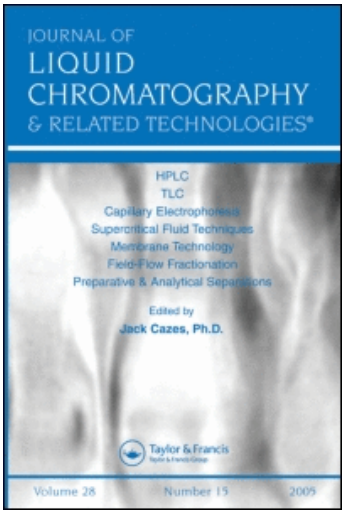

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High-Performance Liquid Chromatographic Separation of Equilin, Estrone, and Estrone Derivatives with Cyclodextrins as Mobile Phase Additives

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To cite this Article Spencer, Brian J. and Purdy, William C.(1995) 'High-Performance Liquid Chromatographic Separation of Equilin, Estrone, and Estrone Derivatives with Cyclodextrins as Mobile Phase Additives', Journal of Liquid Chromatography & Related Technologies, 18: 20, 4063 — 4080
To link to this Article: DOI: 10.1080/10826079508013745
URL: http://dx.doi.org/10.1080/10826079508013745

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(20), 4063-4080 (1995)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF EQUILIN, ESTRONE, AND ESTRONE DERIVATIVES WITH CYCLODEXTRINS AS MOBILE PHASE ADDITIVES

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ABSTRACT

β-Cyclodextrin and two derivatized β-cyclodextrins were utilized as mobile phase additives for the liquid chromatographic resolution of equilin and estrone, as well as estrone from 2-hydroxyestrone, 4-hydroxyestrone, and 16α-hydroxyestrone on a C_{18} stationary phase. β-Cyclodextrin proved to be suitable for the separations of these steroids but the modified β-cyclodextrin provided better resolution. A comparison was made of the separation and retention obtained with β-cyclodextrin with that of the modified β-cyclodextrins. Apparent inclusion complex strengths for estrone and its derivatives with each cyclodextrin were calculated. The effects of mobile phase cyclodextrin concentration and methanol content on selectivity and capacity factor were examined.

INTRODUCTION

Cyclodextrins are cyclic oligosaccharides formed by the enzymatic degradation of starch. They are comprised of α-(1,4)-linked D(+)-glucopyranose units which form a toroidal-shaped molecule having a hydrophobic cavity and a hydrophillic exterior face. The most common form of cyclodextrin used in high performance liquid chromatography is β-cyclodextrin, which consists of seven glucose units and forms a cavity of diameter 7.8 Å.

Cyclodextrins are used in high performance liquid chromatography to provide inclusion complex interactions between the cyclodextrin cavity and solute. They are employed both as a bonded stationary phase and as a mobile phase additive. They are used predominantly in the separation of optical isomers as the chiral environment at the cavity rim can be used as a chiral selector [1,2]. Cyclodextrins, however, can also be used for non-chiral separations for eluents that are difficult to separate, such as similarly-structured molecules [3]. The differences in inclusion complex strengths between solutes and the cyclodextrin cavity, as well as differences in the interaction with the rim functional groups, can result in improved chromatographic separation. It has been found that steroids have a structure suitable for the formation of inclusion complexes with β-cyclodextrins [4-6]. The formation of these inclusion complexes has allowed for

β-cyclodextrins to be utilized in the separation and analysis of steroids by HPLC [7-10].

The work presented here focuses on the use of β-cyclodextrin and modified β-cyclodextrins as mobile phase additives in the separation of steroid molecules. For the modified cyclodextrins, the hydroxyl groups on the rim of the cyclodextrin cavity are replaced with either methyl or hydroxyethyl groups which increases the hydrophobic character of the cyclodextrin cavity relative to the hydrophillic exterior. These differences will change the inclusion complex strength which can lead to greater selectivity.

