ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 1305–1311

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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

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Received for review 13 September 1995; revised manuscript received 1 December 1995

Abstract

The effect of temperature on the retention and multiple separation of hydrocortisone, testosterone, 17α-methyltestosterone, prednisone, cortisone and 17α-hydroxyprogesterone in reversed-phase liquid chromatography has been studied. Capacity factors (k') of the steroids were measured using a 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 5–80°C). The plots of capacity factors vs. reciprocal of absolute temperature are nonlinear in every 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.

Keywords: Cyclodextrin-modified mobile phases; Liquid chromatography; Optimization criteria; Steroids; Temperature effect

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

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[1] Presented at the Fifth International Symposium on Drug Analysis, September 1995, Leuven, Belgium.

PII S0731-7085(96)01766-9

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 the 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. 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 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 steroisomers [9–11]. Despite the number of papers dealing with various applications of CDs in chromatography knowledge of the steroselectivity 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 seem 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]. 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 [8,12,15–18]. 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 chromatogapher makes an initial selection of the separation paramters (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 a trial-and-error basis. This method often fails when many separation parameters are taken into account. An alternative approach is application of a mathematical optimization criterion. Different factors that have been used as criteria for optimization of chromatograms and many of the important problems encountered when dealing with the separation of multicomponent mixtures were discussed and published elsewhere [19–26].

Generally, any criterion based on peak separation can serve as the basis for an 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. [24], 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 [23,24] 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.

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

Steroid	a	b	r
17α-Hydroxyprogesterone	1.37 (0.09)	−1.1 (0.3)	0.987
Testosterone	1.09 (0.09)	−0.6 (0.3)	0.977
17α-Methyltestosterone	1.0 (0.1)	0.04 (0.3)	0.962
Cortisone	0.58 (0.09)	−0.6 (0.3)	0.930
Prednisone	0.49 (0.09)	−0.4 (0.3)	0.895
Hydrocortisone	0.3 (0.1)	0.3 (0.3)	0.684

