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Chromatographic behaviour of selected steroids and their inclusion complexes with β-cyclodextrin on octadecylsilica stationary phases with different carbon loads

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Abstract

Retention and separation studies of selected estrogens, progestogens and their inclusion complexes with β-cyclodextrin were conducted using two C_{18} HPLC columns with different carbon loads. The difference in carbon load between investigated octadecylsilica packing materials was about 50%. The mobile phases were composed of a 30% v/v acetonitrile–water mixture without and with addition of β-cyclodextrin at a concentration of 12 m*M*. The experimental data revealed that retention of the steroids was significantly reduced on the column with the lower carbon load. Moreover, it was found that this column offers better separation power and shorter analysis time at the temperatures studied. However, the calculated values of the retention factor ratios ($k_{0\text{mMCD}}/k_{12\text{mMCD}}$) of the steroids were similar for both columns investigated. This observation suggests that the stationary phase structure appears to have little effect on the formation of host–guest complexes if the complexation process is localised to the chromatographic mobile phase. From a practical point of view, when the mobile phase is modified with β-cyclodextrin, the separation of the steroids is strongly influenced by temperature. The best chromatographic conditions were determined for the separation of multicomponent samples on the column with lower carbon load. A possible retention mechanism for components of interest in the presence of macrocyclic additives is discussed.

Keywords: Stationary phases, LC; Carbon loads; Retention mechanisms; Temperature effects; Hold-up time; Steroids; Cyclodextrins; Estrogens; Progestogens

1. Introduction

The variety of chemically-bonded stationary phases for high-performance liquid chromatography (HPLC) currently available on the market, leave chromatographers with the problem of proper column selection [1,2]. Within numerous silica-based stationary phases, the alkyl-phase columns, due to their stability, efficiency and separation power, remain a common choice for a majority of reversed-phase separations. If taking only the stationary phase into account, the result of HPLC separation is affected by a number of factors including the chemical nature and density of the bonded ligand, the structural and chemical heterogeneity of the silica surface, pore size and their distribution on the support and the amount of residual silanol groups as

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well as metal impurities in the silica supports [3–8]. Unfortunately, available information about the physico-chemical properties of stationary phases is usually insufficient for optimum column selection for a particular separation. Moreover, even if the stationary phases are nominally identical, great differences in separation power and total analysis time can be observed [1].

It is well known that selectivity of a chromatographic system, which is equipped with an alkyl-phase column, can be achieved by varying the binary water–organic mobile phase composition. In this case, both the mobile and stationary phases seem to play an active role in establishing the retention mechanism of components of interest under reversed-phase conditions [9,10]. However, if macrocyclic additives (e.g. cyclodextrins) are used to modify the chromatographic mobile phase, the principles of the retention mechanism are more controversial in the scientific literature [11–18].

Cyclodextrins (CD) are cyclic, toroidal-shaped oligosaccharides that contain six or more saccharide units in a macrocyclic ring [19]. The most common, native cyclodextrins are composed of six, seven and eight glucopyranose units 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. Therefore, this class of compounds has great potential for host–guest interactions. Similar to other macrocyclic compounds, such as crown ethers, calixarenes, cyclophanes or macrocyclic antibiotics, they can form inclusion complexes with organic molecules in solutions. The CD complexation processes are highly stereoselective 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 [11,20,21].

Steroids are an important group of compounds that play a part in many biological processes and have many therapeutic applications. Estrogens and progesterone derivatives are an example of such steroids that have wide clinical interest. During pregnancy, fetal growth retardation and the risk of perinatal death can be identified by estimation of urinary estrogens [22]. The measurement of urinary estriol is one of the most useful tests for the assessment of feto-placental function during pregnancy [23]. For a long time, the measurement of urinary progesterone derivatives was considered to be the key indicator in the assessment of fetal development [24]. Estrogens, progestogens and their derivatives are widely used as birth-control compounds or growth promotants in the food industry and agriculture. Recently, these compounds have been considered as endocrine disrupters in the natural environment [25]. Because both classes of sex hormones are chemically stable and show high physiological activity at very low concentrations, they can be considered as potentially dangerous pollutants of the aquatic environment [26]. However, the great diversity in the structure of estrogens and progesterone derivatives and their wide range of polarities present special problems for the simultaneous analysis of both classes of steroids in one sample [27,28].