This paper describes and characterizes the separation of equilin and estrone, two very similarly structured estrogens of importance to the pharmaceutical industry. The direct analysis of equilin and estrone without cyclodextrin has been reported [11]; however the use of cyclodextrins as a mobile phase additive greatly improves the resolution of these two molecules. To date there has been no report on the use of cyclodextrins in the separation of equilin and estrone. Also presented in this work is the separation of estrone from three of its related derivatives/metabolites, specifically 16α-hydroxyestrone (16α-HE), 2-hydroxyestrone (2-HE), and 4-hydroxyestrone (4-HE). A comparison of the separation obtained with unmodified β-cyclodextrin, methyl-β-cyclodextrin, and hydroxyethyl-β-cyclodextrin as mobile phase additives will be discussed along with the effects of varying cyclodextrin and methanol concentration.

EXPERIMENTAL

Apparatus

Chromatography was performed using a liquid chromatographic system consisting of a model M6000-A pump (Waters Assoc., Milford, MA, U.S.A.), a Model 7125 injector containing a 10 µL loop (Rheodyne, Cotati, CA, U.S.A.), and a Model LC290 UV detector (Perkin Elmer, Norwalk, CN, U.S.A.). The chromatograms were recorded on a Model DE120 strip chart recorder (Goerz Electro, Austria). The column used with the mobile phase additives was a 5 µm Zorbax ODS (150 x 4.6mm I.D.), purchased from Chromatographic Specialties (Brockville, ON, Canada). The β-cyclodextrin column was a Cyclobond I (250 x 4.6 mm I.D.), purchased from Advanced Separation Technologies (Whippany, NJ, U.S.A.). When not in use, the columns were stored in 100% methanol.

Chemicals

Equilin, estrone, 2-hydroxyestrone, 4-hydroxyestrone, and 16α-hydroxyestrone were obtained from Sigma (St. Louis, MO, U.S.A.). β-Cyclodextrin (β-CD), heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD), and hydroxyethyl-β-cyclodextrin (HE-β-CD) were obtained from Aldrich (Milwaukee, WI, U.S.A.). HPLC-grade methanol was purchased from Fisher (Fair Lawn, NJ, U.S.A.). Monobasic potassium phosphate, dibasic

potassium phosphate, and phosphoric acid were obtained from A&C Chemicals (Montreal, QC, Canada). Water was doubly distilled and deionized.

Procedures

The mobile phase was prepared by mixing methanol with potassium phosphate buffer. Cyclodextrin was dissolved and the mixture was degassed and filtered through a 0.45 μm membrane filter. The pH was lowered with 10% phosphoric acid and raised with dibasic potassium phosphate. The solutes were dissolved in methanol to give a concentration of about 1 mg/ml and the typical injection volume was 2 μl. The wavelength of detection was 200 nm.

Retention times were determined by averaging at least three separate determinations. A reproducibility study was conducted where six injections had an RSD of less than 1% for the capacity factor and of less than 2.5% for the resolution factor.

RESULTS AND DISCUSSION

In this investigation, the chromatographic separation of equilin and estrone, as well as estrone from three of its metabolites/derivatives, was

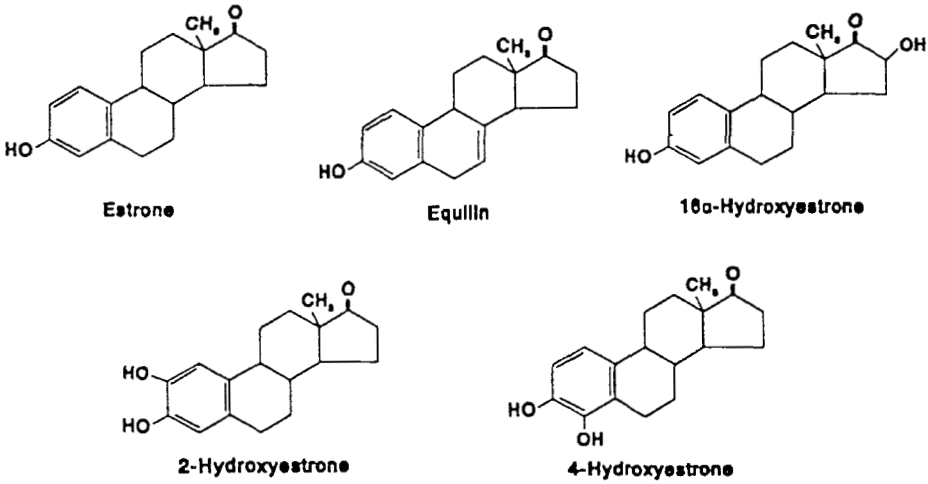

FIGURE 1 Steroids studied

examined using β-cyclodextrin, DM-β-cyclodextrin, and HE-β-cyclodextrin as mobile phase additives with a C_{18} stationary phase. Figure 1 presents the five molecules under investigation.