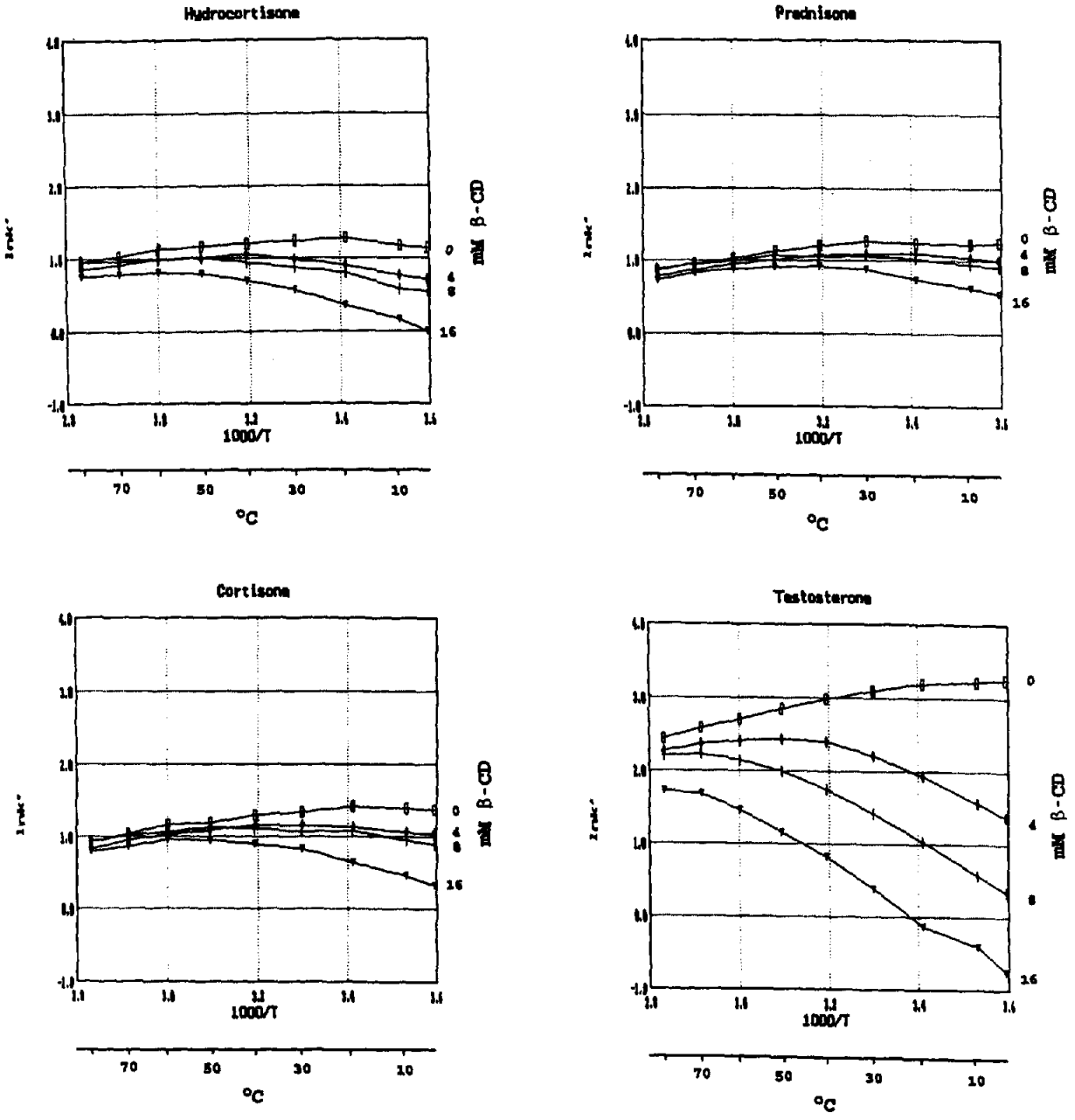

Fig. 1. (Part 1).

This work is a continuation of earlier contributions [8,12,18] concerning the influence of both cylcodextrin concentration and temperature on chromatographic retention and separation. The discussion, however, is extended for the first time to multiple separations.

2. Experimental

2.1. Reagents

Hydrocortisone was obtained from Polfa Enterprise (Pabianice, Poland); testosterone and 17α-

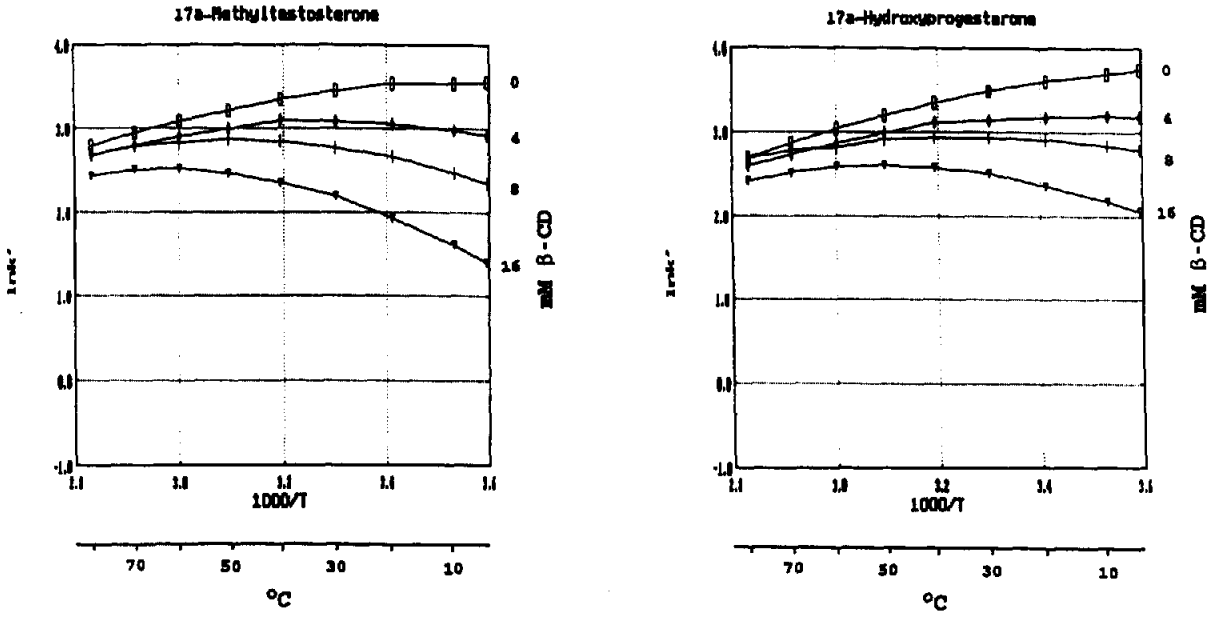

Fig. 1. Plots of k' vs. $1000/T$ for investigated steroids at different concentrations of β-CD in the mobile phase.

methyltestosterone were kindly provided by Polfa Enterprise (Jelenia Góra, Poland). Prednisone, cortisone and 17α-hydroxyprogesterone were obtained from Aldrich Chemical Company, Inc. 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 the 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 concentrations of 2, 4, 8 and 16 mM.

2.2. Chromatography

For chromatographic separations, 5 μm octadecyl Bakerbond (Baker Inc. 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 240 nm. A Rheodyne Model 7125 injection valve and a 20 μl loop were used for sample introduction. The flow rate was set at 1 ml min^{-1} (for a mobile phase containing 16 mM β-CD) and 2 ml min^{-1} (for mobile phases without and with the addition of 4 and 8 mM β-CD).

