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Separation of steroids using temperature-dependent inclusion chromatography

Pawel K. Zarzycki[a,*], Roger Smith[b]

[a]*Medical University of Gdansk, Faculty of Pharmacy, Hallera 107, 80-416 Gdansk, Poland*

[b]*Mothers and Babies Research Center, Endocrine Unit, John Hunter Hospital, Newcastle, New South Wales 2310, Australia*

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Abstract

The influence of temperature on retention and separation of estrogens, progesterone derivatives and β-cyclodextrin in reversed-phase high-performance liquid chromatography has been studied. Steroids were detected using direct UV detection at 240 and 280 nm. Detection of β-cyclodextrin was achieved using a post-column indirect photometric method. Chromatographic experiments were performed using an acetonitrile–water mobile phase (30%, v/v) and a wide range of column temperatures from 0 to 80°C with 20°C steps. Linear Van't Hoff plots were observed for steroids and β-cyclodextrin when an unmodified binary mobile phase was applied. The retention of steroids was strongly influenced by temperature when the mobile phase was modified with β-cyclodextrin at a concentration of 12 m*M*. Particularly, for 17β-estradiol and 20α-hydroxyprogesterone a strong deviation from the linear Van't Hoff plots and a remarkable affinity for β-cyclodextrin was observed. Polynomial regression calculations were performed to fit the set of experimental data points. Using third-order polynomial equations, minimum separation factor values (α_{min}) were calculated for temperatures from −10 to +100°C with 1°C steps. The best chromatographic conditions for separation of multicomponent samples were chosen. A possible retention mechanism for solutes in the presence of macrocyclic additives is discussed. The results presented describe the role of temperature in high-performance liquid chromatography systems in which the mobile phase is modified with an inclusion agent.

Keywords: Temperature effect; Inclusion complexes; Steroids; Cyclodextrins

1. Introduction

Generally, in classical reversed-phase liquid chromatography solute retention is inversely related to temperature. The dependence of the logarithms of the retention parameter ($\ln k$) on temperature is known as the Van't Hoff plot [1,2]. When the retention mechanism is the same over the temperature range investigated a plot of $\ln k$ versus $1/T$ yields a straight line [3,4]. Nevertheless, any reversible process that alters the enthalpy or entropy of adsorption in principle gives rise to non-linear Van't Hoff plots. Among others, changes in conformation and changes in the extent to which the mobile phase interacts with either the solute or the stationary phase are examples of such reversible behavior [5,6]. Moreover, the presence of multiple types of retention mechanisms or multiple types of binding sites also leads to nonlinearity of the Van't Hoff plots. Particularly, if the mobile phase is modified with an

*Corresponding author. Tel.: +48-58-3493-131; fax: +48-58-3493-130.

E-mail address: pawel_k_z@hotmail.com (P.K. Zarzycki).

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inclusion agent multiple types of interaction between solutes and modifier as well as stationary or mobile phase components can be expected. Therefore, the effect of temperature on retention might be very complex and usually, even in simple chromatographic systems it is not known how temperature affects efficiency, selectivity or other separation conditions [7–9].

Cyclodextrins (CD) are a group of toroidal-shaped oligosaccharides that contribute to several guest-associated phenomena in solution [10]. The three most commonly employed natural cyclodextrins contain six, seven and eight glucopyranose units in macrocyclic rings and are denoted as α-, β- and γ-cyclodextrin, respectively. The interior of CD cavities is relatively hydrophobic because all the hydroxyl groups are on the outside of the molecule. Moreover, the C2–OH and C3–OH groups form a ''belt'' of hydrogen bonds, making the molecule more rigid. The CD complexation processes are highly selective and can be considered as the method of choice for resolution of various isomers: structural, geometrical, diastereomeric and enantiomeric. Particularly in chromatography, cyclodextrins are commonly used as chiral selectors and for improving separation of other stereoisomers [11–13]. The primary factors in CD-guest complexes are Van der Waals forces, hydrophobic interactions and hydrogen bonding [14,15]. However, other factors such as shape of the guest molecules may also be important [16,17].

Despite the number of papers dealing with various applications of cyclodextrins in chromatography, knowledge of the retention behavior of solutes in the presence of inclusion agents in the mobile phase is poor. Additionally, in liquid chromatography (LC) little attention has been focused on temperature. Only a few workers have studied the effect of temperature on selectivity in LC phases modified with cyclodextrins and modeling of the chromatographic process with cyclodextrins as mobile phase components is still in progress [18–25]. Moreover, most of the published papers concern the influence of temperature on the separation of two solutes, although in reality, chromatograms are usually multicomponent [26–28].