Despite the number of papers dealing with various applications of cyclodextrins in chromatography, knowledge of the retention behaviour of steroids in the presence of inclusion agents in the mobile phase is poor. Our previous studies indicate that retention of steroid-cyclodextrin inclusion complexes can be varied between two lines formed by the Van't Hoff plot of the cyclodextrin and the Van't Hoff plot of the uncomplexed solute. On the basis of this observation we have proposed a simple strategy for the optimisation of steroid separation using temperature as the critical parameter for selectivity in the chromatographic system [17,18].

This paper is a continuation of our earlier contributions concerning optimisation of the isocratic HPLC separation of the key steroid hormones in the presence of a mobile phase macrocyclic modifier [18,29–31]. The individual steroids were selected because of their importance during pregnancy. Estriol, estradiol and estrone are the principal estrogens in human pregnancy. Measurement of estetrol is considered as an index of fetal well-being, especially in pathological pregnancies. 17α- and 20α-Hydroxyprogesterone are the main metabolites of progesterone which can be easily detected by UV-HPLC detectors [23,24].

The objective of this work is to study the chromatographic behaviour of selected estrogens and progestogens as well as their inclusion complexes with β-cyclodextrin using stationary phases with different carbon loads. The results of this study advance the understanding of the retention mechanism of inclusion complexes in different chromatographic conditions.

2. Experimental

2.1. Chemicals

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

2.2. Chromatography

All chromatographic studies were carried out with the LiChrospher RP18 (Cat. No.: 54776) and Supelcosil LC-18 (Cat. No.: 58298) HPLC analytical columns. Both columns were obtained from Supelco (Bellefonte, PA, USA). The liquid chromatograph consisting of an analytical solvent pump (Programmable Solvent Module 126), UV–Vis spectrophotometer (Diode Array Detector Module 168) and System Gold (DOS version 601) were products of Beckman Instruments (San Ramon, CA, USA). A Beckman 210A Injection Valve and a 20-μl loop were used for sample introduction.

The column temperature was controlled with an accuracy of ±0.01 °C using an Alltech Water Jacket (Alltech Associates, Deerfield, IL, USA) connected to a Polystat (Cole Parmer, Chicago, IL) digital circulating thermostat. 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 relative standard deviation (CV%) values of the retention factor measurements were below 1.5%. The flow-rate of the mobile phase was set at 1 ml min^{-1}. Due to the detection technique sodium nitrate (10 μg ml^{-1}) was used for the determination of hold-up times, following the methods described elsewhere. Mobile phases were filtered through a 0.2-μm membrane and degassed prior to use.

The retention parameters of steroids were measured using a 30% (v/v) acetonitrile–water mobile phase, unmodified and modified with β-cyclodextrin at a concentration of 12 m*M*. 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.

3. Results and discussion

As model compounds for chromatographic investigations, six estrogens and two progestogens were chosen (Table 1). The retention studies were carried out using binary mobile phases composed of 30% acetonitrile–water (v/v) that was either unmodified or modified with β-cyclodextrin at 12 m*M* concentration. The separation of steroids was studied on two silica-based C_{18} HPLC columns. Their physicochemical properties are summarised in Table 2. Both

Table 1
List of the solutes investigated

No.	Steroid	Abbreviation
1	Estetrol	E4
2	Estriol	E3
3	17β-estradiol	E2
4	17α-estradiol	17αE
5	Estrone	E1
6	Equilin	EQ
7	20α-Hydroxyprogesterone	20αOHP
8	17α-Hydroxyprogesterone	17αOHP

Table 2
General properties and physical characteristics of the HPLC columns and ODS packing materials [32,33]

	LiChrospher RP-18 (column 1)	Supelcosil LC-18 (column 2)
Column length (cm)	25	25
Internal diameter (mm)	4.6	4.6
Particle size (μm)	5	5
Particle shape	Spherical	Spherical
Pore size (Å)	100	120
Pore volume (ml/g)	1.25	0.6
Surface area (m^2/g)	350	170
Surface coverage ($\mu mol/m^2$)	3.61	3.1
Carbon load (%)	21.0	11.0
Endcapping	No	No

columns have the same internal dimensions, and are packed with the same sized spherical particles (5 μm) of sorbent. In both cases, the surface coverage by the octadecylsilane ligands is similar. The surface area, pore size or volume can affect the efficiency of the chromatographic process. However, we did not observe significant changes in steroid peak shape, symmetry and the values of the theoretical plate height for solutes chromatographed on the two columns. The percentage of carbon load is a measure of the number of C_{18} chains that are attached to the particles of the stationary phase. This is a function of the interrelationship between surface area of the silica particles, surface coverage and the chain length of the bonded functional groups. Therefore, the substantial difference in the carbon load between the investigated octadecylsilica packing materials (ca. 50%) can be explained by the differences in surface area and pore volume.

