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Effect of temperature on separation of estradiol stereoisomers and equilin by liquid chromatography using mobile phases modified with β-cyclodextrin*

H. LAMPARCZYK† and P.K. ZARZYCKI

Medical Academy, Faculty of Pharmacy, Gen. J. Hallera 107, PL-80-416 Gdańsk, Poland

Abstract: The unusual temperature effect on the retention of 17α-estradiol, 17β-estradiol and equilin in reversed-phase liquid chromatography has been observed. Capacity factors (k') of the steroids were measured using mobile phase with different concentrations of β-cyclodextrin (from 0 to 16 mM) in a fixed mobile phase composition (acetonitrile–water) and wide range of column temperatures (from 5° to 80°C). The plots of capacity factors vs reciprocal of absolute temperature are nonlinear in each case. At subambient temperatures the capacity factors decreased with temperature decrease. This effect is more evident for the natural 17β-estradiol than for its 17α-isomer.

Keywords: *Liquid chromatography; cyclodextrin modified mobile phases; temperature effect; estradiol stereoisomers; equilin.*

Introduction

Plots of the logarithms of capacity factors against the reciprocal of absolute temperature are usually linear and are known as Van't Hoff plots [1–3], shown in equation (1)

$$\ln k' = \Delta H/T + \Delta S + \ln \phi, \qquad (1)$$

where k' denotes capacity factor, ΔH enthalpy change, ΔS entropy change and ϕ phase ratio of the column.

Nevertheless, any reversible process which alters the enthalpy or entropy of adsorption, in principle gives rise to nonlinear Van't Hoff plots. Among others, changes in conformation, or changes in the extent to which the mobile phase interacts with either the analyte or stationary phase are examples of such reversible behaviour [4, 5]. Moreover, the presence of multiple types of retention mechanisms or multiple types of binding sites also leads to non-linearity of the Van't Hoff plots. Particularly in the case of chiral recognition the multiple types of retention as well as the importance of conformation can be expected and, therefore, the effect of temperature on retention might be very complex.

Cyclodextrins (CDs) are toroidal shaped cyclic oligomers of α-1,4-D-glucopyranose units and they are well known as chiral selectors [6, 7]. The name "cyclodextrins" currently comprises a large group of α-, β-, and γ-CDs together with their numerous derivatives, however, β-cyclodextrin (β-CD) is presently the most used CD. In LC CDs have been used both chemically bonded to a stationary phase and added to the mobile phase [8, 9].

Despite the number of papers dealing with various applications of CDs in chromatography, including chiral separations, the knowledge of stereoselectivity and structural relationships between CDs and guest molecules is poor. Many factors seem to be responsible for the separation including type of CD used [10], its concentration in mobile phase [10, 11] and type of mobile phase itself [10–12]. Generally, in LC little attention has been focused on temperature. Hence, only a few workers have studied the effect of temperature on stereoselectivity in LC phases modified with CDs [10, 13–16].

Therefore, in this work we report the influence of mobile phase composition, concentration of β-CD and temperature on separation of 17α-estradiol, 17β-estradiol and equilin

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†Author to whom correspondence should be addressed.

which have been chosen as model compounds. Stereoisomers, 17α- and 17-β-estradiol differ only in the position of the hydroxyl group at C-17, and as it was recently found their retention behaviour is strongly influenced by the composition of the mobile phase [17].

Experimental

Reagents

Steroid standards (17α-estradiol, 17β-estradiol and equilin) were obtained from Aldrich Chemical Company, Inc. β-CD was supplied by Chinoin (Budapest, Hungary) and purified by recrystallization from boiling water. Sodium nitrate was obtained from a commercial supplier. Acetonitrile (Merck, Darmstadt, Germany) was of LC grade. Water was purified by double distillation. Mobile phases were filtered through a 1.5 μm membrane prior to use.