Without cyclodextrins or other additives in the mobile phase, the resolution of equilin and estrone is poor. The eluents' peaks were wide with no baseline separation. However, the presence of cyclodextrins in the mobile phase improves the separation of the two estrogens. Figure 2 compares the separation of equilin and estrone when the mobile phase additive was β-cyclodextrin, DM-β-cyclodextrin, and HE-β-cyclodextrin. β-Cyclodextrin provides baseline separation of the two steroids; however the resolution is greatly improved when DM- or HE-β-cyclodextrin is the mobile

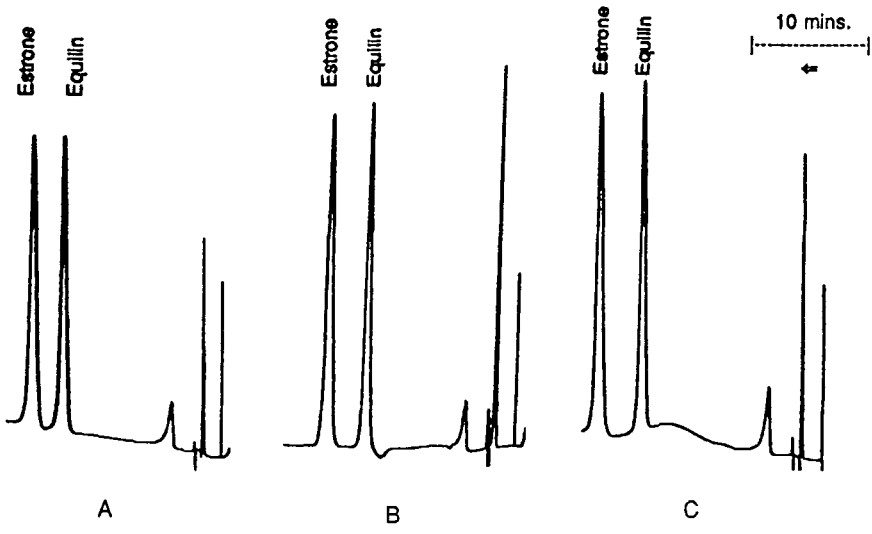

FIGURE 2 Comparison of cyclodextrin type in the mobile phase on separation of equilin and estrone. Mobile phase is 45:55 0.05M KH_2PO_4:methanol plus 5 mg/ml of indicated cyclodextrin. (A) β-CD; (B) DM-β-CD; (C) HE-β-CD.

phase additive. Evidently, the inclusion complex strength of equilin over estrone was changed significantly enough to give an improvement in resolution.

Retention times decrease when some form of cyclodextrin is present in the mobile phase, an indication that inclusion complexes are formed. Table 1 summarizes the separation and retention of equilin and estrone with various cyclodextrins in the mobile phase. A comparison is made with a β-cyclodextrin stationary phase using a comparable mobile phase organic modifier composition. Analogous separation on a β-cyclodextrin column

TABLE 1

Retention and Separation of Equilin and Estrone Comparing Different Cyclodextrins

Mobile Phase Additive	$k'_{EQUILIN}$	$k'_{ESTRONE}$	α
No Cyclodextrin	15.8	17.6	1.11
β-Cyclodextrin	9.09	10.9	1.20
DM-β-Cyclodextrin	8.43	10.9	1.29
HE-β-Cyclodextrin	10.6	13.5	1.27
β-Cyclodextrin Stat. Phase	2.33	1.93	1.21

* All mobile phases were composed of 45:55 0.05M KH_2PO_4:methanol with 5 mg/ml of the indicated cyclodextrin. In the case of the β-cyclodextrin stationary phase, the mobile phase is 60:40 0.05M KH_2PO_4:methanol.

required very short retention times. Increasing the aqueous ratio in the mobile phase to increase solution retention resulted in broad peaks. The elution order of equilin and estrone is reversed when comparing the cyclodextrin mobile phase to a cyclodextrin stationary phase, further evidence of the formation of inclusion complexes.

