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High performance liquid chromatography with cyclodextrin and calixarene macrocycle bonded silica stationary phases for separation of steroids

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Abstract

β-Cyclodextrin, *p-tert*-butyl-calix[8]arene and chloropropyl bonded silica stationary phases have been prepared and were applied at the same time to develop a chromatographic procedure to separate steroids. In order to select the best type of stationary phase for the analysis, similar preparation processes of the two kinds of macrocycle stationary phases with the same spacer were adopted respectively. The chromatographic behaviors and retention mechanisms of the two kinds of macrocycle stationary phases for steroids were systematically studied and compared with those of chloropropyl bonded silica and ODS. The effect of mobile phase variables, such as methanol content, pH value of buffer, ionic strength and buffer composition on chromatographic behaviors was investigated. The results showed that the retention mechanisms of the four stationary phases for steroids were obviously different, and excellent separation was achieved on β-cyclodextrin bonded silica stationary phase (β-CD-BS), as a consequence of the structure and the properties of the stationary phase. The retention process on β-CD-BS exhibited inclusion complexation, hydrogen-bonding and weak hydrophobic interaction, while for *p-tert*-butyl-calix[8]arene bonded silica stationary phase (CBS), π–π and hydrogen-bonding besides hydrophobic interaction played an important role.

Keywords: *p-tert*-Butyl-calix[8]arene bonded silica stationary phase; β-Cyclodextrin bonded silica stationary phase; Steroid; Separation

1. Introduction

Various new bonded stationary phases were continuously explored and applied with the development of HPLC. For a given sample analysis, the selection of an appropriate stationary phase is often the most important step, which determines the quality of the final result. One of the factors playing a predominant role in the separation process is the interaction between the solute and the stationary phases. Specific surface property and molecular structure of the bonded stationary phase are the most important characteristics of stationary phase.

The macrocycle molecules bonded stationary phases based on supramolecular interaction have been receiving much attention due to especial separation selectivity. Crown ether and cyclodextrin bonded stationary phases have achieved a great success especially cyclodextrin for chiral separation. In general, the separation selectivity of system is improved by introducing cyclodextrin into HPLC because of inclusion complexation, hydrogen-bonding interaction, etc. besides hydrophobicity. In addition, calixarene as third host of supramolecule has also recently been applied in chromatography.

Steroid hormones are important for controlling human body functions as a part of the endocrine system together with neuronal systems and the immune system. Numerous synthetic sex steroid hormones have been used as therapeutic agents. The therapeutic doses required in the treatment of many diseases are often much larger than those employed

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in contraception. In addition, some hormonal disorders such as adrenocortical adenoma and cushing syndrome are controlled by corticoids [1]. Therefore, the separation and determination of sex steroid hormones along with corticoids are very important. However, because of the great diversity in the structure and polarity of sex hormones and corticoids, for the simultaneous analysis of both classes of steroids some special problems are often encountered. At the present time, the reversed-phase high performance liquid chromatography (HPLC) using ODS as the stationary phase is one of the most widely used analytical methods for the separation of steroids. However, gradient elution was often used in these analysis methods [2], which would lead to solvent being largely consumed with excessive peak tailing. Gonzalo-Lumbreras and Izquierdo-Hornillos [3–5] separated steroids by the optimization method involved the use of binary, ternary and quaternary mobile phases; the operation is complicated and time consuming.

Some workers have attempted to study on improving separation of steroids by introducing supramolecular interaction. Shimada et al. [6] separated neurosteroids on ODS column using α-, β-, γ-CD as mobile phase additives. Zarzycki and coworkers [7–10] have proposed a strategy that optimized the steroid separation using temperature as the critical parameter and improved selectivity using mobile phases modified with β-CD in liquid chromatography. Subsequently, they [11] investigated the chromatographic behavior of selected steroids and their inclusion complexes with β-cyclodextrin on octadecylsilica stationary phases with different carbon loads. Flood et al. [12] reported the characterization of inclusion complexation and separation of steroids with cyclodextrins as mobile phase additives by high performance liquid chromatography. Cserháti and Forgács [13] studied the effect of cyclodextrin derivatives on the retention of 18 steroid drugs on β-CD polymer coated silica column using methanol–water mixture as elution. In addition, calixarene as third host of supramelocule has also been reported to the separation of steroids. Gebauer et al. [14] separated 17 α/β-estradiol stereoisomers on calixarene-boned silica gels. Recently, Sokoließ et al. [15] studied and found that calixarene bonded stationary phases were more selectivity than the Kromasil C_{18} phase on the separation of gestagenic and androgenic steroids due to specific interactions with the steroids of similar hydrophobicity. However, study on the separation of steroids on cyclodextrin and calixarene bonded stationary phases at the same time and comparison of the retention mechanisms with same solutes as probes was not reported.