Steroids are an important group of compounds playing a part in many biological processes and having many therapeutic applications. Especially, estrogens and progesterone derivatives are of clinical interest for many reasons. During pregnancy, fetal growth retardation and the risk of perinatal death can be identified by estimation of urinary estrogens [29]. The measurement of urinary estriol is one of the most useful tests for the assessment of feto-placental function during pregnancy. For a long time, the measurement of urinary progesterone derivatives was considered to be the key indicator in the assessment of fetal development [30]. However, the great diversity of estrogens and progesterone derivative structures and their wide range of polarities present special problems for the simultaneous analysis of both classes of steroids in one sample [31].

Previous chromatographic studies show that the Van't Hoff plots of steroids are nonlinear in every case when the mobile phase is modified with β-cyclodextrin and a wide range of temperatures investigated [25–28]. This study reports a simple strategy for the optimization of separation of a battery of estrogens and progesterone derivatives using temperature as the critical parameter for selectivity in the chromatographic system. In addition, we demonstrate that the retention of an inclusion modifier plays an essential role in the chromatographic behavior of the solutes investigated. The results of this study advance the understanding of the retention mechanism of inclusion complexes at different temperatures.

2. Experimental

2.1. Chemicals

Estriol, 17β-estradiol, 17α-estradiol, estrone, equilin, 20α-hydroxyprogesterone (4-pregnen-20α-ol-3-one) and β-cyclodextrin, were obtained from Sigma (St. Louis, MO, USA). Estetrol and 17α-hyroxyprogesterone (4-pregnen-17α-ol-3,20-dione) were purchased from Steraloids (Newport, RI, USA) and Ikapharm (Ramat-Gan, Israel), respectively. Acetonitrile 99.7% HPLC grade, was a product of APS Ajax Finechem (NSW, Australia). Sodium nitrate, sodium carbonate and phenolphthalein were obtained from a commercial supplier and used as received. Water was purified by double distillation.

2.2. Chromatography

HPLC studies were carried out with a 250×4.6-mm LiChrospher RP18 (5 μm) column obtained from Supelco (Bellefonta, PA, USA). The liquid chromatograph consisting of an analytical solvent pump (Programmable Solvent Module 116), UV–Vis spectrophotometer (Diode Array Detector Module 168) and System Gold V601 were product of Beckman Instruments (San Ramon, CA, USA). A Beckman 210A Injection Valve and a 20-μm loop were used for sample introduction.

The column temperature was controlled using an Alltech Water Jacket (Alltech Associates, Deerfield, IL, USA) connected to a circulating thermostat adjustable from 0 to 80°C with an accuracy of ±0.5°C. The chromatographic column and column inlet filter (Knauer, Berlin, Germany) were thermostated at least 1 h before and during the experiment in order to obtain a proper temperature equilibrium.

The retention factors (k) were calculated in the usual manner and are based on the average of at least five independent determinations of each solute. The flow-rate was set at 1 ml min^{-1}. The void volume was determined by injecting the sodium nitrate solution at a concentration of 10 μg ml^{-1}. Mobile phases were filtered through a 1.5-μm membrane and degassed prior to use.

The retention parameters for each steroid were measured from 0 to 80°C with 20°C steps using a 30% (v/v) acetonitrile–water mobile phase, unmodified and modified with β-cyclodextrin at a concentration of 12 mM. Stock solutions of steroids were prepared in acetonitrile at a concentration of 0.5 mg ml^{-1}. From these stock solutions, appropriate injection standard solutions at a concentration of 20 μg ml^{-1} were prepared by mixing the required volume of the stock solution and the chromatographic mobile phase components.

The injection standard solution of β-cyclodextrin was prepared in the mobile phase (30%, v/v) at a concentration of 13.6 mg ml^{-1} (12 mM). The retention behavior of β-cyclodextrin was studied using the indirect photometric detection method [32–34]. As a post-column detection reagent an alkaline solution of phenolphthalein at a concentration of 60 μM in a mixture of acetonitrile–water (30%, v/v) and sodium carbonate 0.01 M was applied.