Figure 3 shows an example of how the various β-cyclodextrin forms affect the resolution of estrone, 2-hydroxyestrone, 4-hydroxyestrone, and 16α-hydroxyestrone. The unmodified β-cyclodextrin provides suitable separation of the 2- and 4-hydroxyestrone isomers however there is barely baseline separation of the 4- and 16α-isomers. Replacing the β-cyclodextrin with DM-β-cyclodextrin allows for better separation of the 4- and 16α-

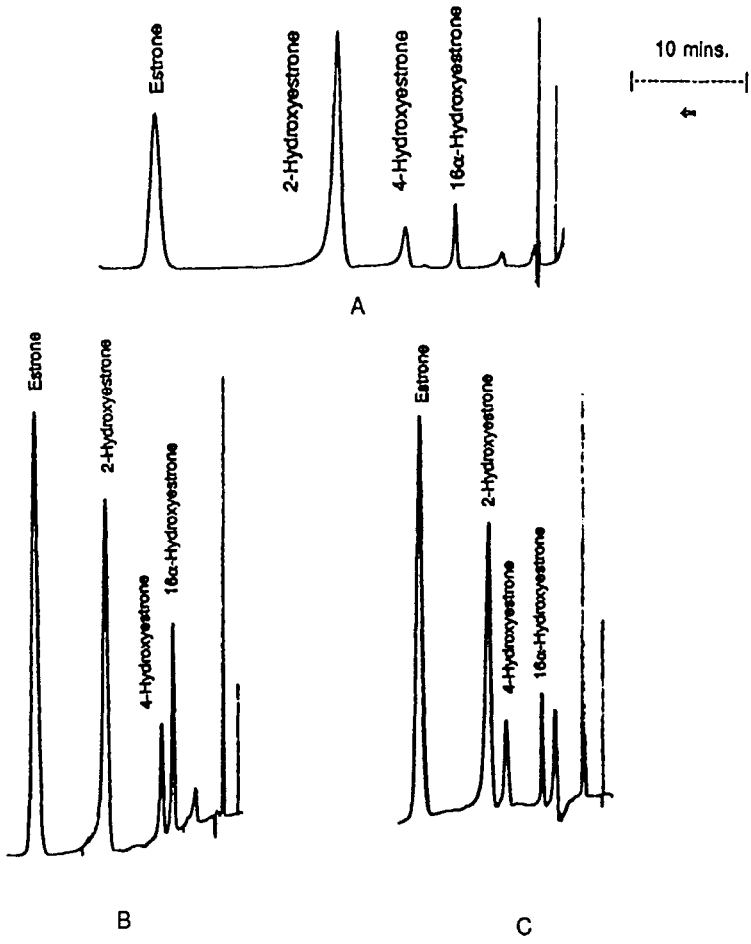

FIGURE 3 Comparison of cyclodextrin type in the mobile phase on separation of estrone from its derivatives. Mobile phase is 55:45 0.05M KH_2PO_4:methanol plus 5 mg/ml of indicated cyclodextrin. (A) HE-β-CD; (B) β-CD; (C) DM-β-CD.

isomers but does not separate the 2- & 4-isomers as well as the unmodified β-cyclodextrin. HE-β-cyclodextrin as the mobile phase additive yields the best separation for estrone, 16α-hydroxyestrone, 2-hydroxyestrone, and 4-hydroxyestrone. In all cases, estrone is well separated from its hydroxy