In the present work, new β-cyclodextrin and *p-tert*-butyl-calix[8]arene bonded stationary phases with the same spacer have been first prepared by similar processes and used simultaneously for the separation of steroids. The chromatographic separation of some steroids on CBS and β-CD-BS were studied in comparison with CPS and ODS. In the chromatographic separation processes, the influence of mobile phase variables such as methanol content, pH value, ionic strength and buffer composition on the retention behavior and mechanism of steroids on the stationary phases was also investigated.

2. Experiment

2.1. Chemicals and reagents

Silica (Kromasil) and ODS (Kromasil C_{18}, 5 μm spherical, 1.054 mmol g^{-1}) were purchased from Akzo Nobel (Sweden). Physico-chemical characteristics of the bare silica adsorbent are presented in Table 1. Chloropropyltriethoxylsilane was purchased from Wuhan University Chemical Plant (Wuhan, China), *p-tert*-butyl-calix[8]arene was prepared according to a procedure reported [16]. β-Cyclodextrin was purchased from Academic Institution of Zymosis (Jiangsu, China). Steroid samples – ethinylestradiolum (EE), nandrodone phenylpropionate (NPP), dexamethasoni natrii phosphas (DNP), hydroxyprogesterone caproate (HPC), diethylstilbestroum (DEE) – were afforded by Drug Appraisal Office of Beijing (China). The structures of steroids are given in Fig. 1. Other reagents are of A.R. grade unless indicated otherwise.

2.2. Instruments

The liquid chromatography system consisted of a Model LC-60AT pump (Shimadzu, Japan), a Reodyne 7725 injector with 20 μl sample loop, UV detector (Science Instrument of Shanghai, China) and TL9900 chromatographic station (Teleh, Beijing, China). Element analyzer (UOD-1106, Italy) was used.

2.3. Preparation of p-tert-butyl-calix[8]arene and β-cyclodextrin bonded silica stationary phases

2.3.1. Preparation of p-tert-butyl-calix[8]arene bonded silica stationary phase (CBS) [17]

Active silica gel (2 g) was suspended in 100 ml dry toluene containing 2 ml chloropropyltriethoxysilane and three drops triethylamine was added. The mixture was stirred and heated to reflux under nitrogen atmosphere for 24 h. The solid was filtered and washed in sequence with toluene, acetone,

Table 1
Physico-chemical structure of bare silica

Characteristics	Value
Mean particle size, d_p (μm)	5
Particle shape	Spherical
Specific surface area, S_{BET} ($m^2\ g^{-1}$)	310
Pore volume, V_p ($cm^3\ g^{-1}$)	0.82
Mean pore diameter, D (nm)	10
Concentration of OH group, α_{OH} (μmol m^{-2})	7.1
Trace amounts of metals (ppm)	
$C_{M,Na}$	28
$C_{M,Fe,A}$	<10

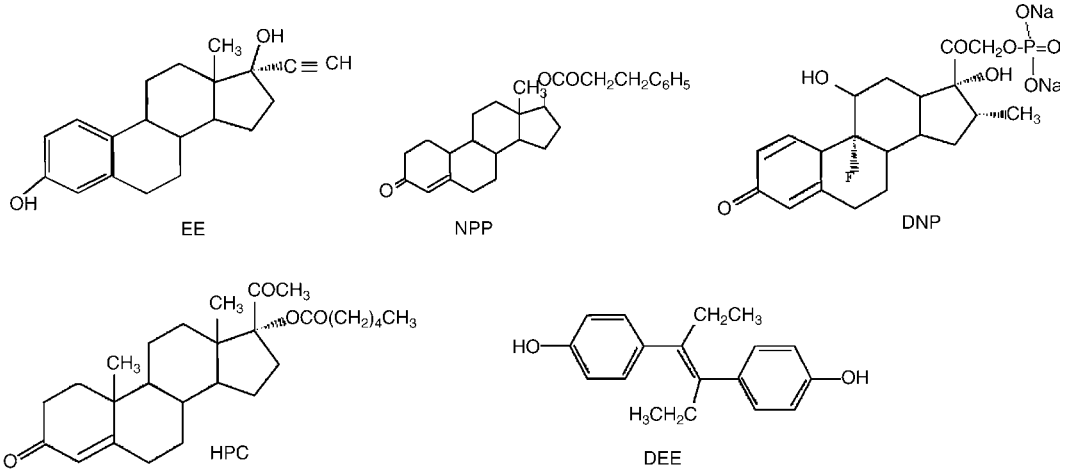

Fig. 1. The structure of steroid hormone medicines to be used.