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 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 [18].

3. Results and discussion

The influences of temperature and concentration of β-CD on the retention and separation of six steroids have been studied. Hydrocortisone, prednisone, cortisone, testosterone, 17α-methyltestosterone and 17α-hydroxyprogesterone were chosen as model compounds. The steroids were chromatographed at different column temperatures, from 5–80°C, with concentrations of 0, 4, 8 and 16 mM β-CD.

Using mobile phase without β-CD a nearly linear Van't Hoff plot is observed for 17α-hydroxyprogesterone, testosterone, 17α-methyltestosterone and cortisone (see Table 1). For prednisone and hydrocortisone, however, both the slope values (a) and the correlation coefficients (r) are low and therefore nonlinearity of the Van't Hoff plots should be assumed. In previous studies, in the case of unmodified mobile phases, an excellent linearity was observed for series of estrogens [18] and substituted naphthalenes [12]. Hence, it can be concluded that the linear behaviour is more evident for analytes in which conformation changes during the chromatographic process are less possible. Nevertheless, some of the saturated steroid molecules are particularly susceptible to changes in conformation and, therefore, this might be the explanation for the nonlinearty of their Van't Hoff plots.

The influences of the β-CD concentration and temperature on the capacity factors for each solute are exemplified in Fig. 1. After modification of the mobile phase with β-CD (particularly in the low-temperature region) nonlinearity of the Van't Hoff plots is observed in each case and is more evident than in unmodified mobile phases. A similar strong deviation from Van't Hoff plots has been described and discussed in previous papers for 1,8-dimethylnaphthalene [12], norgestrel optical isomers [8], estradiol stereoisomers and equilin [18]. Previous study and the current experimental material suggests that in the high temperature region the degree of complexation with β-CD for all investigated compounds is low and therefore the typical Van't Hoff plot is observed.

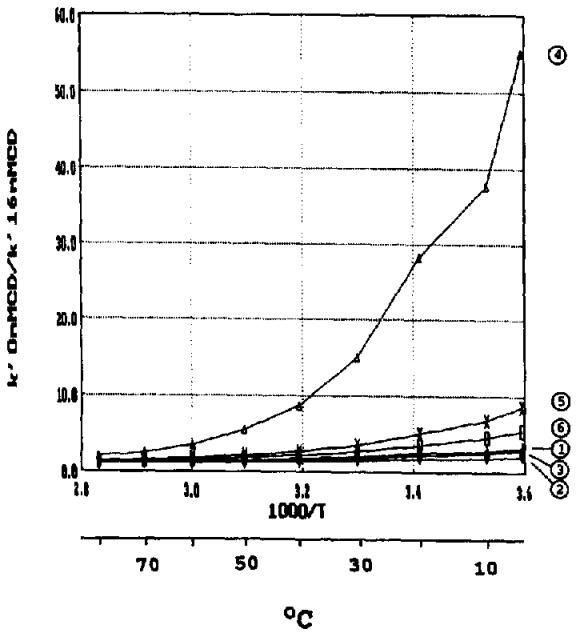

Fig. 2. The capacity factor ratio ($k'_{0\ mM\beta\text{-CD}}/k'_{16\ mM\beta\text{-CD}}$) vs. $1000/T$ for hydrocortisone (1), prednisone (2), cortisone (3), testosterone (4), 17α-methyltestosterone (5), and 17α-hydroxyprogesterone (6).

Nevertheless, the addition of β-CD to the mobile phase decreases the chromatographic retention of the investigated solutes over the entire temperature region. However, as can be seen in Fig. 1, decrease of retention is more evident in the subambient temperature region.

The temperature at which the deviation from linear Van't Hoff behaviour begins is strongly affected both by the stereochemistry of the solute molecule and by the concentration of β-CD (Fig. 1). For all analytes the deviation is most evident at the highest concentration of β-CD (16 mM). Comparing solute molecules the strongest deviation is observed in the case of testosterone. In order to illustrate these differences more precisely, the capacity factor ratio ($k'_{0\ mM\beta\text{-CD}}/k'_{16\ mM\beta\text{-CD}}$) was plotted against the reciprocal of absolute temperature in Fig. 2. This ratio is directly related to the number of moles of analyte in the mobile phase modified with β-CD divided by the number of moles of analyte in the unmodified complexation with β-CD. As can be seen in Fig. 2, at 5°C

the degree of complexation is strongest for testosterone molecules (55.2) and then for 17α-methyltestosterone (8.5), 17α-hydroxyprogesterone (5.4), hydrocortisone (3.1), cortisone (2.8) and prednisone (2.0). It is obvious that greater complexation decreases retention, because the adsorption of cyclodextrin complexes on the stationary phase is less than the adsorption of the corresponding free molecules. However, at 80°C all the studied compounds have very similar capacity factor ratios, which range between 1.1 and 2.0.