2.3. Calculations

For evaluation of the data, an IBM-compatible PC was used. Linear and polynomial regression calculations as well as non-linear curve-fitting were performed using the Excel for Windows spreadsheet program and the multiple linear regression program described by Orvis [35].

3. Results and discussion

In this work as model compounds six estrogenic steroids (estetrol, estriol, 17β-estradiol, 17α-estradiol, estrone, equilin) and two progesterone derivatives (17α-hydroxyprogesterone and 20α-hydroxyprogesterone) were chosen (Fig. 1). All

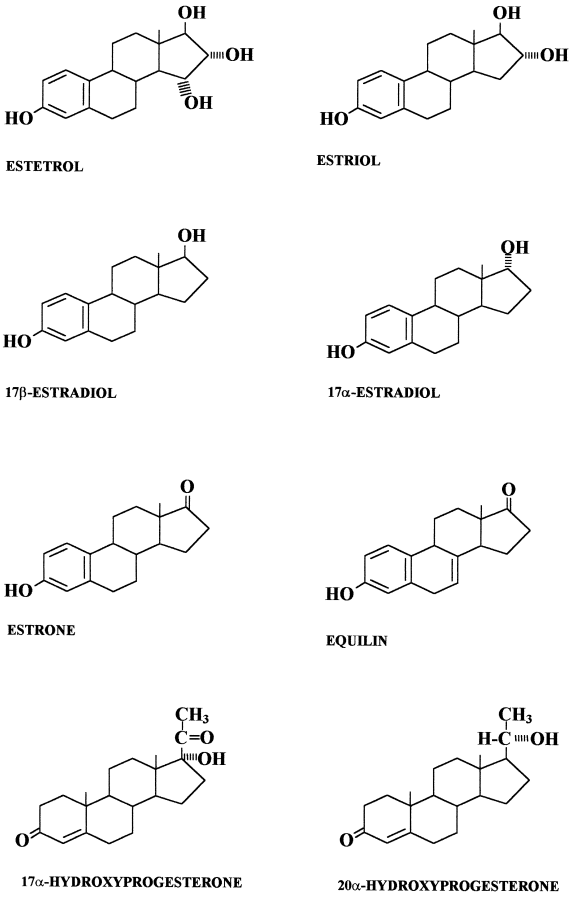

Fig. 1. Chemical structures of investigated steroids.

steroids were chromatographed using a wide range of column temperatures from 0 to 80°C in steps of 20°C and acetonitrile–water mobile phases unmodified and modified with addition of β-cyclodextrin in a concentration of 12 m*M*. As can be seen from the data presented in Table 1, a linear relationship between the retention parameter of solutes and the reciprocal of absolute temperature is observed when the mobile phase is unmodified by an inclusion agent. The linear behavior of solutes suggests that for each solute the retention mechanism is the same over the temperature range investigated [3,4]. It is easy to check that selectivity of the chromatographic system is similar over the whole range of column temperatures. As expected, decreasing the temperature increased the retention of the solutes. The elution order of all steroids remained unchanged for temperatures from 20 to 60°C. At a low temperature region close to 0°C, the chromatographic system was non-selective for both estradiol stereoisomers as well as for equilin, estrone and 17α-hydroxyprogesterone. In the high-temperature region the retention times of all steroids was shortest; however, the separation between estrone, equilin and estradiol stereoisomers was still very poor.

Fig. 2 illustrates experimentally and calculated retention profiles of steroids (small diamond points) using the mobile phase modified with 12 m*M* of β-cyclodextrin. As can be seen from the plots presented in Fig. 2 and from determination coefficients of the third-order polynomial equations presented in Table 2, the calculated curves meet all the measured data points very well. In all cases strong deviations from linear Van't Hoff plots were observed and selectivity of the chromatographic system strongly depended on column temperature. Below temperatures of 40°C retention of all steroids decreased rapidly. A remarkable affinity of the inclu-