Determination of the column hold-up time (void time, dead time) for a chromatographic system coupled to a UV detector is commonly made by measuring the retention time of a low-mass compound which is non-transparent for UV light (e.g. sodium nitrate or nitrite in the case of polar mobile phases and reversed-phase columns or benzene and its derivatives for normal-phase systems). The void time marker should be unretained by the stationary phase and fully permeate all pores of a packing material. The precision of the hold-up time determination may differ for different hold-up time markers, therefore discussions regarding both the definition and measurement of hold-up time is still very active [34,35].

In our studies, the hold-up times were measured by injecting small amounts of sodium nitrate and detection of the void marker peaks at 220 nm. Table 3 presents the differences in the retention of sodium nitrate on each column investigated using acetonitrile–water mobile phases with and without addition of β-cyclodextrin. As can be seen, neither of the investigated mobile phase compositions had an effect on the retention of the hold-up time marker. Therefore, the influence of macrocyclic additive on hold-up time measurement can be neglected. However, the difference in retention of the hold-up time marker on column 1 and column 2 is significant (11.2%) and is compatible with a difference in surface coverage of the column investigated (14.2%). Because both support materials lack endcapping, it can be expected that interactions with residual silanol groups could

Table 3
The differences in the retention of the void time marker (sodium nitrate) on each column investigated using acetonitrile–water mobile phases with and without the addition of β-cyclodextrin

	β-CD concentration			
	Column 1		Column 2	
	0 m*M*	12 m*M*	0 m*M*	12 m*M*
Retention time (min)	1.448	1.438	1.624	1.627
STD	0.006	0.009	0.009	0.005
CV%	0.42	0.66	0.52	0.30

STD, standard deviation; CV%, relative standard deviation.

contribute to the retention mechanism of the solute [5,7]. Hence, the difference in retention of sodium nitrate can be explained assuming that small solute molecules can move between the C_{18} chains and interact with the silanol groups which are attached to the stationary phase particles. The experimental data indicate that the contribution of polar silanol groups to the retention of the hold-up time marker is less evident for a column with a higher surface coverage. For that reason, the observed retention time of sodium nitrate on column 1 is less than that observed on column 2.

The experimental data presented in Figs. 1 and 2 have been obtained at a temperature of 26 °C. This had been previously determined as the optimum temperature for separation of a battery of estrogens and progestogens using the LiChrospher RP-18 column [18]. As can be seen from the data displayed in Fig. 1, observed retention times for all steroids chromatographed on column 2 are significantly decreased in comparison to column 1. The decrease

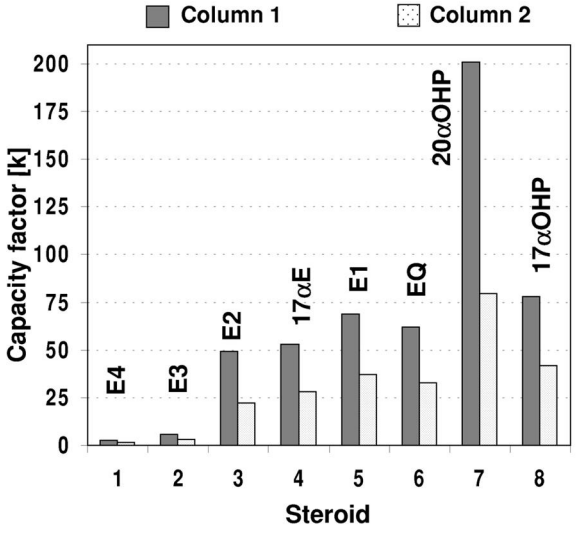

Fig. 1. The retention factors for steroids measured on column 1 (LiChrospher RP18) and column 2 (Supelcosil LC-18) using a mobile phase composed of 30% v/v acetonitrile in water without β-cyclodextrin addition. The *x*-axis co-ordinate corresponds to the individual steroids: (1) estetrol; (2) estriol; (3) 17β-estradiol; (4) 17α-estradiol; (5) estrone; (6) equilin; (7) 20α-hydroxyprogesterone; (8) 17α-hydroxyprogesterone. The temperature of the chromatographic process was kept constant at 26 °C.

