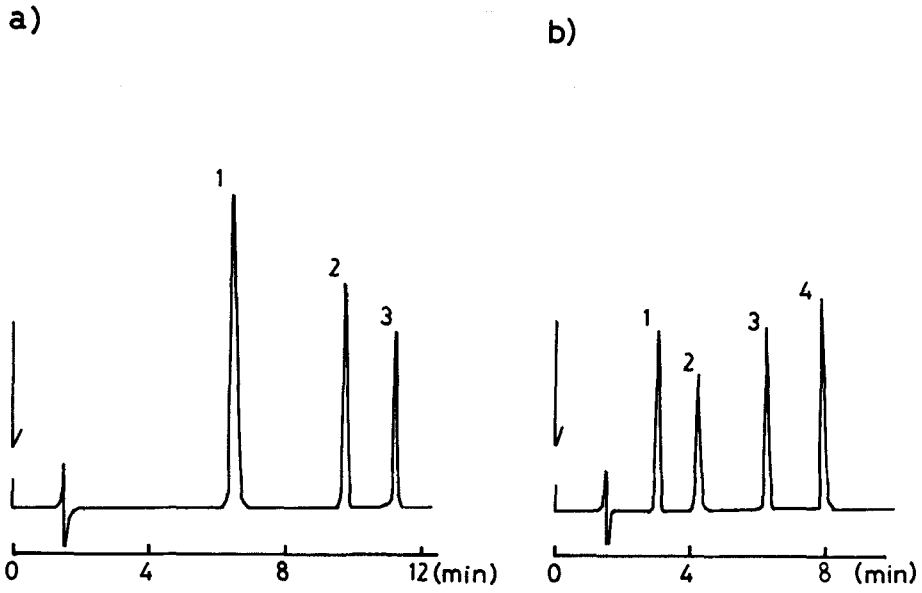

FIGURE 3. Separation of Catechol Estrogens.
a) 1: 2-OHE_2, 4-OHE_2, 2: 2-OHE_1, 3: 4-OHE_1;
b) 1: 4-OHE_2, 2: 2-OHE_2, 3: 4-OHE_1, 4: 2-OHE_1.
Conditions: mobile phase, a) acetonitrile/0.06 M AcONa (pH 4.0) (1:2), b) acetonitrile/0.06 M AcONa (pH 4.0) (1:2) containing 4.4 x 10^{-3} M β-CD; detection, UV (280 nm).

gel 3053 with methanol/0.5% ammonium dihydrogen phosphate (pH 2.5) (11:15) [9]. In that experiment, prolonged time (ca. 40 minutes) was needed to attain the complete resolution (Rs > 1.25) of each peak and the high pressure (ca. 280 kg/cm^2) was required to deliver the mobile phase at a flow rate of 1 ml/min. In order to overcome these problems, the mobile phase fortified with β-CD was used. As shown in Figure 3, the addition of β-CD (4.4 x 10^{-3} M) to the mobile phase improved the resolution of 2-OHE_2 and 4-OHE_2, where acetonitrile was employed as the organic modifier instead of methanol and the pressure was below 150 kg/cm^2.

TABLE 1
Effect of β-CD on the Retention of Guaiacol Estrogens

Guaiacol Estrogens	k' Values	
2-OMeE$_2$	8.5[1)]	4.5[2)]
2-OH 3-OMeE$_2$	10.0	3.0
2-OMeE$_1$	13.8	7.8
2-OH 3-OMeE$_1$	14.8	6.4
4-OMeE$_2$	1.8[3)]	1.7[4)]
3-OMe 4-OHE$_2$	2.9	2.3
4-OMeE$_1$	2.9	3.0
3-OMe 4-OHE$_1$	4.8	4.4

Conditions: solute (each 5 μg); mobile phase, 1) acetonitrile/H_2O (4:5), 2) acetonitrile/H_2O (4:5) containing 8.8×10^{-3} M β-CD, 3) acetonitrile/H_2O (5:4), 4) acetonitrile/H_2O (5:4) containing 3.5×10^{-3} M β-CD; detection, UV (280 nm); t_0=1.3 min.

Separation of Guaiacol Estrogens

The separation of guaiacol estrogens (2-OMeE$_1$, 2-OMeE$_2$, 2-OH 3-OMeE$_1$, 2-OH 3-OMeE$_2$; 4-OMeE$_1$, 4-OMeE$_2$, 3-OMe 4-OHE$_1$, 3-OMe 4-OHE$_2$) has been previously established by reversed-phase HPLC for determining the catechol O-methyltransferase activity using 2-OHE$_1$ or 4-OHE$_1$ as a substrate. However, the proposed method required more than thirty minutes for the complete separation of the products [6]. The addition of β-CD to the mobile phase resulted in a reversal of the elution order and a decrease in the retention time (t_R < 12 min), improving the separation of these compounds (Rs > 1.25) (Table 1).