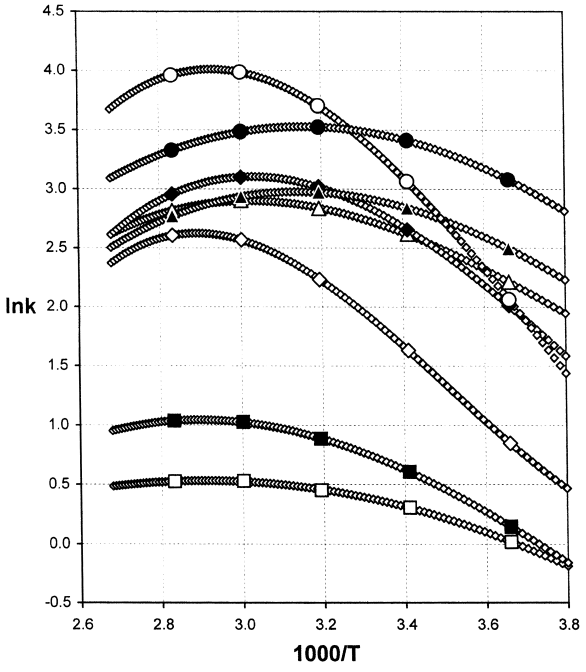

Fig. 2. Plots of measured and calculated (small diamonds) ln *k* values for steroids at different temperatures. Estetrol (□), estriol (■), 17β-estradiol (◇), 17α-estradiol (△), estrone (♦), equilin (▲), 17α-hyroxyprogesterone (●), 20α-hydroxyprogesterone (○).

Table 1
Regression coefficients (intercept, slope) and determination coefficient (r^2) of the regression equation $\ln k = \text{intercept} + \text{slope} \times (1000/T)$ for the investigated compounds chromatographed on a C_{18} stationary phase using acetonitrile–water (30:70, v/v) mixture as eluent[a]

Compound	Intercept	Slope	r^2	SE of *y* estimation
Estetrol	−0.7(0.1)	0.51(0.03)	0.9889	0.02
Estriol	−1.1(0.1)	0.85(0.03)	0.9950	0.02
17β-Estradiol	−1.86(0.09)	1.72(0.03)	0.9991	0.02
17α-Estradiol	−1.2(0.2)	1.56(0.06)	0.9961	0.04
Estrone	−2.4(0.1)	1.98(0.03)	0.9992	0.02
Equilin	−2.77(0.09)	2.06(0.03)	0.9994	0.02
17α-Hydroxyprogesterone	−0.6(0.2)	1.49(0.05)	0.9960	0.04
20α-Hydroxyprogesterone	−0.8(0.2)	1.83(0.05)	0.9979	0.03
β-Cyclodextrin	−0.16(0.06)	−0.26(0.02)	0.9856	0.01

[a] Number of samples, 5; temperature range 0–80°C (the values in parentheses indicate the standard errors of coefficients at a 95% significance level).

Table 2
Regression coefficients (a,b,c,d) and determination coefficient (r^2) of the third-order polynomial equation $\ln k = a + bx + cx^2 + dx^3$ (where $x = 1000/T$) for investigated steroids chromatographed on a C_{18} stationary phase using as eluent an acetonitrile–water (30:70, v/v) mixture modified with β-cyclodextrin at a concentration of 12 mM[a]

Steroid	a	b	c	d	r^2	SE of y estimation
Estetrol	−7.5848	5.7221	−1.0552	0.0163	0.9995	0.01
Estriol	−26.3838	22.9279	−6.0214	0.4748	0.9999	0.005
17β-Estradiol	−107.3301	99.2465	−29.1917	2.7735	0.9999	0.004
17α-Estradiol	−38.2977	34.3219	−9.1804	0.7724	0.9999	0.006
Estrone	−66.2509	57.8406	−15.5139	1.3133	0.9999	0.006
Equilin	−30.6169	25.7409	−6.1760	0.4413	0.9999	0.001
17α-Hydroxyprogesterone	−20.5571	17.0871	−3.5737	0.1830	0.9999	0.003
20α-Hydroxyprogesterone	−83.3929	74.9540	−20.6223	1.7822	0.9999	0.0002

[a] Number of samples, 5; temperature range 0–80°C.

sion agent and the strongest deviation from linear Van't Hoff plots in case of 17β-estradiol and 20α-hydroxyprogesterone was observed.