The mobile phases used were mixtures of acetonitrile–water (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 25:75, 20:80% v/v). The β-CD was added to mobile phase (30:70, v/v, acetonitrile in water) to give a final concentration of 2, 4, 6, 8, 10, 12, 14 and 16 mM. The stock solutions of standards were prepared in acetonitrile at a concentration of 1 mg ml^{-1}. From these stock solutions, appropriate injection standard solutions at a concentration of 100 μg ml^{-1}, were prepared by mixing the required volume of the stock solution and chromatographic mobile phase. The injection volume was 20 μl for all solutions.

Chromatography

The ODS-2 column (120 × 4 mm i.d.; particle size 5 μm) was obtained from Knauer. The liquid chromatograph consisted of an analytical solvent pump (Knauer's A0307), UV–Vis spectrophotometer (A0293), column box and linear recorder (Knauer). A Rheodyne Model 7125 injection valve and a 20-μl loop were used for sample introduction. The UV detector was operated at 280 nm. The flow rate was 1 ml min^{-1}. The void volume was determined by injecting sodium nitrate solution, at a concentration of 10 μg ml^{-1}. The dead retention time was 39.5 s.

The column temperature was controlled by immersing the column in a stirred constant-temperature bath containing ethanol–water (30:70, v/v) used as a heat-exchange medium. The bath was connected to a thermostat adjustable from 5 to 80°C. Temperature was controlled with an accuracy of ±0.5°C. Additionally, the bottle with mobile phase was thermostated 1 h before and during the experiment in order to obtain proper temperature equilibrium. The retention parameters for each solute were measured at temperatures of 80, 75, 70, 60, 50, 40, 30, 25, 20, 15, 10 and 5°C.

The capacity factors were calculated in the usual manner and are based on the average of at least five independent determinations of each solute.

Results and Discussion

The influence of mobile phase composition (without CD) on the apparent capacity factors (k') has been studied using the following acetonitrile:water mixtures; 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 25:75 and 20:80% v/v. There were no dramatic differences in the k' vs % acetonitrile curves for the three drugs studied. In general, the retention time decreased as the concentration of acetonitrile was increased. Due to low solubility of β-CD in organic solvents, a mobile phase containing 30% v/v of acetonitrile in water was chosen for further studies.

The analytes were chromatographed at different column temperatures from 5 to 80°C. The example chromatograms are shown on Figs 1–3. When unmodified mobile phase was used (Fig. 1) the retention time is very long, particularly at lower temperature. At low (2 mM) concentration of β-CD (Fig. 2) the retention times become approximately two times shorter and in concentration of 16 mM of β-CD, the retention time of the last solute (equilin) not extending 6 min (Fig. 3).

The influences of the β-CD concentration and temperature on the capacity factors for each solute are exemplified in Figs 4–6. Plots of the logarithms of the capacity factors against the reciprocal of absolute temperature show a linear Van't Hoff behaviour when unmodified mobile phase was used (see Table 1) and for modified mobile phases in higher temperatures. The temperature at which the deviation from linear Van't Hoff behaviour begins is strongly affected by the stereochemistry of the solute molecule and by the concentration of CD. Comparing 17α-estradiol with 17β-estradiol (Figs 4, 5) it can be found that for the