TABLE 2

Comparison of Cyclodextrins for Estrone and its Derivatives

Mobile Phase Additive	$k'_{ESTRONE}$	$k'_{2\text{-}HE}$	$k'_{4\text{-}HE}$	$k'_{16\alpha\text{-}HE}$	$\alpha_{2\text{-}\&4\text{-}HE}$
No Cyclodextrin	88.8	44.5	45.5	17.2	1.02
β-Cyclodextrin	16.3	10.3	5.49	4.63	1.88
DM-β-CD	13.0	7.92	6.63	3.92	1.19
HE-β-CD	29.4	15.6	10.6	6.92	1.47

• All mobile phases are 55:45 0.05M KH_2PO_4:methanol with 5 mg/ml of the indicated cyclodextrin.

derivatives. Table 2 shows the relationship between capacity factor for estrone and its three derivatives and the type of cyclodextrin used in the mobile phase. Table II also lists how each type of cyclodextrin affects the separation factor for the similarly structured 2- and 4-hydroxyestrones. As was noted in Figure 3, β-cyclodextrin provides the best separation of the 2- and 4-isomers.

Effect of Cyclodextrin Concentration

Figure 4 shows the effect of mobile phase DM-β-cyclodextrin concentration on the separation of equilin and estrone. The addition of even a small amount of DM-β-CD results is an considerable increase in resolution.

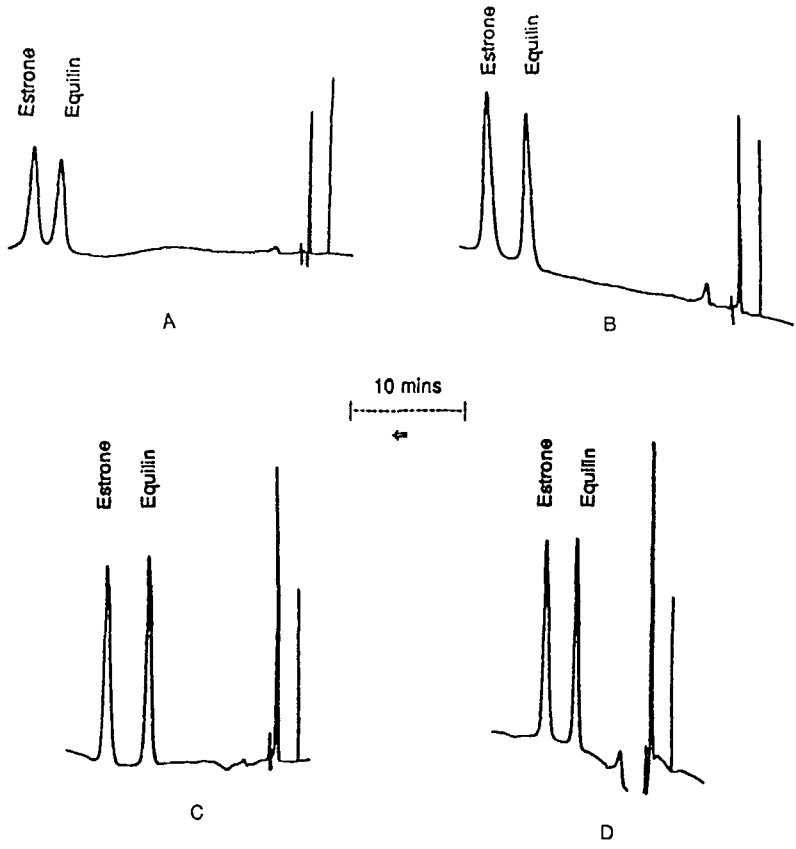

FIGURE 4 Effect of cyclodextrin concentration on the separation of equilin and estrone. (A) 0 mg/ml; (B) 1 mg/ml; (C) 5 mg/ml; (D) 13 mg/ml methyl-β-cyclodextrin. Mobile phase is 45:55 0.05M KH_2PO_4.

As the cyclodextrin concentration is increased, the capacity factors for equilin and estrone decrease dramatically indicating the formation of relatively strong inclusion complexes. The reduction in capacity factor is accompanied by an increase in separation factor. Table 3 lists the effect of

TABLE 3

Effect of DM-β-Cyclodextrin Concentration on Separation of Equilin and Estrone

[DM-β-CD] (mg/ml)	α	Rs
0.0	1.11	1.25
1.0	1.16	2.34
2.0	1.21	2.70
5.0	1.31	2.94
10.0	1.38	3.05
13.0	1.38	3.06

* All mobile phases were composed of 45:55 0.05M KH_2PO_4:methanol with the indicated amount of cyclodextrin.