methanol, distilled water and acetone. The product (chloropropyl bonded silica, CPS) as precursor of following reaction was dried at 100 °C under vacuum for 8 h.

p-tert-Butyl-calix[8]arene (1 g) and sodium hydride (0.1 g) were stirred and heated under reflux in 100 ml dry toluene for 1 h, and then superlatant liquid was transferred to 250 ml three neck flask, CPS (2 g) and 100 ml dry toluene were added. The mixture was stirred and heated under reflux for 48 h under catalyst in nitrogen atmosphere. The reaction was stopped and the product was filtered and washed in sequence with toluene, acetone, methanol, distilled water and acetone. Subsequently, CBS was obtained, and dried at 100 °C under vacuum for 8 h, then cooled to room temperature in a desiccator.

2.3.2. Preparation of β-cyclodextrin bonded silica stationary phase (β-CD-BS) [18]

The preparation process is similar to that of CBS. At first, the chloropropyl bonded silica as precursor was prepared, and then the sodium salt of β-cyclodextrin reacted with CPS in dry DMF. The condition of reaction and operation of the preparation of β-CD-BS were of the same as those of CBS. The schematic structures of the three stationary phases prepared are illustrated in Fig. 2.

2.4. Chromatographic procedures

The bonded phases (CBS, β-CD-BS, CPS and ODS) were respectively packed into a 15 cm × 4.6 mm (i.d.) stainless-steel column by slurry technique. The mobile phase used was methanol–water or methanol-buffer. Before use, the mobile phase was generally filtered through a G-4 filtered glass and degassed in an ultrasonic bath for 5 min. The flow-rate of mobile phase was set at 0.8 ml/min. The wavelength used for detection was 254 nm. The samples were dissolved in methanol. All measurements were carried out at ambient temperature (25 ± 2 °C) and repeated at least twice.

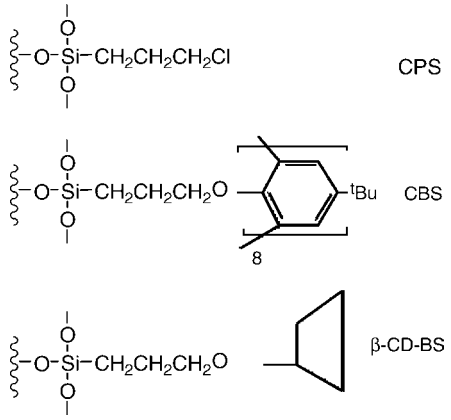

Fig. 2. The schematic structures of the prepared stationary phases.

3. Results and discussion

3.1. Preparation and characterization of CBS and β-CD-BS

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 well as metal impurities in the silica supports. To minimize influence of silica matrix, all stationary phases were prepared by using the highest quality silica. In addition, ODS packing also came from the same company and brand. Moreover, in order to avoid other distribution coming from the preparation processes of the two macrocycle bonded stationary phase, we try to take same condition of reaction and operation process to prepare β-CD-BS and CBS. Table 2 presents the bonded amount of organic ligands based on elemental analysis data. CPS is the intermediate or the first stage product of modification pro-

Table 2
The degree of silica coverage with organic ligands based on elemental analysis

Packings	Spacers at first stage of modification		Macrocycle bonded at second stage of modification	
	C (%)	Bonded amount (mmol g^{-1})	*C* (%)	Bonded amount (mmol g^{-1})
CPS	3.21	0.092	–	–
CBS	3.21	0.092	8.98	0.060
β-CD-BS	3.35	0.096	4.88	0.013

cess as the spacer bonded phase, its bonded amount exhibits only small differences and excellent reproducibility at every time preparations. In addition, from the molecular structure of stationary phase, the stability of stationary phase is enhanced by ether bond and residual silanols are shielded by macrocycle connected with silica gels by three methylenes short spacer. Therefore, the difference of matrix of the two kinds of macrocycle stationary phases is negligible. According to the carbon content of macrocycle bonded stationary phases after subtracting that of CPS, the bonded amounts of macrocycle for the second stage of the modification were calculated. Consequently it can be expected that there is active participation of both macrocyclic molecules bonded in the separation process [19] although in the bonded density of the two macrocycle stationary phases there exists difference to some extent.