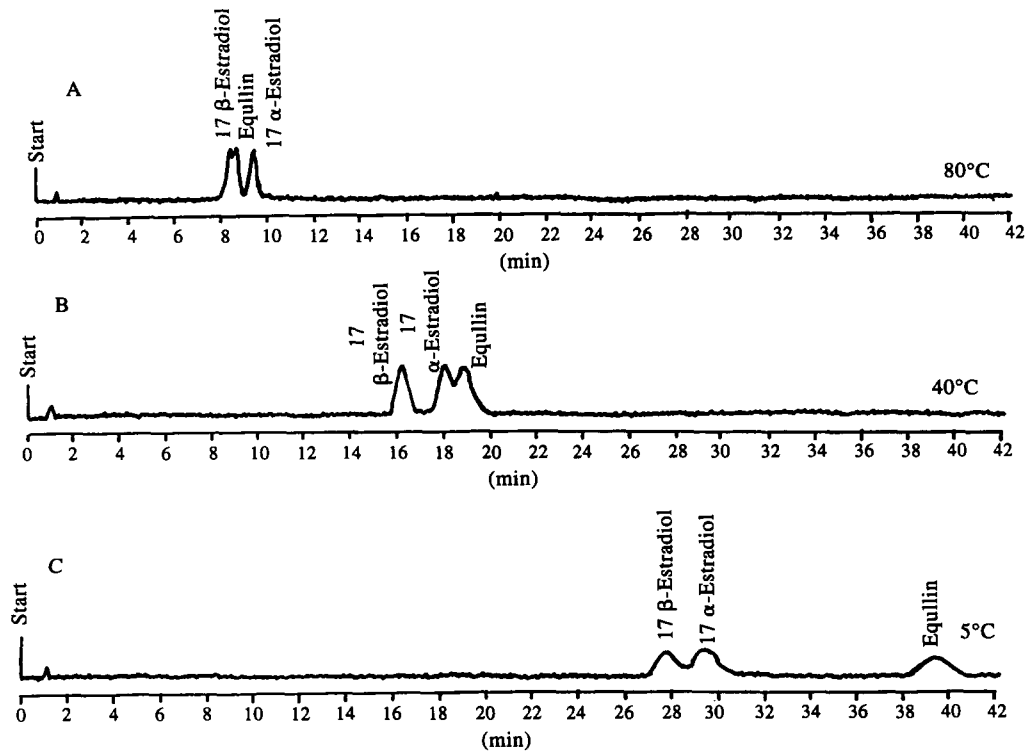

Figure 1
Separation of steroids at 80, 40 and 5°C. Mobile phase, acetonitrile–water 30:70 (v/v) without the addition of β-cyclodextrin. Column Knauer-ODS-2 (120 × 4.6 mm i.d.).

natural 17β-estradiol the deviation is much stronger. Similar nonlinearity of the Van't Hoff plots, when CD modified mobile phase was used, has been recently observed by us for 1,8-dimethylnaphthalenes and norgestrel optical isomers [10, 14]. The earliest report on the influence of temperature on norgestrel optical isomers separations was written by Gazdag *et al.* [16], but these studies were performed in a very narrow temperature range (25–50°C) and therefore the conclusions reached by these authors are incomplete. Considering the current experimental material and the results described in previous papers, the following suggestions on the separation mechanism on molecular level can be given. In the temperature range 80° to approximately 50°C, the degree of complexation with β-CD is very low and therefore the typical Van't Hoff plot is observed. The phenomenon that retention decreases with temperature increase was observed many times in LC and in gas-chromatography as well. On a molecular level it can be easily explained by faster migration of the solute molecules through the chromatographic column and their lower affinity to the stationary phase. Below approx. 50°C the inclusion mechanism starts to be important. As evidence, deviation from the Van't Hoff plot is observed, the retention time is shorter while the resolution is maintained. On a molecular level, retention behaviour in this temperature range, can be probably interpreted as being due to slower rotation of the guest and host molecules and hence a steric fit is possible. Recently, Cabrera and Labuda [18] have studied enantioselective LC separation of pharmaceuticals, oxazepam and Prominal, using chemically bonded β-CD stationary phase. They observed linear Van't Hoff plots but large differences in the chiral discrimination behaviour, described by $\ln \alpha$ vs $1/T$. They conclude that separation of Prominal enantiomers is entropy controlled whereas for oxazepam enthalpy controlled. This simple thermodynamic interpretation well confirms the basic ideas [4, 5]. On the other hand however, in the case of mobile phases modified by the addition of β-CD, the observed Van't Hoff plot is nonlinear [14] and, therefore, this phenomenon seems to be more complex.

