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The influence of temperature on the multiple separation of estrogenic steroids using mobile phases modified with β-cyclodextrin in high-performance liquid chromatography[1]

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Abstract

The effect of temperature on the retention and multiple separation of six estrogenic steroids in reversed-phase liquid chromatography has been studied. Capacity factors (k') of estriol, 17β-estradiol, 17α-estradiol, d-equilenin, equilin and estrone were measured using mobile phase modified with different concentrations of β-cyclodextrin (from 0–16 mM), a fixed solvent composition (acetonitrile-water) and a 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 when mobile phase modified with β-cyclodextrin was used. Particularly strong nonlinearity was observed at lower temperature and at higher β-cyclodextrin concentration. The complex chromatograms were evaluated using optimization parameters such as capacity factor of the last-eluted peak (k'_{max}), the smallest resolution between adjacent peaks ($R_{s,min}$) and relative resolution product (r). The results presented describe precisely the role of temperature in high-performance liquid chromatography systems in which mobile phases modified with cyclodextrin were used. Moreover, the elution order of estrogenic steroids on modified and unmodified mobile phases has been discussed. © 1997 Elsevier Science B.V.

Keywords: Cyclodextrin; Mobile phase additives; Liquid chromatography; Optimization criteria; Estrogens; Temperature

1. Introduction

Generally, in classical reversed-phase liquid chromatography solute retention is inversely related to temperature. The dependence of the logarithms of the capacity factor (ln k') on temperature is given by Eq. (1) and is known as Van't Hoff plot [1–3].

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

where k' denotes capacity factor, ΔH enthalpy change, ΔS entropy change and ϕ phase ratio of the column. This fundamental equation can be easily explained by assuming that ΔH, ΔS and ϕ are independent of temperature. When the retention mechanism is the same over the temperature range investigated and the above parameters are

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constant, the resulting plot of ln k' against $1/T$ yields a straight line [3,4].

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 [5,6]. 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. Particulary 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 may be very complex [7,8].

Cyclodextrins (CDs) are toroidal-shaped cyclic oligomers of α-1,4-D-glucopyranose units, which contribute to several guest-associated phenomena in solution. In chromatography, CDs are commonly used as chiral selectors and for improving separation of other stereoisomers [9–11]. Despite the number of papers dealing with various applications of CDs in chromatography knowledge of the stereoselectivity and structural relationships between CDs and guest molecules is poor. The inclusion properties of CDs do not depend solely on the size and steric arrangement of potential guests. Many other factors seems to be responsible for the separation, including the type of CD used [12], its concentration in the mobile phase [13,14] and the type of mobile phase [13,14]. Moreover, the peak resolution of ionic compounds in presence of cyclodextrins strongly dependeds on the pH of the mobile phase. In this case, control of the solutes ionization is necessary to optimize a separation process [15–17]. On the other hand however, the term pH in organic-water system is meaningless [18]. Considering, that steroids are generally nonpolar compounds the temperature effect should better defined when unbuffered water is used as mobile phase. Generally, in LC little atention has been focused on temperature. Hence, only a few workers have studied the effect of temperature on stereoselectivity in LC phases modified with CDs [8,12,19–22]. Moreover, most of the published papers concern the influence of temperature on the separation factor of two solutes, although in reality, chromatograms are usually multicomponent.

One way to resolve this problem is the intuitive method, wherein the chromatographer makes an initial selection of the separation parameters (such as mobile phase composition, temperature, concentration of modifier in the mobile phase and so on) based on the chemical nature of the solutes and then refines this selection on trial-and-error basis. This method often fails when many separation parameters are taken into account. An alternative approach is application of a matematical optimization criterion. Different factors that have been used as criteria for optimization of chromatograms and many of important problems encountered when dealing with the separation of multicomponent mixtures were discussed and published elsewhere [23–30].

Generally, any criterion based on peak separation can serve as the basis for initial separation search. However the most fruitful approach seems to be application of three parameters, i.e. k'_{max}, $R_{s,min}$ and r. The first parameter (k'_{max}) is defined as the capacity factor of the last-eluted peak. The second ($R_{s,min}$), which has been successfully used by Haddad et al. [28], denotes the smallest resolution between adjacent peaks where resolution (R_s) is defined as the difference in retention times (t_R) of the two peaks divided by the standard deviation (σ) of these peaks:

$$R_s = \frac{t_{R2} - t_{R1}}{2(\sigma_2 - \sigma_1)} \tag{2}$$

The relative retention product (r), was introduced by Drouen and co-workers [27,28] and is defined as:

$$r = \frac{\prod_{i=1}^{n-1} R_{s_{i+1,i}}}{\left(\left(\sum_{i=1}^{n-1} R_{s_{i+1,i}}\right)(n-1)\right)^{n-1}} \tag{3}$$

where R_s is the resolution measured between pairs of adjacent peaks and n denotes the number of solutes.