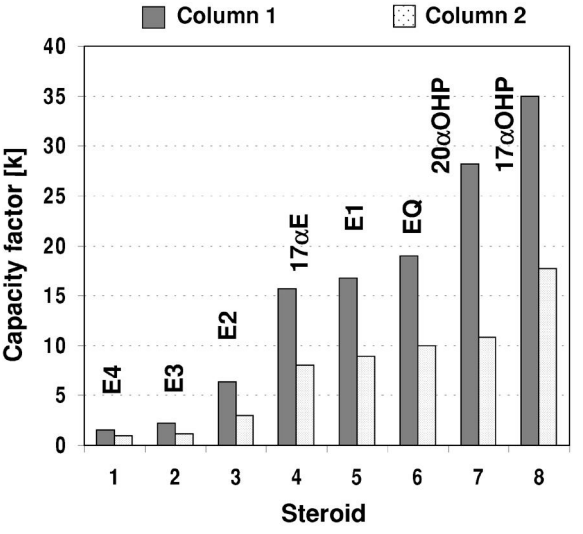

Fig. 2. The retention factors for steroids chromatographed on column 1 and column 2 using a mobile phase with the addition of β-cyclodextrin at a concentration of 12 m*M*. The *x*-axis co-ordinate and experimental conditions are as defined in Fig. 1.

in retention ranges from 37.1% (estetrol) to 60.4% (20α-hydroxyprogesterone). This result is in contrast to retention studies conducted on the hold-up time marker. It is generally assumed that under reversed-phase conditions, the main interactions occur between the individual steroids and the stationary phase C_{18} chains. Therefore, the decrease in the steroid retentions on column 2 appears to be associated with the carbon load of the stationary phase. A similar phenomenon occurs, when the mobile phase is modified with cyclodextrin (Fig. 2). The addition of the macrocyclic modifier strongly affects the elution order and retention time of the steroids. The total analysis time is reduced sixfold compared with separations conducted on a mobile phase without the macrocyclic additive. This indicates that strong interactions exist between free steroids and cyclodextrin molecules, within the mobile phase.

The data in Fig. 3 give the retention factor ratios ($k_{0\text{mMCD}}/k_{12\text{mMCD}}$) calculated for both columns from the retention data obtained using mobile phases with and without cyclodextrin. This parameter is directly related to the mol solute present in the mobile phase [30]. It is noteworthy that the values of the retention factor ratios are similar for both columns investi-

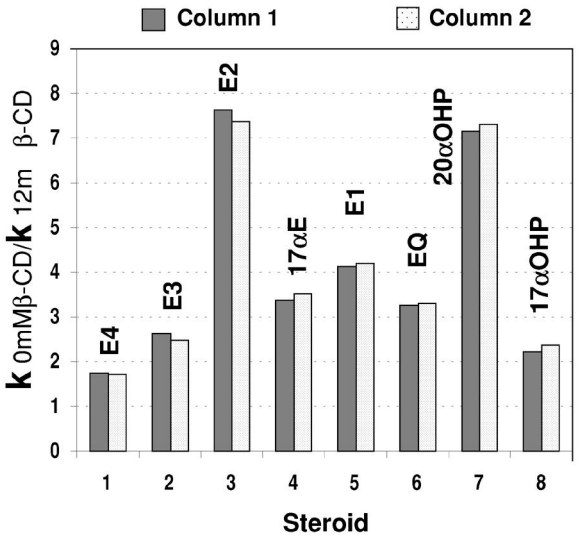

Fig. 3. The ratios of retention factors (k_{0mMCD}/k_{12mMCD}) of steroids investigated calculated for column 1 and column 2.

gated. This observation indicates that the intensity of the host–guest interaction is similar on both columns investigated and properties of the stationary phases appear to have little effect on the formation of host–guest complexes, which occur in the mobile phase. Therefore, the selectivity of chromatographic systems in which a major part of the cyclodextrin molecules remain in the mobile phase, i.e. the retention time of the macrocyclic modifier is close to void volume [13,18], can be controlled by two independent mechanisms. First, the interaction of steroids with C_{18} chains of the stationary phase and, second as a result of the intensity of host–guest interactions in the mobile phase.

The addition of β-cyclodextrin to the mobile phase results in a system that is highly temperature-dependent [17,18]. Fig. 4 displays the separation of the steroid mixture using varied experimental conditions. The first two chromatograms (A and B) were recorded at 26 °C on columns 1 and 2, respectively. Optimum temperature for separation of the steroids on the column with a low carbon load was determined using the method described previously [18]. Chromatogram C is the result of the separation, using column 2 at temperature 29 °C. As can be seen, for both temperatures investigated (chromatogram B and C) all the solutes have shorter retention times on the low carbon load column than on the column with a high carbon load (chromatogram A). The calculated separation factor values between the adjacent peaks on chromatograms A–C are presented in Table 4. It can be seen that the separation of estetrol and estriol is more efficient using the column with a high carbon load (chromatogram A). However, the manipulations of temperature with respect to column 2 (chromatogram C) provided a chromatographic system suitable for fast and efficient separation. The effect of temperature optimization is best observed by improvements in the selectivity between equilin and 20α-hydroxyprogesterone as well as 17α-estradiol and estrone.