The retention behaviour of estradiol stereoisomers and equilin has been studied by Olsson *et al.* [17]. They have used bonded β-CD

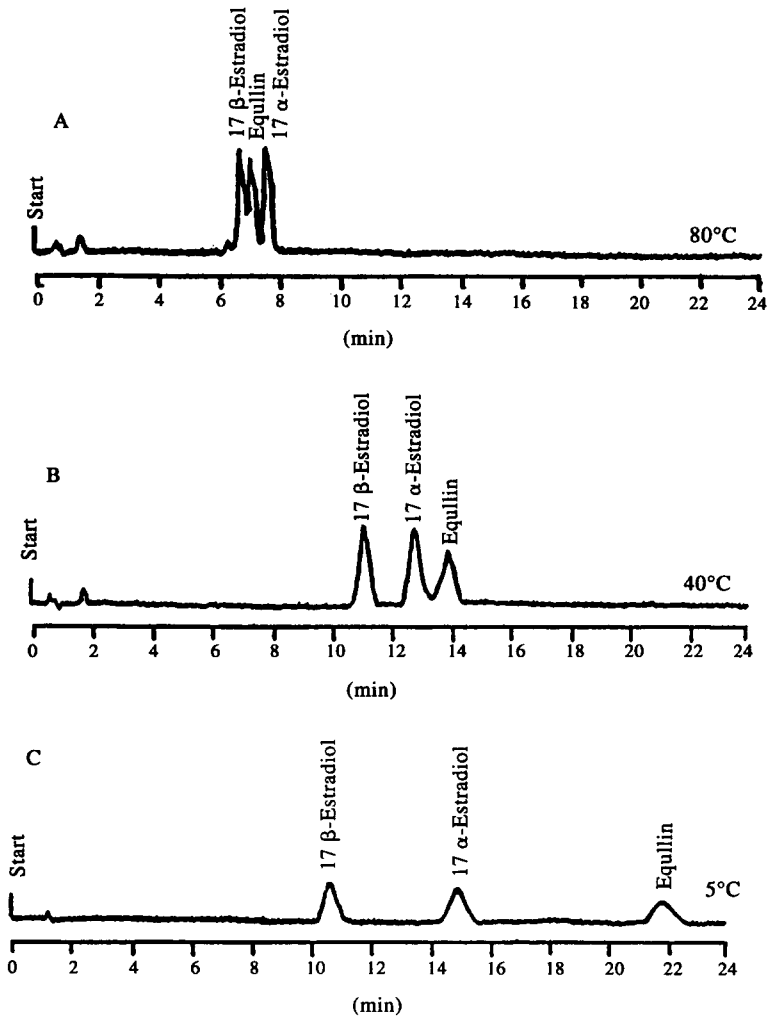

Figure 2
Separation of steroids at 80, 40 and 5°C. Mobile phase, acetonitrile–water 30:70 (v/v) modified with 2 mM β-cyclodextrin. Other chromatographic conditions as in Fig. 1.

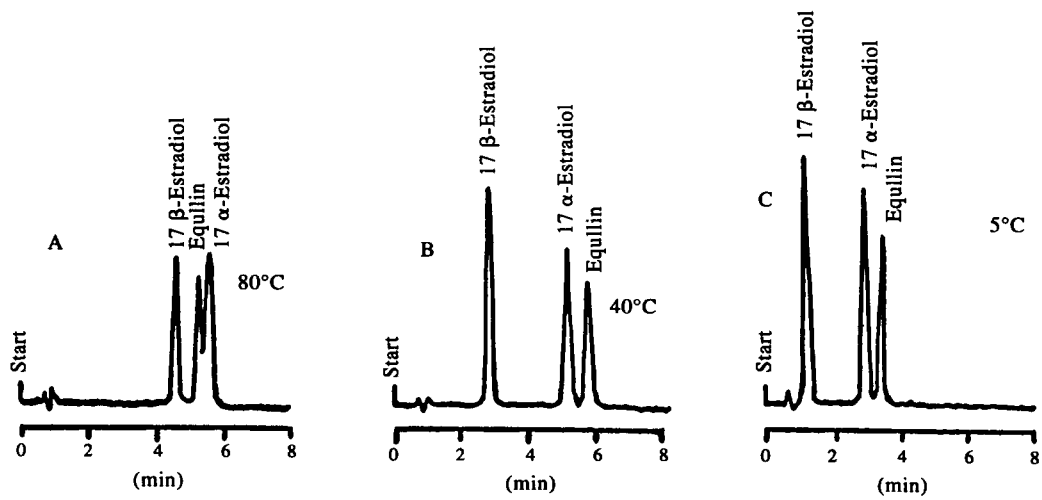

Figure 3
Separation of steroids at 80, 40 and 5°C. Mobile phase, acetonitrile–water 30:70 (v/v) modified with 16 mM β-cyclodextrin. Other chromatographic conditions as in Fig. 1.