This work is a continuation of our earlier contributions [8,12,22,31] concerning the influence of both cyclodextrin concentration and temperature

on chromatographic retention and multiple separation.

2. Experimental

2.1. Reagents

Equilin and d-equilenin were obtained from Sigma. Estriol, 17α-estradiol, 17β-estradiol and estrone were reached from Aldrich. Acetonitrile (HPLC grade) and β-CD were purchased from Merck (Darmstadt-Germany). Sodium nitrate was obtained from a commercial supplier. Water was purified by double distillation. Mobile phases were filtered through a 1.5 μm membrane and degassed prior to use.

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 10 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. The β-CD was added to mobile phase (30:70 v/v, acetonitrile in water) to give final concentration of 2, 4, 8 and 16 mM. It is notable that a maximum solubility of β-cyclodextrin in a mixture containing of 30% acetonitrile in water is near 22.9 mM at 22°C [32,33]. For that reason, the concentration of 16 mM can be easy obtain by shaking the binary mobile phase with suitable amount of β-cyclodextrin in ultrasonic water bath for 1 h.

2.2. Chromatography

For chromatographic separations, 5 µm octadecyl Bakerbond (Baker, Phillipsburg, NJ) was used, packed in a Vertex column (120 mm × 4.6 mm i.d.) obtained from Knauer (Berlin-Germany). The liquid chromatograph, consisting of an analytical solvent pump, UV-VIS spectrophotometer and linear recorder, was a product of Knauer. The UV detector was operated at 280 nm. A Rheodyne Model 7125 injection valve and a 20 µl loop were used for sample introduction. The flow rate was set at 2 ml min^{-1} for mobile phases without and with the addition of 4 and 8 mM of β-CD. Due to relatively high viscosity of mobile phase modified with 16 mM β-cyclodextrin (especially at low temperature region) the flow rate in this case was set at 1 ml min^{-1}. It is noteworthy, that it was not observed large difference in separation efficiency of chromatographed steroids using the flow rate 1 and 2 ml min^{-1}.

The void volume was determined by injecting sodium nitrate solution, at a concentration of 10 µg ml^{-1}. The dead retention times for flow rates of 1 ml min^{-1} and 2 ml min^{-1} were 17.02 s and 35.63 s respectively. The retention parameters for each solute were measured at of 80, 70, 60, 50, 40, 30, 20, 10 and 5°C. The capacity factors (k') were calculated in the usual manner and are based on the average of at least five independent determinations of each solute. The column thermostat and other experimental details have been described in a previous paper [22].

3. Results and discussion

In classical liquid chromatography the separation of multicomponent mixture of estrogens is difficult and often incomplete. Additionaly, the LC method is still not fully satisfactory with respect to speed of analysis. Estriol, 17β-estradiol, 17α-estradiol, d-equilenin, equilin and estrone have been chosen as a model compounds to illustrate the influence of temperature and concentration of β-CD on the chromatographic retention and multiple separations of steroids. Molecular structures of investigated estrogens are shown on Fig. 1. All compounds chosen belong to the group of female sexual hormones. However, estriol, 17β-estradiol and estrone are human hormones, while d-equilenin and equilin are horse hormones. Nevertheless, both groups were been applied in human medication. As it can be seen on Fig. 1 they displayed various polarity due to number of hydroxyl groups and aromaticity of the B ring. 17α-Estradiol is not a natural product. However, it was included to our investigation in order to check chromatographic differences with its natural stereoisomer, 17β-estradiol.

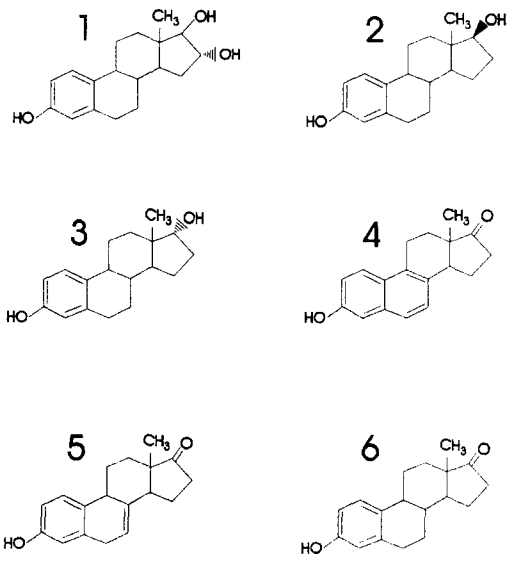

Fig. 1. Chemical structures of investigated estrogens; estriol (1), 17β-estradiol (2), 17α-estradiol (3), d-equilenin (4), equilin (5) and estrone (6).