3.2. Chromatography of steroid hormone

3.2.1. Effect of methanol content

The effect of mobile phase composition on the retention of steroids was investigated by changing the ratio of methanol to water in the mobile phase as shown in Fig. 3. The retention of solutes decreased with increasing the methanol content of mobile phase on the three stationary phases. The results indicated that all the stationary phases exhibited hydrophobicity and can behave as reversed-phase packings. Though a good linear correlation existed between log *k* and methanol content in mobile phase on CPS, the linear correlation is relatively poor on the macrocycle stationary phases. After linear regression, the R^2 values are 0.992–0.993, 0.971–0.984, and 0.985–0.993 on CPS, CBS and β-CD-BS, respectively. The linear relation of the three stationary phases is CPS > CBS > β-CD-BS. That is, the retention of solutes on CPS lies in hydrophobic interaction, while the hydrophobic interaction is not the only factor in the retention and separation of these compounds on macrocycle stationary phases. Making a comparison of the retention of solutes under the same mobile phase on the three

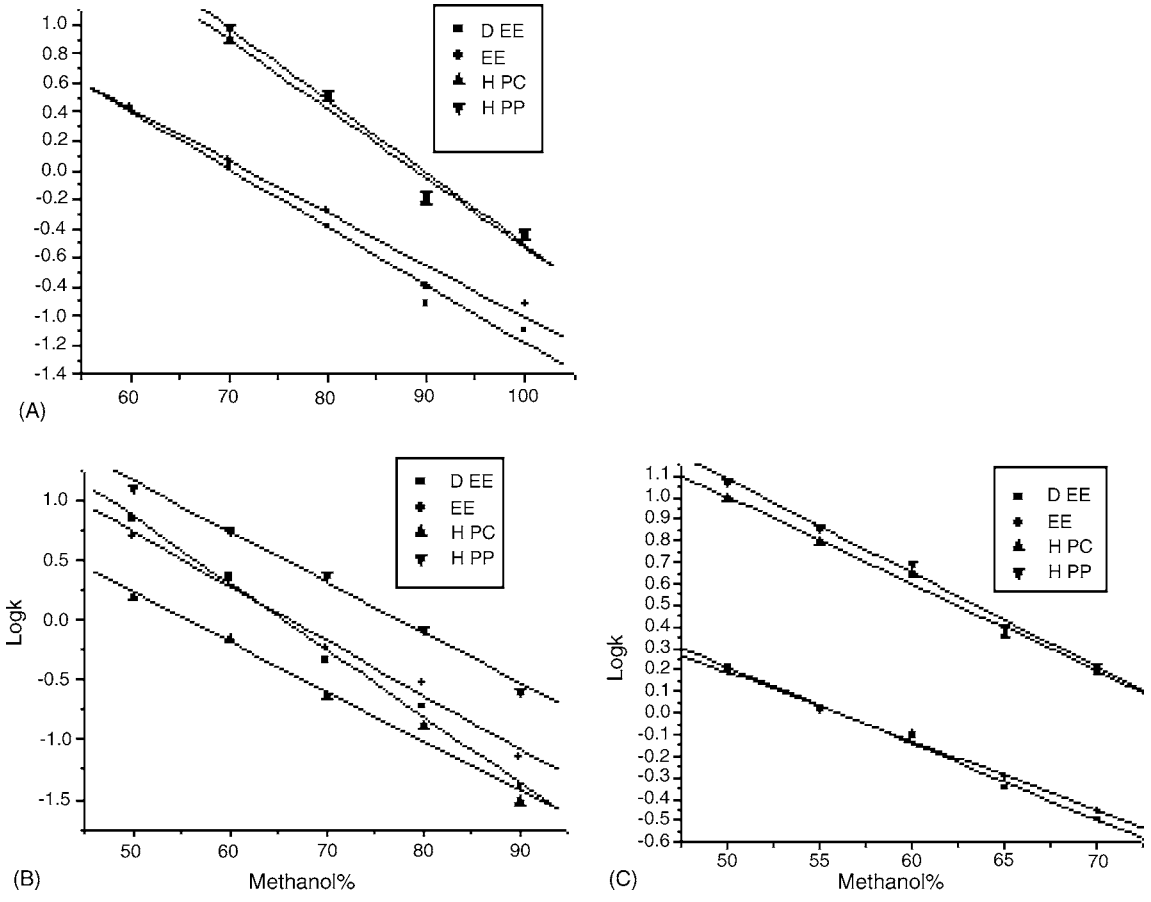

Fig. 3. Influence of methanol content on the retention factors (log *k*) of steroids on CBS (A), β-CD-BS (B) and CPS (C).