4. Conclusions

The experimental data have shown that retention of the steroids chromatographed on octadecylsilica packing material with a low carbon load is significantly reduced in comparison to a column filled with a high carbon load stationary phase. Generally, low carbon load sorbent offers better separation and a shorter analysis time of the solutes investigated. However, the calculated values of the retention factor ratios of the steroids are similar for both columns studied. Therefore, the stationary phase structure appears to have little effect on the formation of host–guest complexes. It is noteworthy that the retention of the solutes on the investigated reversed-phase chromatographic systems can be controlled in two independent ways. These are the interaction of steroids with the C_{18} chains of the stationary phase and the intensity of host–guest interactions in the mobile phase. From a practical point of view, the total analysis time of the mixture investigated can be reduced twofold by using a column with a low carbon load and sixfold by addition to the mobile phase of β-cyclodextrin at a concentration of 12 m*M* and temperatures ranging from 26 to 29 °C.

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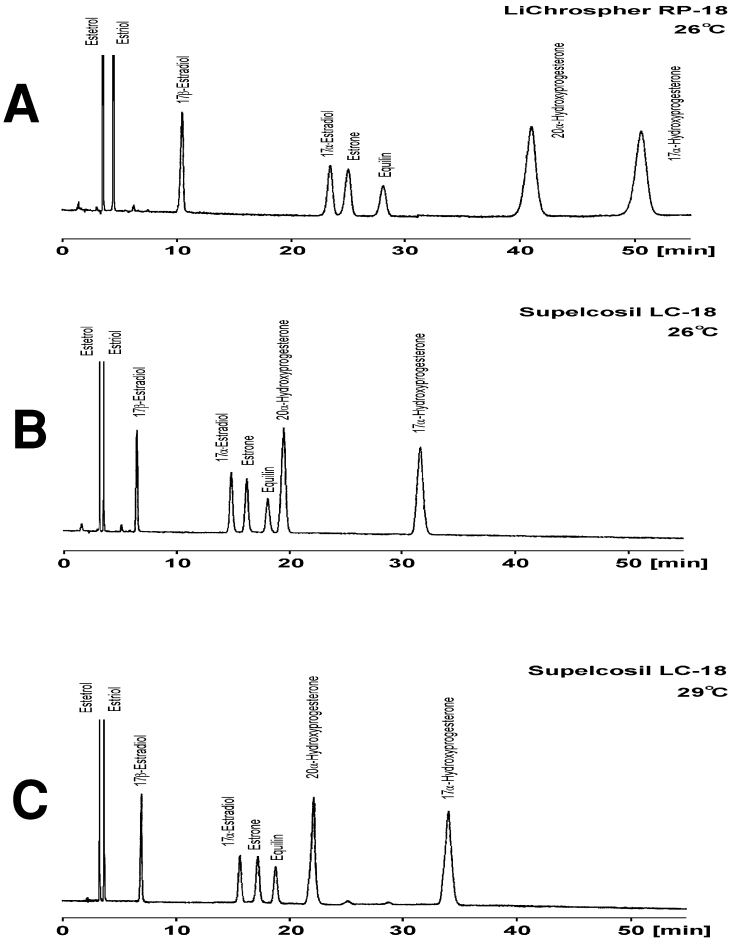

Fig. 4. Separation of steroids using columns with different carbon loads and a binary mobile phase (30% v/v acetonitrile–water) modified by β-cyclodextrin at a concentration of 12 m*M*. Chromatograms A and B were executed at a temperature of 26 °C on column 1 and column 2, respectively. Chromatogram C was conducted on column 2 at the optimum temperature of 29 °C.

Table 4
The separation factor values (α) observed for adjacent peaks of steroids on each column investigated using a mobile phase containing 12 m*M* β-cyclodextrin

Steroids	Column 1 at 26 °C	Column 2 at 26 °C	Column 2 at 29 °C
Estetrol/estriol	1.41	1.22	1.24
Estriol/17β-estradiol	2.95	2.50	2.60
17β-Estradiol/17α-estradiol	2.44	2.67	2.63
17α-Estradiol/estrone	1.07	1.11	1.11
Estrone/equilin	1.13	1.12	1.10
Equilin/20αOHP	1.48	1.09	1.20
20αOHP/17αOHP	1.24	1.63	1.58

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