The estrogens were chromatographed at different column temperatures, from 5 to 80°C, and concentrations of 0, 4, 8 and 16 mM β-CD. When unmodified mobile phase was used the observed retention time was very long, particularly at lower temperature. A linear Van't Hoff behaviour was observed in whole temperature range (see Table 1).

The results of experiment confirms our earlier assumptions that the linear behaviour is more evident for analytes in which the conformation changes during chromatographic process are less possible. When mobile phases modified with β-CD were used the deviation from linear Van't Hoff plot is strongly affected by the stereochemistry of the solute molecule and by the concentration of cyclodextrin (particularly in the low-temperature region). Moreover, the addition of β-cyclodextrin to the mobile phase decreases the chromatographic retention of the investigated solutes on whole temperature region, however decrease of retention is more evident at subambient temperature region.

Table 1
Regression coefficients (a, b) and correlation coefficient (r) of the regression equation $\ln k' = a(1000/T) + b$ for steroids, using an unmodified acetonitrile-water mixture as the mobile phase

Steroid	a	b	r
d-Equilenin	2.13 (0.05)	−3.9 (0.2)	0.998
Equilin	2.02 (0.06)	−3.4 (0.2)	0.997
Estrone	1.94 (0.07)	−3.1 (0.2)	0.996
17β-Estradiol	1.61 (0.07)	−2.4 (0.2)	0.994
17α-Estradiol	1.47 (0.09)	−1.8 (0.3)	0.988
Estriol	0.67 (0.07)	−1.3 (0.2)	0.967

The values in parentheses indicate the standard error at 95% significance level.

From a practical point of view, changes in temperature gives the possibility to have a dramatic influence on retention and separation of chromatographed steroids. Because, the optimum separation for a multicomponent mixture is often a compromise between maximum resolution and minimum analysis time, therefore to find the best separation of chromatographed steroids three optimization criteria-i.e. the capacity factor of last-eluted peak (k'_{max}), the smallest resolution between adjacent peaks ($R_{s,min}$) and the relative resolution product (r), were simultaneously compared. The smalest k'_{max} value provides faster analysis, hence this parameter should be as small as possible. The resolution ($R_{s,min}$) between the pair of peaks most difficult to separate should obviously be as large as possible. The relative resolution product (r) defined by Eq. (3) ranges from 0 for two coinciding peaks to a maximum value of unity when the distances between all peak maxima are equal. However, as was noted by Drouen et al. [27], a value of $r = 1$, does not necessarily mean good separation; it only described the 'symmetry' of a chromatogram. Therefore, to judge the final result it is important to consider a number of optimization parameters simultaneously. Fig. 2 illustrates the retention of the last-eluted compound (k'_{max}) of the chromatographed mixture at each temperature and concentration of β-cyclodextrin. As can be seen, the retention decreases rapidly when cyclodextrin is added to the mobile phase with most of the drop occuring below 8 mM of β-CD. On the temperature axis, retention increases rapidly as the temperature and concentration of cyclodextrin

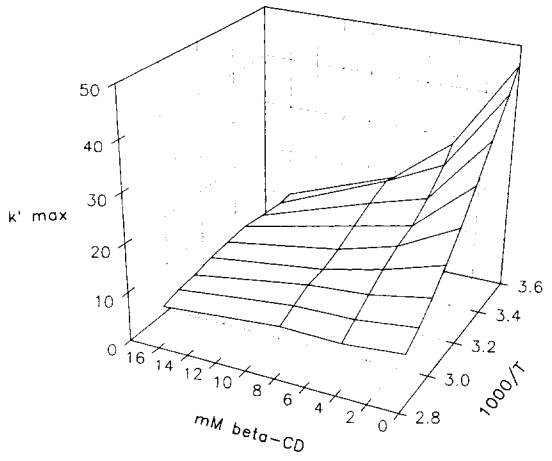

Fig. 2. The capacity factor of the last eluted peak (k'_{max}) as a function of temperature and β-cyclodextrin concentration.

is lowered. Fig. 3 shows the peak resolution ($R'_{s,min}$) as a function of the temperature and the β-cyclodextrin concentration. The surface on the 3D-plot is strongly folded. However, as can be seen the separation can be greatly improved using mobile phase with high concentration of β-cyclodextrin at the low temperature region. The surface reaches maximum at temperature of 20°C and at 16 mM concentration of β-cyclodextrin. Fig. 4 shows three dimensional plot of temperature, β-CD concentration and relative resolution product (r). The r criterion increases to 0.35 at