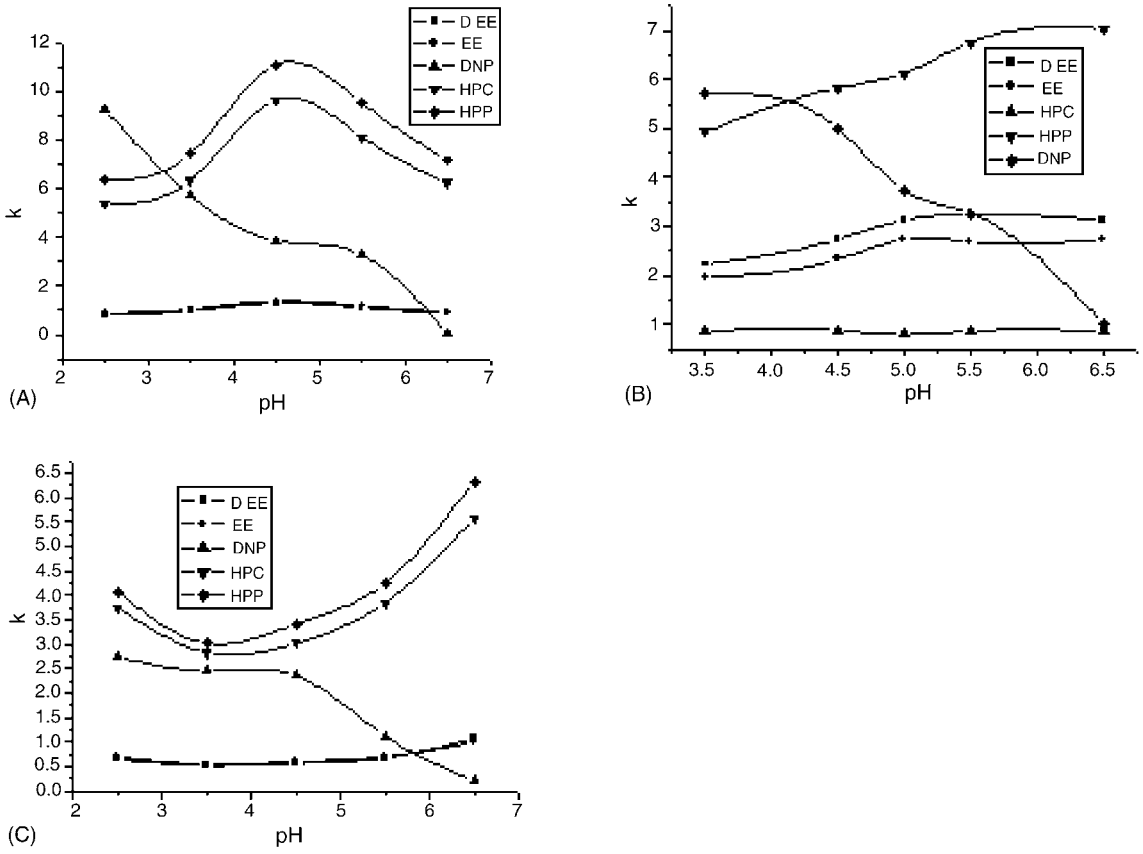

Fig. 4. Influence of pH on retention factors (*k*) of steroids on CBS (A), β-CD-BS (B) and CPS (C). Mobile phase:methanol-buffer (0.02 M KH_2PO_4 pH4.5) ratios were (A) 70:30, (B) 50:50, (C) 55:45.

packings, it can be found that the retention of CBS is the strongest among the three stationary phases, and the retention of solutes on the two other stationary phase are similar. The reasons may be as follows: at first, the hydrophobicity of *p-tert*-butyl-calix[8]arene is the strongest among the three stationary phases because of the weak hydrophobicity of chloropropyl group and β-CD. Secondly, the bonding density of CBS is larger than that of β-CD-BS.

3.2.2. Effect of pH values in buffer

Effect of pH values was shown in Fig. 4, the retention of DNP depended remarkably on the pH value of buffer on the three stationary phases. The retention factor decreased drastically with increasing pH value of buffer. The phenomenon can be explained as follows: the retention value increased due to DNP as molecular form at low pH value. The higher hydrophobicity of neutral molecule is responsible for stronger retention. As increasing the pH value over pK_a of the solute, DNP was ionized, the retention of DNP as ionic form decreased quickly at high pH value.

Gonzalo-Lumbreras and Izquierdo-Hornillos [4] studied the effect of pH on the retention of steroids by ODS and found that no significant changes in the retention of steroids examined were observed. While the influences of pH value on the retention of steroids except for DNP is different on the three stationary phases prepared by us, which implies that pH value of buffer exhibits different influence on the properties of stationary phases. As can be noticed in Fig. 4A, for CBS, the retention values of solutes increased gradually with increasing pH value of buffer and arrived at maximum retention value at pH 4.5 [20], and then the retention values decreased with further increasing of pH value. The phenomenon may relate to the existed form of *p-ter*t-butyl-calix[8]arene phenols on CBS, which influences two main factors to produce the retention of solutes, namely hydrophobicity and π–π interaction. On one hand, the retention of steroids increased with increasing π–π interaction between the *p-ter*t-butyl-calix[8]arene and the solutes because the phenolic anions had higher electron density in benzene ring than their neutral molecules with ionization of phenolic hydroxyl groups of CBS. On the other hand, as the pH value was over 4.5, the phenolic hydroxyl groups of the *p-ter*t-butyl-alix[8]arene were ionized further. The hydrophobicity of ionized CBS decreased, which led to decreasing the retention of the solutes. The two opposite effects led to the fact that retention values reach their maximum about pH 4.5.