Table 1
Regression coefficients (a, b) and correlation coefficient (r) of the regression equation log $k' = a(1000/T) + b$ for steroids, using unmodified acetonitrile–water mixture as mobile phase. The numbers in parentheses indicate the standard error at 95% significance level

Steroid	a	b	r
17α-estradiol	0.6893 (0.03)	−0.8285 (0.08)	0.9935 (0.02)
17β-estradiol	0.7248 (0.02)	−0.9886 (0.07)	0.9959 (0.02)
Equilin	0.9021 (0.02)	−1.4790 (0.05)	0.9986 (0.01)

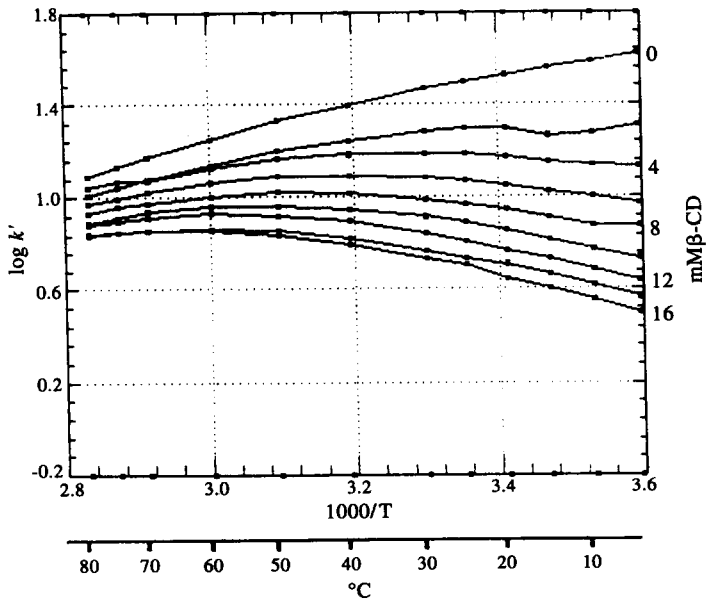

Figure 4
Plots of log k' vs $1000/T$ for 17α-estradiol at different concentrations of β-cyclodextrin in the mobile phase.

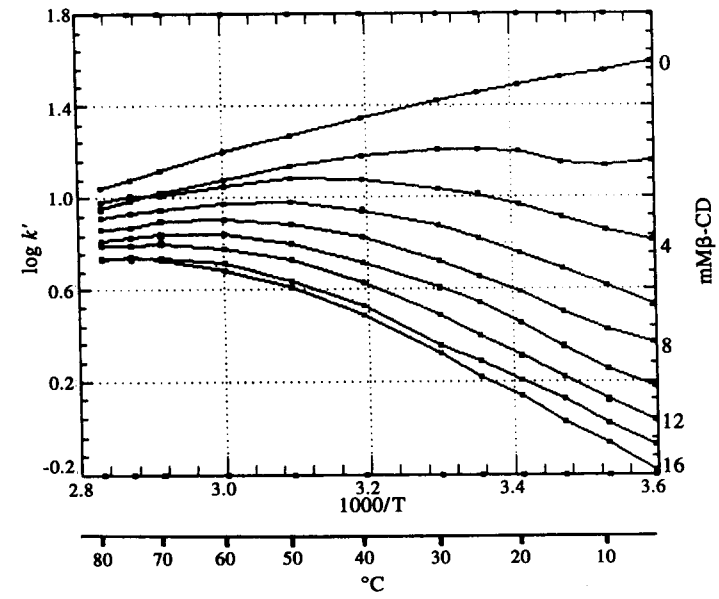

Figure 5
Plots of log k' vs $1000/T$ for 17β/estradiol at different concentrations of β-cyclodextrin in the mobile phase.