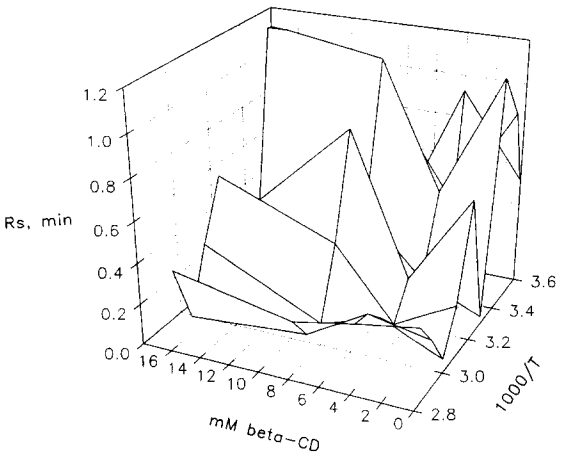

Fig. 3. The peak resolution ($R_{s,min}$) as a function of temperature and β-cyclodextrin concentration.

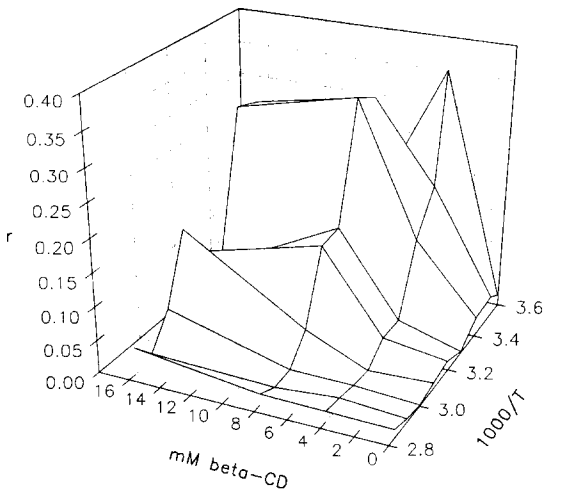

Fig. 4. The relative resolution product (r) as a function of temperature and β-cyclodextrin concentration.

5°C and at 4 mM concentration of β-cyclodextrin.

Examples of chromatograms obtained using 0, 4, 8 and 16 mM concentrations of β-CD at temperature of 20°C, are shown on Fig. 5. As can be seen, an excellent separation of six-component mixture can be obtained, using 16 mM of β-CD at temperature of 20°C (Fig. 5D), what fully confirms the previous selection achieved on the basis of optimization criteria. It is noteworthy, that the elution order of the separated compounds is changed when different chromatographic conditions were used.

From a practical point of view it is always important to predict the elution order on a given chromatographic system. The retention behaviour on unmodified mobile phase is easy to predict on the basis of the qualitative polarity concept. As can be seen on Fig. 5A and Fig. 6A, firstly estriol with tree OH groups is eluted. Then two steroids with two OH groups, i.e. 17β-estradiol and 17α-estradiol. As the last elute tree compounds with one hydroxyl- substituent. However, d-equilenin, with naphtalene-like structure can be consider as most polar, then equilin with one double bond in ring B and at last estrone which have fully saturated ring B. The observed retention order fully confirms this prediction in ambient 20°C as well

as in subambient 5°C temperatures. Moreover, such a retention behaviour has been observed many times in the case of polycyclic aromatic hydrocarbons metabolites, which have structures very similar to steroids [34].