The influences of pH value on the retention of steroids except DNP are small on β-CD-BS as shown in Fig. 4B. As seen, the retention values of steroids increased slightly with increasing pH. At neutral mobile phase, the reten-

tion values reach maximum value, which may be related to inclusion interaction between neutral organic molecule and bonded β-CD. The studies on the complexation of steroids with cyclodextrin were almost performed with neutral water–methanol/acetonitrile as mobile phases [12,21].

At the same time, we investigated the effect of pH value on retention of solutes on CPS as shown in Fig. 4C. It can be found that the plot is different completely from others except DNP. No obvious effect of pH value on the retention of EE and DEE is observed. Other solutes, for example, HPC and NPP exhibit stronger retention at low and high pH values, which may relate to hydrogen-bonding interaction to be increased. At low pH values, there exists hydrogen-bonding interaction due to residual silanol of silica surface; with increasing pH values of buffer, the hydrogen-bonding interaction decreased. However, at high pH over pK_a of hydroxyl group of silica surface about 3.0, the dipole–dipole interaction resulted in increasing the retention of solutes due to the ionization of residual silanol.

From the above analysis, it can be known that the chromatographic behaviors of three packings are much different from each other. The hydrogen-bonding and hydrophobic interactions play an important role in the retention process on CPS. When *p-tert*-butyl-calix[8]arene is attached to CPS, it happens to increase hydrophobic and π–π interaction between solutes and stationary phase; however the retention of solutes on β-CD-BS are based on inclusion complexation, hydrogen-bonding and hydrophobic interactions.

3.2.3. Effect of ionic strength

The influence of ionic strength in mobile phase on retention factors was investigated by increasing KH_2PO_4 buffer concentration (from 0.005 to 0.04 mol/l) at constant pH (4.5). Under these conditions no significant changes in the retention of steroids on CPS and β-CD-BS were observed [3,4], while the retention of steroids on CBS changed with the concentration of ionic strength as shown in Fig. 5. The retention values of solutes increased with increasing the ionic strength of mobile phase at low concentration range. While the retention values decreased slightly with further increasing buffer concentration. The maximum value of retention is obtained at 0.02 mol/l KH_2PO_4 solution as buffer for CBS. The reason was probably as follows: the concentration of K^+ increased at limited range, the retention of steroids increased on CBS because K^+ complexation resulted in a better pre-organization of the basket-like of *p-tert*-butyl-calix[8]arene which makes solutes move easily into hydrophobic cavity. Such a similar phenomenon that calyx[4]arene stationary phase shows high selectivity for Na^+ by "ionic induce fit" has been observed by Gebauer [22]. On the other hand, the concentration of K^+ is too much, hydrophobic cavity of *p-tert*-butyl-calix[8]arene is taken up by K^+ so that it is difficult for solutes to be close to stationary phase which led to decreasing the retention of solutes.

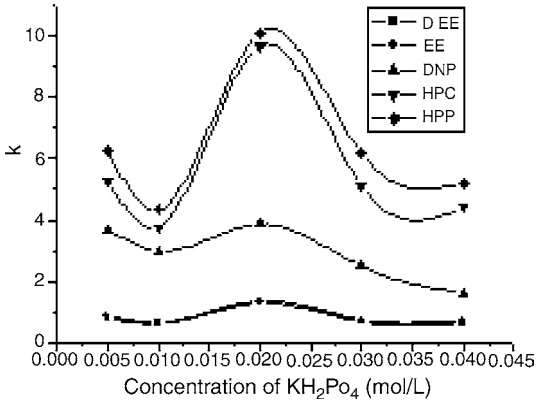

Fig. 5. Influence of ion strength on retention factors (*k*) of steroids on CBS. Mobile phase:methanol-buffer (0.02 M KH_2PO_4 pH 4.5) = 70:30.

Table 3
Retention factors (*k*) of solutes in different buffers on CBS

Ion types	DEE	EE	DNP	HPC	NPP
K^+	1.28	1.36	3.86	9.65	11.09
Na^+	1.10	1.13	3.72	8.24	9.95
NH_4^+	1.02	1.07	5.56	6.40	7.50

Mobile phase:methanol-buffer (0.02 M MH_2PO_4 pH 4.5) = 70:30.