TABLE 2
Effect of β-CD on the Detector Response[1)]

β-CD Concentration ($\times 10^{-3}$ M)	Detector		
	UV	ECD	FL
0	1.0	1.0	1.0
0.9	1.0	0.9	1.1
2.2	1.0	0.8	1.2
4.4	1.0	0.5	1.2

1) Peak area ratio relative to the value obtained with 0 M β-CD. Conditions: solute, E_3 (UV, 5 μg; ECD, FL, 200 ng); mobile phase, acetonitrile/H_2O (1:4) containing β-CD; detection, UV (280 nm), ECD (800 mV), FL (λex 280 nm, λem 310 nm).

Separation of Estrogen Glucuronides

The chromatographic separation of E_3 16G and 17G is a difficult problem due to their close similarity in physical properties. The separation of these two has been attained previously by HPLC on TSK-GEL LS-410 ODS-SIL with tetrahydrofuran/0.7% disodium hydrogen phosphate (pH 3.0) (1:6). The proposed method, however, required more than thirty minutes and high pressure (ca. 300 kg/cm^2) at a flow rate of 1 ml/min [10]. The addition of β-CD (4.4×10^{-3} M) to the mobile phase (organic modifier: acetonitrile) gave a significant resolution (Rs 1.1) of these two (t_R < 10 min), where the pressure required was below 150 kg/cm^2. A typical chromatogram of these compounds and other related estrogen glucuronides is shown in Figure 4. The present method is applicable to the determination of estrogen glucuronides in biological fluids.

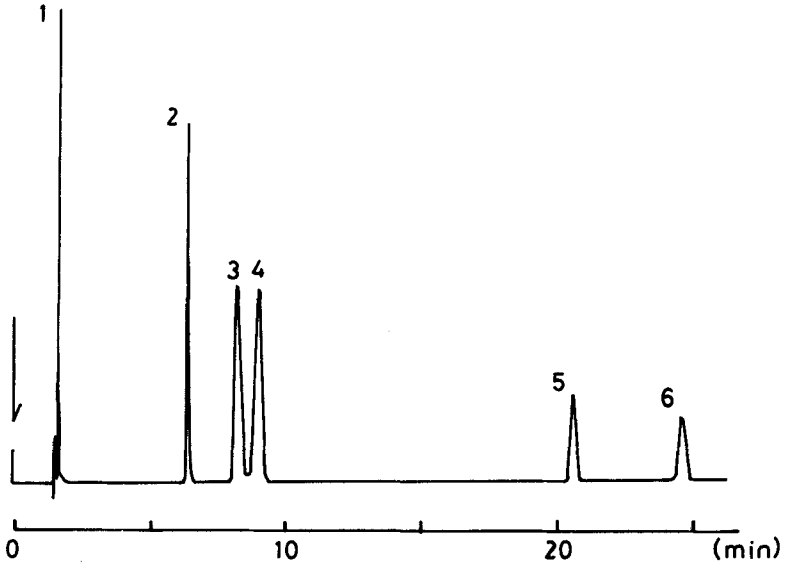

FIGURE 4. Separation of Estrogen Glucuronides.
1: E_3 3G (solvent front), 2: E_2 3G, 3: E_3 16G, 4: E_3 17G, 5: E_1 3G, 6: E_2 17G.
Conditions: mobile phase, acetonitrile/0.024 M AcONa (pH 4.0) (1:18) containing 4.4×10^{-3} M β-CD; detection, UV (280 nm).

Effect of β-CD on the Detector Response

It has been reported that CD serves to enhance and stabilize the fluorescence intensity of dansyl amino acids on a silica gel layer [11]. The fluorescence enhancement was also observed upon inclusion of coumarin derivatives with β-CD [12]. On the other hand Matsue et al. reported that the complexation with β-CD retards the electron transfer rate [13]. These findings prompted us to examine the effect of β-CD concentration in the mobile phase on the detector response. The results obtained are listed in Table 2. The fluorescence intensity was enhanced by the addition of β-CD to the mobile phase whereas the reversed effect was

observed with ECD. The coexistence of CD did not interfere with ultraviolet detection.

In conclusion the use of β-CD in the mobile phase affords an advantage to the separation of isomeric estrogens and their fluorescence detection in the reversed-phase HPLC. β-CD is readily available and exerts no effects on the reproducibility of retention time and column life.

Further applications of this method to other steroids and related compounds are being conducted in these laboratories, and the details will be reported elsewhere.

ACKNOWLEDGEMENTS

The authors are indebted to Misses E. Himuro, Y. Matsuda (Kanazawa University) and E. Haniuda (Tohoku University) for their technical assistances. Thanks are also due to Mrs S. Nagatomo (Nihon Shokuhin Kako Co., Ltd.), T. Nemoto (Kao Co.), and S. Honma (Teikoku Hormone Mfg. Co.) for supplying the materials. This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan.

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