DM-β-cyclodextrin on the separation of equilin and estrone. The immediate increase in separation and resolution factors is followed by a levelling off area where increases in cyclodextrin concentration no longer affect resolution. Each steroid has a different affinity for the cyclodextrin cavity, thus varying the concentration will alter the elution rate of each solute along the column. The presence of the double bond on the B-ring in equilin, which is absent in estrone, will result in a more rigid structure compared to estrone. This is an indication that the steroid geometry plays a role in inclusion complex formation as the double bond is the only difference between the two compounds. The more rigid structure of equilin forms a stronger inclusion complex than the more flexible estrone.

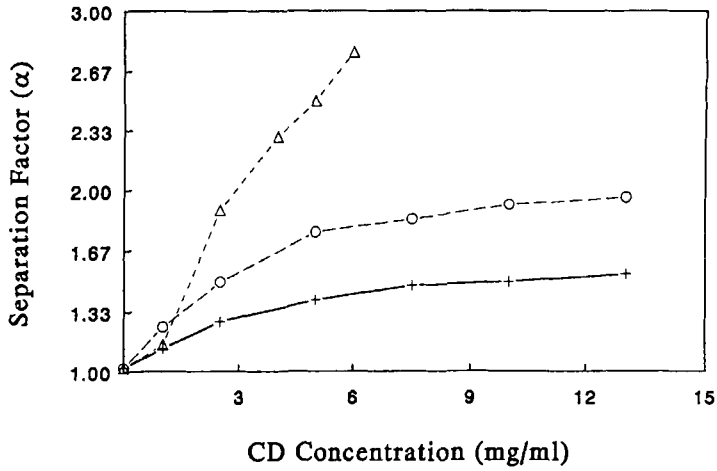

FIGURE 5 Effect of cyclodextrin concentration of the separation of 2- and 4-hydroxyestrone.Mobile phase is 60:40 0.05M KH_2PO_4:methanol plus indicated amount of cyclodextrin. + = DM-β-CD; Δ = β-CD; O = HE-β-CD.

Figure 5 shows the effect of mobile phase concentration of the three different types of cyclodextrins on the resolution of 2- and 4-hydroxyestrone. β-Cyclodextrin had the greatest effect on resolution, achieving a dramatic increase in resolution. Due to its limited solubility, β-cyclodextrin has a maximum mobile phase concentration of 6 mg/ml at this methanol concentration. Increases in separation were also noted for DM-β-cyclodextrin and HE-β-cyclodextrin as mobile phase additive, but not of the same extent as β-cyclodextrin.

Using the relationship between capacity factor and cyclodextrin concentration developed by Fujimura *et al.* [12], we can calculate the

apparent formation constant (K_f) for the inclusion complex from the following equation:

$$\frac{1}{k'} = \frac{1}{k_0'} + \frac{[CD]\,K_f}{k_0'}$$

where [CD] is the cyclodextrin concentration in the mobile phase and k_0' is the capacity factor for a solute that does not form an inclusion complex. A plot of 1/k' *versus* cyclodextrin concentration gives a straight line from whose slope the formation constant can be determined. It should be noted that these apparent formation constants are dependant on the methanol concentration in the mobile phase as a change in the mobile phase polarity will affect the inclusion complex strength [7]. Table 4 summarizes the apparent formation constants of estrone and its derivatives with the three types of cyclodextrin studied. The methanol content of the mobile phase was 45% v/v. Three of the four steroids formed a stronger inclusion complex with the unmodified β-cyclodextrin while 2-HE formed a stronger complex with the DM-β-CD. In all cases, inclusion complex strength was weaker with the HE-β-cyclodextrin than with the other two forms. The difference in inclusion complex strength between 2- and 4-HE suggests that inclusion complex formation occurs with the A-B rings of the steroids. However, the differences in inclusion complex strength between estrone and

TABLE 4

Apparent Formation Constants for Estrone and it's Derivatives

Mobile Phase Additive	K_f (M) Estrone	2-HE	4-HE	16α-HE
β-Cyclodextrin	622	409	1138	482
DM-β-Cyclodextrin	713	481	849	466
HE-β-Cyclodextrin	376	318	714	278

* All mobile phases were composed of 55:45 0.05M KH_2PO_4:methanol

16α-hydroxyestrone implies that an inclusion complex also forms with the C-D rings, so selectivity can be based on differences in both ring systems.