3.2.4. Effect of cation in buffer

From the discussion above, it can be understood that CBS shows especial selectivity for K^+. In order to confirm further the complexation with K^+, the influence of different buffer cations were investigated on the retention of steroids on CBS. KH_2PO_4, NaH_2PO_4 and $NH_4H_2PO_4$ were used as buffer in the mobile phase. The retention factors of solutes in the mobile phase with different cations on CBS are given in Table 3. As anticipated, K^+ can enhance the retention of solutes and separation selectivity of CBS. Such phenomenon has not been observed on β-CD-BS, CPS and ODS.

3.2.5. Separation of steroids

In general, as reported by many researchers [20], the separation selectivities of stationary phases for solutes were compared under the same mobile phase. The comparison of efficiency is limited due to different optimal mobile phases being existed for different packings. In order to make a more practical comparison, we compared the retention of steroids under the same mobile phase and optimal mobile phases on the four packings. Under the same chromatographic condition, the retention data of the solutes are shown in Table 4. The separation of steroids was not achieved by using CPS

Table 4
Retention factors (*k*) of solutes on CBS, β-CD-BS, CPS and ODS

Packings	DEE	EE	DNP	HPC	NPP
CBS	1.13	1.10	3.72	8.24	9.95
β-CD-BS	0.52	0.47	2.02	0.23	2.45
CPS	0.26	0.32	1.06	1.32	1.40
ODS	3.16	3.27	1.03	–	–

Mobile phase:methanol (0.02 M NaH_2PO_4 pH 4.5) = 70:30; on ODS HPC and NPP were not eluted in 90 min.

due to the weak hydrophobicity; while HPC and NPP were eluted over 90 min on ODS due to stronger hydrophobic interaction. On CBS and β-CD-BS, the steroids were separated selectively except the solute pair DEE and EE.

The optimizing resolution for a multi-component mixture is often a compromise between maximum resolution and minimum analysis time, therefore to find the best chromatographic separation of steroids, the retention factor of last eluted peak (k_{max}) and the smallest resolution between adjacent peaks ($R_{s,min}$) were simultaneously compared [10,23]. The smallest k_{max} value provides faster analysis. The smallest resolution ($R_{s,min}$) between the pair of solutes to be difficultly separated should obviously be as large as possible. Therefore, it is important to consider the optimization of parameters at the same time for obtaining the best separation. The retention behavior of DEE and EE on CBS, CPS and ODS are similar, and R_s value between two solutes is zero on CBS, CPS and ODS. Therefore, it is necessary to compare the sub-minimal resolution ($R_{s,submin}$). After optimization of mobile phases, the typical chromatograms are given on the four columns as shown in Fig. 5. To evaluate reasonably the chromatograms, the separation data of steroids corresponding Fig. 6 are shown in Table 5. From the data, it can be found

Table 5
Separation data of steroids on CBS, β-CD-BS, CPS and ODS

	CBS	β-CD-BS	CPS	ODS
k_{max}	5.17	5.87	8.22	10.90
$R_{s,min}$	0.00	1.15	0.00	0.00
$R_{s,submin}$	1.16	1.29	1.08	2.29

The conditions of mobile phases are the same as shown in Fig. 5.

that the optimum separation was achieved on β-CD-BS having smaller k_{max} under preferable $R_{s,min}$, and the solutes were baseline separated selectively including the solutes pair DEE and EE. In comparison with the other packings, the solutes of steroids except DEE were baseline separated having the largest $R_{s,submin}$, but the k_{max} had also maximum value on ODS, which implies the longest analysis time. Although the $R_{s,submin}$ on CBS was lower than that on ODS, the solute pair to be difficultly separated was still baseline separated closely, and half of the analysis time was saved. Therefore, for rapid analysis, CBS is selected rather than ODS for the separation of steroids.

In addition, it can be found that the solute pairs to be difficultly separated change with packings. The solute pair of HPC and NPP is difficult to be separated by CBS and CPS,