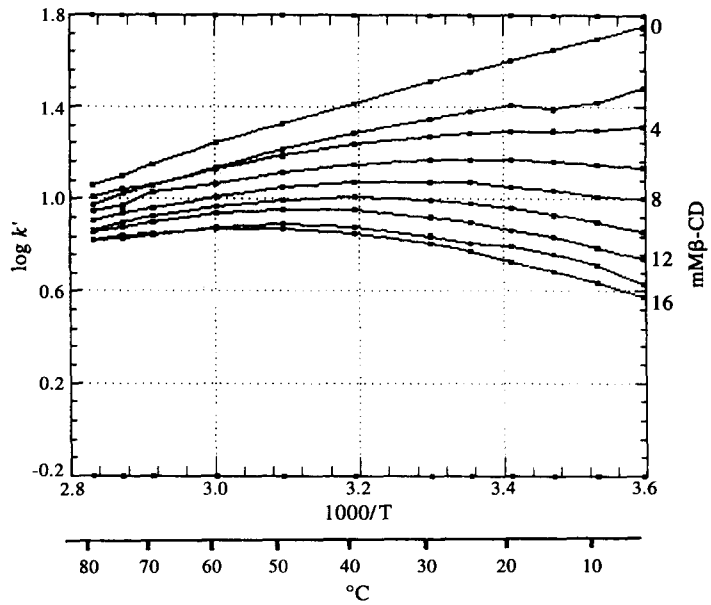

Figure 6
Plots of log k' vs $1000/T$ for equilin at different concentrations of β-cyclodextrin in the mobile phase.

Table 2
Resolutions (R_s) for estradiol stereoisomers and equilin at various temperatures and concentrations of β-cyclodextrin (β-CD) in the mobile phase

	Temperature (°C)	β-estr./α-estr. [R_s]	α-estr./equilin	β-estr./equilin
0 mM βCD	5	0.88	4.47	5.24
	40	1.63	0.66	5.24
	80	1.61	1.16	0.45
2 mM βCD	5	4.18	5.34	9.15
	40	1.91	1.13	3.04
	80	1.95	1.18	0.77
6 mM βCD	5	7.32	3.47	10.42
	40	3.94	1.45	5.28
	80	1.76	0.84	0.92
12 mM βCD	5	7.06	2.05	9.75
	40	5.06	1.28	6.57
	80	2.09	0.67	1.36
16 mM βCD	5	5.34	1.19	6.69
	40	5.90	1.32	7.38
	80	2.91	0.59	2.35

stationary phase at ambient temperature, however, in this case the peak tailing was severe, although slightly improved when acetylated β-CD was applied. In our experiment at the conditions of low temperature and high concentration of β-CD, as it can be seen on Fig. 3, the peak shape is excellent. Table 2 lists the resolutions (Rs) defined as the differences in retention of the two peaks divided by the average peak width at the base, expressed in time units, $R_s = 2(t_{R_2} - t_{R_1})/(W_1 + W_2)$. As it can be seen these parameters in lower temperature range are excellent.

From a practical point of view two parameters are very important i.e. resolution and retention time. As it can be seen on Figs 1–6, in the case of mobile phases modified with the addition of β-CD, the retention time is shortest in low temperature and high concentration of β-CD. The influence of column temperature and β-CD concentration on resolution is more complex. From the results listed in Table 2 it

can be concluded that the best resolution is achieved when mobile phase modified with 6 mM of β-CD at the temperature of 5°C was applied. Nevertheless, the Rs value is higher than 1 in all temperature ranges below 40°C. From a practical point of view it is easier to vary column temperatures than to operate with mobile phases of very high β-CD concentration. Both the column and the mobile phases can be thermostated within a temperature range from 5 to 40°C without problems. Therefore, when an extremely short retention time is not required, the column temperature of 5°C and β-CD concentration within 6–12 mM might be recommended for routine analysis.

Although, separation using chemically bonded chiral stationary phases seems to be preferred, there are several things to benefit from using chiral selectors in the mobile phase. Because the inclusion process is performed in mobile phase the retention time is often shorter maintaining stereoselectivity. The type and concentration of the stereochemical selector can also be changed and stationary phases with different properties can be used.

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