It is noteworthy, that the degree of deviation from linear plots and the temperature region at which the deviation from linear Van't Hoff behavior begins, is strongly affected by the stereochemistry of the chromatographed molecules. Similar strong deviations from Van't Hoff plots were previously observed for a series of polycyclic aromatic hydrocarbons and different classes of steroids [17,27]. Recently, Morin and co-workers have reported peculiarities of the retention mechanism for a series of imidazole derivatives in an RP-LC system modified with β-cyclodextrin in the presence of high concentration of *n*-nonylamine [36,37]. The authors described unusual nonlinearity of Van't Hoff plots confirming that the studied system is very complex. Other authors reported that competition for penetration into the cyclodextrin cavity between separated compounds and mobile phase additives, e.g., sodium dodecyl sulfate or 2-methyl-2-propanol molecules can determine the selectivity of chromatographic systems at different temperatures [38,39]. The behavior of solutes in the presence of cyclodextrin can be explained by considering that a decrease in temperature increases CD complexation, which is well documented experimentally [40]. In chromatographic systems a decrease of retention is followed by an increase of solute complexation by inclusion modifier of the mobile phase. This behavior can be explained by assuming that the retention of β-cyclodextrin is considerably lower than the retention of solutes chromatographed and the predominating mechanism for retention is the formation of guest-β-cyclodextrin complexes in the mobile phase. The data presented in Fig. 3 show that at the wide range of temperatures investigated the retention of β-cyclodextrin is significantly lower than the retention of the steroids chromatographed using an unmodified mo-

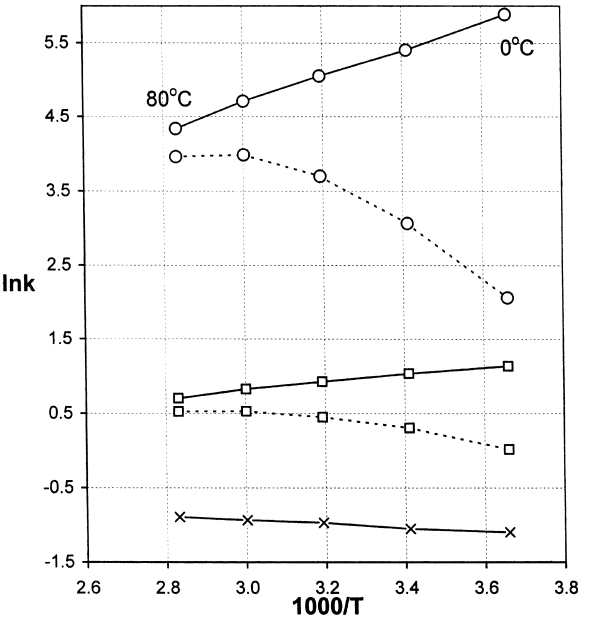

Fig. 3. Influence of temperature on chromatographic retention of β-cyclodextrin (×), estetrol (□) and 20α-hydroxyprogesterone (○) using an unmodified acetonitrile–water mobile phase and modified (dotted lines) with β-cyclodextrin at a concentration of 12 m*M*.

bile phase. The results seem to indicate that retention of inclusion complexes can be varied between two lines formed by the Van't Hoff plot of the β-cyclodextrin and Van't Hoff plot of the uncomplexed steroid. Additionally, in the low temperature region the inclusion modifier action is more efficient due to high values of the binding constant of the complexes created and large differences in retention of β-cyclodextrin and uncomplexed solutes.

From a practical point of view, changes in the temperature of chromatographic systems with mobile phases modified with inclusion agents may have a substantial influence on the separation of solutes. To evaluate the best selectivity of the investigated system, the minimum separation factor values (α_{min}) between adjacent peaks for a wide range of temperatures from −10 to +100°C were calculated. According to the data presented in Fig. 4 the temperature changes produced significant differences in the separation of steroids. The best selectivity of the chromatographic system (maximum values of α_{min} parameter) can be expected at the temperatures 47 and 26°C. Examples of chromatograms (Fig. 5) and data presented in Table 3 confirm the selection achieved on the basis of calculated minimum separation factor values. For both column temperatures excellent separation of an eight component mixture is observed. Because the optimum separation for multi-

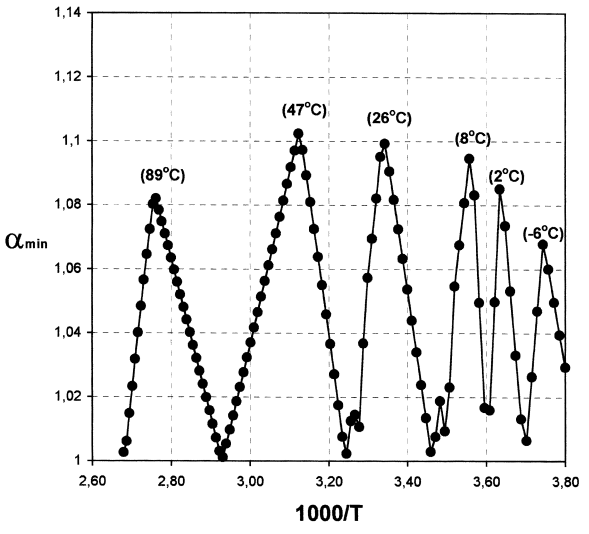

Fig. 4. Relationship between calculated α_{min} values and column temperature from −10°C to +100°C.