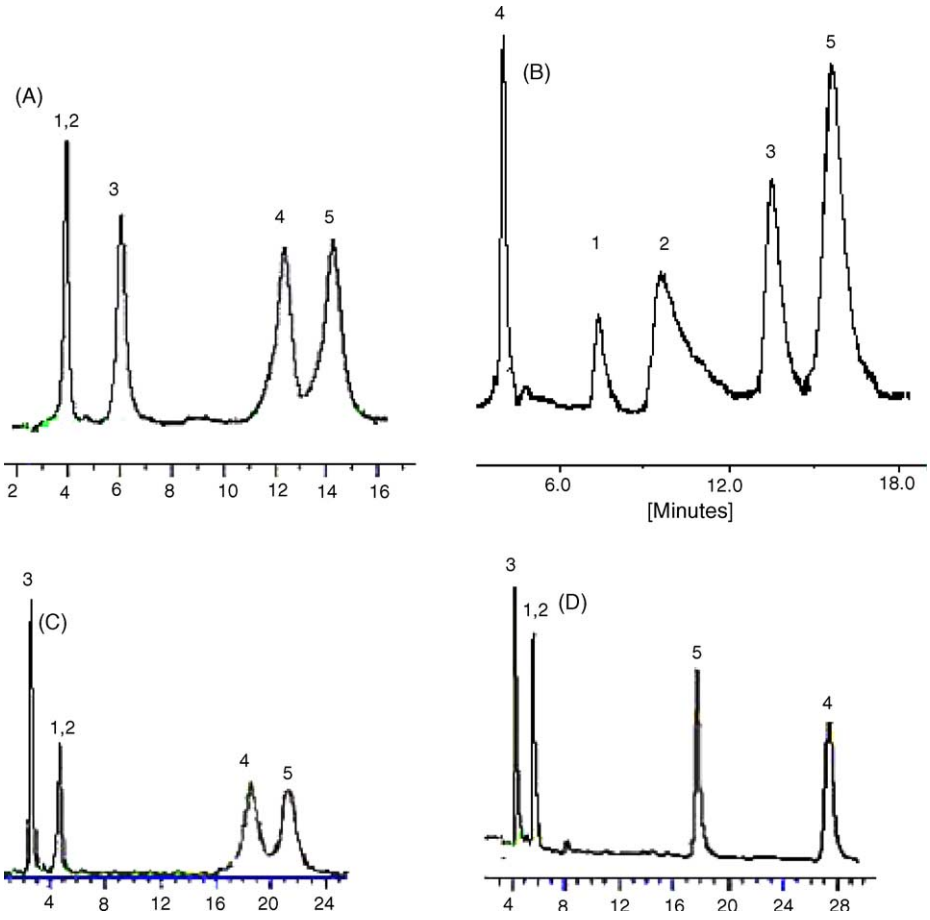

Fig. 6. Separation chromatograms of steroid on CBS (A), β-CD-BS (B), CPS (C) and ODS (D). Mobile phase:methanol-buffer ratios were (A) (0.04 M KH_2PO_4 pH 4.5) = 70:30, (B) (0.02 M KH_2PO_4 pH 4.5) = 60:40, (C) (0.02 M KH_2PO_4 pH 6.5) = 50:50, (D) (0.02 M KH_2PO_4 pH 4.5) = 85:15; (1) EE, (2) DEE, (3) DNP, (4) HPC, (5) NPP.

while the solute pair of NDP and EE turns to be difficultly separated by ODS, which hints that different retention mechanisms exist. For polar and weak hydrophobic solutes, the separation selectivity on CBS is superior to ODS because other interactions such as hydrogen-bonding, π–π interactions exist besides hydrophobic interaction.

The four stationary phases exhibit different separation selectivity and elution order for the solutes examined. The stronger retention of DNP on CBS results from dipole–dipole interaction under the chromatographic condition. NPP was eluted after HPC on CBS and CPS opposite to ODS. The reason for this phenomenon is more likely to be the better formation of π–π interaction among phenyl of solute and cyclic cavity of *p-tert*-butyl-calix[8]arene or lone-pair electron of chlorine. While HPC was first eluted on β-CD-BS for larger molecular size, which exhibits the inclusion complexation of β-CD.

From the discussion above, it can be seen that the hydrophobicity and π–π interaction of calixarene bonded stationary phase are stronger than those of cyclodextrin bonded stationary phase, while the inclusion complexation of the latter is stronger than that of the former. In addition, it can be found that β-CD-BS is more compatible than CBS for the separation of steroids although the bonding density of β-CD-BS is lower than that of CBS. Therefore it is thought that the ability of supramolecule recognition to β-CD is excellent over *p-tert*-butyl-calix[8]arene.

4. Conclusion

β-Cyclodextrin and *p-tert*-butyl-calix[8]arene bonded silica stationary phases have been prepared and were applied for the separation of steroid hormones. The influence of mobile phase variables, such as content of organic modifier, pH value of buffer, ionic strength and buffer composition on the retention of steroids was studied. It was demonstrated that optimizing resolution for these solutes were obtained by using β-CD-BS. It can be concluded that the inclusion interaction played an important role in separating the steroids on β-CD-BS. In addition, from the retention behavior of steroids in high performance liquid chromatography, it can be concluded that two macrocycle supramolecule bonded stationary phases exhibited different molecular recognition mechanism and ability for steroids.

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