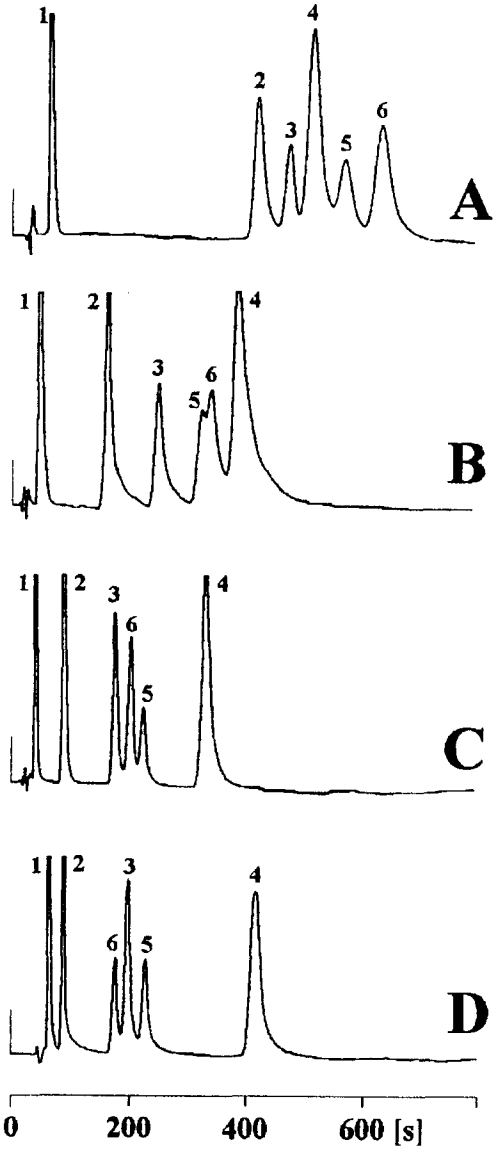

Fig. 5. Chromatograms of six-component mixture at 20°C and different concentrations of β-CD; 0 mM (A), 4 mM (B), 8 mM (C) and 16 mM (D). Eluent: acetonitrile:water 30:70 (v/v). Ultraviolet detector (280 nm). Solutes: estriol (1), 17β-estradiol (2), 17α-estradiol (3), d-equilenin (4), equilin (5), estrone (6).

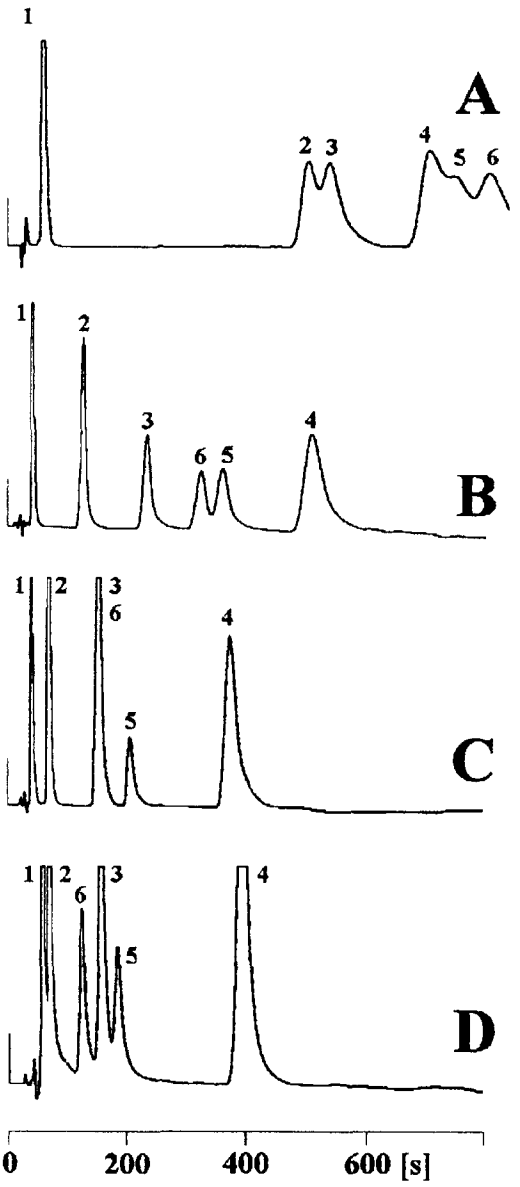

Fig. 6. Chromatograms of six-component mixture at 5°C and different concentrations of β-CD; 0 mM (A), 4 mM (B), 8 mM (C) and 16 mM (D). Eluent: acetonitrile:water 30:70 (v/v). Ultraviolet detector (280 nm). Solutes: estriol (1), 17β-estradiol (2), 17α-estradiol (3), d-equilenin (4), equilin (5), estrone (6).

As it can be seen on Fig. 5 and Fig. 6B–D the addition of β-CD strongly influence the retention order. Under extreme conditions, i.e. addition of 16 mM of β-CD (Fig. 5D and Fig. 6D) the most affected steroids are estrone, which elute as a third compound, and 17β-estradiol, while d-equilenin only weakly interacts with β-CD. Hence it can be concluded, that ability of a steroid to interact with β-CD is due to planarity of its molecule. Therefore, the elution order differ at different cyclodextrin concentrations.

4. Conclusions

In the case of mobile phases modified with the addition of β-CD, the retention time is shorter in low temperature and high concentration of β-CD. Moreover, at subambient temperature region the selectivity of the chromatographic system is greatly improved, even for very complex multiple separations. Simultaneous application of three optimization criteria, i.e. k'_{max}, $R_{s,min}$ and r, provides an useful tool for searching of the best chromatographic conditions in the case of multiple separations.

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