From a practical point of view, 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 the last-eluted peak (k'_{max}), the smallest resolution between adjacent peaks ($R_{s,min}$) and the relative resolution product (r)—were simultaneously compared. The smallest k'_{max} value provides the fastest 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. [23], 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.

The numerical data of the optimization criteria are collected in Table 2. As can be seen, in the case of 5°C and 16 mM concentration of β-CD, k'_{max} reaches its minimum value, whereas $R_{s,min}$ and r have maximum values. This means that under these conditions the separation will be the best attainable during an extremely short analysis time.

Examples of chromatograms obtained using 0, 4, 8 and 16 mM concentrations of β-CD are shown in Fig. 3. The temperature for each chromatogram presented was selected in such a manner that $R_{s,min}$ has a maximum value. As can be seen, excellent separation of a six-component mixture can be obtained, using 16 mM β-CD at 5°C (Fig. 3D), which fully confirms the previous selection achieved on the basis of optimization criteria. It is noteworthy that the elution order of the separated compounds changes when different chromatographic conditions are used.

Table 2
Optimization paramters (k'_{max}, $R_{s,min}$, r) for investigated steroids at various temperatures and concentrations of β-CD in the mobile phase

T(°C)	B-CD concentration (mM)											
	0			4			9			16		
	k'_{max}	$R_{s,min}$	r	k'_{max}	$R_{s,min}$	r	k'_{max}	$R_{s,min}$	r	k'_{max}	$R_{s,min}$	r
5	42.54	0.36	0.02	24.18	0.20	0.03	16.31	0.06	0.01	7.87	0.99	0.30
10	40.36	0.25	0.02	24.33	0.10	0.02	17.17	0.01	0.00	8.87	0.81	0.19
20	37.24	0.23	0.01	24.04	0.11	0.02	18.45	0.08	0.00	10.69	0.47	0.12
30	32.92	0.17	0.00	23.08	0.27	0.01	18.83	0.05	0.01	12.42	0.30	0.03
40	28.32	0.01	0.00	22.62	0.11	0.00	18.87	0.31	0.02	13.23	0.13	0.00
50	24.86	0.10	0.00	19.93	0.01	0.00	18.52	0.34	0.01	13.58	0.24	0.02
60	21.78	0.22	0.01	18.18	0.23	0.00	16.88	0.01	0.00	13.36	0.41	0.02
70	18.96	0.03	0.00	16.35	0.26	0.01	16.21	0.04	0.00	12.44	0.15	0.00
80	16.22	0.15	0.01	14.55	0.16	0.00	14.74	0.10	0.00	11.42	0.14	0.00

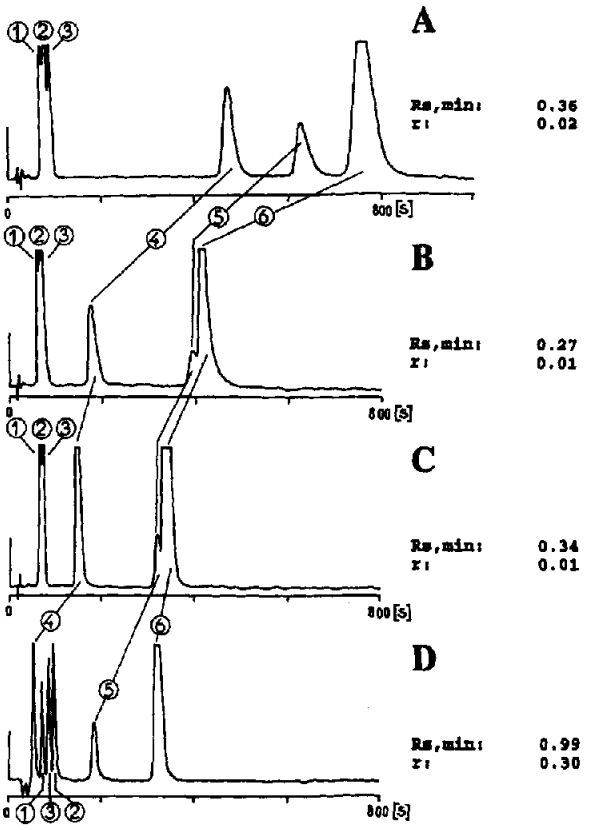

Fig. 3. Chromatograms of six-component mixture at 0 mM β-CD, 5°C (A); 4 mM β-CD, 30°C (B), 8 mM β-CD, 50°C (C) and 16 mM β-CD, 5°C (D). Eluent; acetonitrile:water 30:70 (v/v). Ultraviolet detector (240 nm). Solutes: hydrocortisone (1), prednisone (2), cortisone (3), testosterone (4), 17α-methyltestosterone (5) and 17α-hydroxyprogesterone (6).

4. Conclusions

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

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