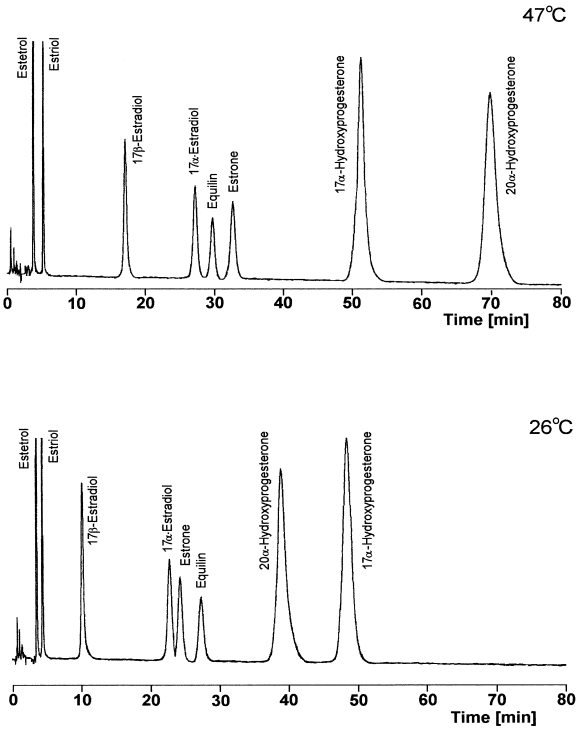

Fig. 5. Separation of steroids at 47 and 26°C using a mobile phase modified with 12 m*M* of β-cyclodextrin.

component mixtures is often a compromise between maximum separation and minimum analysis time, the temperature of 26°C can be recommended for simultaneous analysis of the investigated steroids in biological samples.

4. Conclusions

The retention of the steroids investigated is strongly influenced by temperature when the mobile phase is modified with β-cyclodextrin. Particularly, for 17β-estradiol and 20α-hydroxyprogesterone a strong deviation from linear Van't Hoff plots and remarkable affinity for β-cyclodextrin have been observed. At a wide range of temperatures investigated the retention of β-cyclodextrin is significantly lower than the retention of steroids chromatographed using an unmodified mobile phase. The experimental data indicates that retention of inclusion complexes can be varied between two lines formed by the Van't

Table 3
Measured[(I)] and calculated[(II)] separation factors (α) for adjacent peaks of separated steroids at temperatures 47 and 26°C

47°C			26°C		
Steroids	$\alpha^{(I)}$	$\alpha^{(II)}$	Steroids	$\alpha^{(I)}$	$\alpha^{(II)}$
Estriol Estetrol	1.60	1.59	Estriol Estetrol	1.41	1.42
17β-Estradiol Estriol	4.08	4.19	17β-Estradiol Estriol	2.94	3.09
17α-Estradiol 17β-Estradiol	1.64	1.64	17α-Estradiol 17β-Estradiol	2.44	2.38
Equilin 17α-Estradiol	1.10	1.10	Estrone 17α-Estradiol	1.07	1.10
Estrone Equilin	1.11	1.11	Equilin Estrone	1.13	1.11
17α-Hydroxyprogesterone Estrone	1.59	1.57	20α-Hydroxyprogesterone Equilin	1.45	1.48
20α-Hydroxyprogesterone 17α-Hydroxyprogesterone	1.37	1.37	17α-Hydroxyprogesterone 20α-Hydroxyprogesterone	1.25	1.19

Hoff plot of the β-cyclodextrin and the Van't Hoff plot of the uncomplexed solute.

From a practical point of view, the temperature changes produce significant differences in the separation of steroids. The best selectivity of the studied chromatographic system was observed at the temperatures 47 and 26°C. For both column temperatures excellent separation of an eight component mixture was obtained.

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