Inclusion complex strength alone cannot be used to predict retention in this system as the solutes may also interacting distinctively with the stationary phase.

Effect of Methanol Concentration

The retention profiles of all five steroids followed the reverse-phase model where the capacity factor of all the steroids decreased with increasing mobile phase methanol concentration [13]. The resolution of equilin and estrone also decreased with increasing methanol concentration. Increasing the organic content of the solvent will weaken the strength of the inclusion

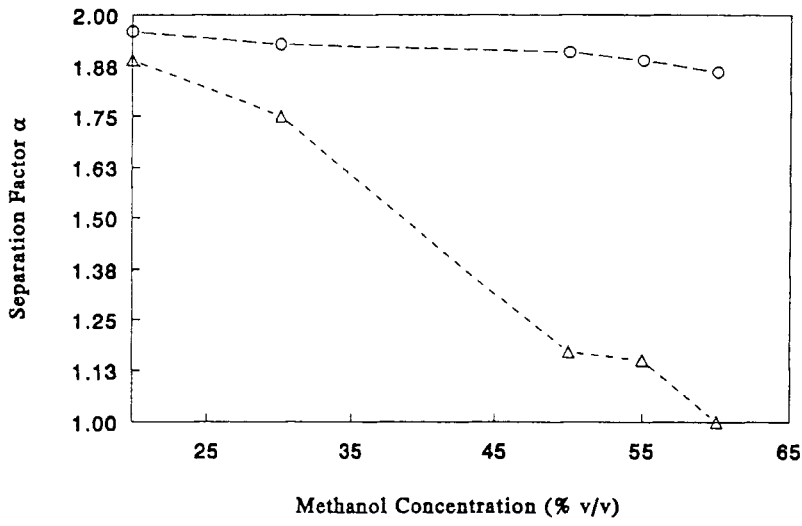

FIGURE 6 Effect of methanol concentration on the separation of 2- & 4-hydroxyestrone and 4- & 16α-hydroxyestrone. Mobile phase contains 4 mg/ml DM-β-cyclodextrin. O = $\alpha_{4\text{-},16\alpha\text{-HE}}$; Δ = $\alpha_{2\text{-},4\text{-HE}}$.

complex formed between the guest and the cyclodextrin [14]. Figure 6 shows the effect of methanol concentration on the separation of 2- & 4-HE and 4- & 16α-HE using DM-β-cyclodextrin as the mobile phase additive. The separation of the 2- and 4-hydroxy isomers declines with increasing methanol concentration until there is no separation at 60% methanol content. For the separation of 4- and 16α-HE, however, the reduction in retention is not accompanied by a loss in separation as the methanol concentration did not alter their relative inclusion complex strengths.

CONCLUSION

Cyclodextrins as a mobile phase additive have proven to be useful in the separation of two similarly structured steroids, equilin and estrone. There was an increase in resolution when β-cyclodextrin was used as a mobile phase additive. There was a further increase in resolution when the cyclodextrin was modified with methyl or hydroxyethyl groups. β-Cyclodextrin, DM-β-cyclodextrin, and HE-β-cyclodextrin were also suitable as mobile phase additives in the separation of estrone, 16α-hydroxyestrone, 2-hydroxyestrone, and 4-hydroxyestrone. Inclusion complexes can be formed with the A-B rings and/or with the C-D rings of the steroids.

ACKNOWLEDGEMENTS

The authors are indebted to the Natural Science and Engineering Research Council of Canada and the Fonds Pour la Formation de Chercheurs et l'Aide à la Recherche for the financial support of this work.

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Received: April 20, 1995
Accepted: May 9, 1995