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Study on the chromatographic behavior of water-soluble vitamins on *p-tert*-butyl-calix[8]arene-bonded silica gel stationary phase by HPLC

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Abstract

In this paper, the chromatographic behavior of some water-soluble vitamins was studied on a new *p-tert*-butyl-calix[8]arene-bonded silica gel stationary phase (CABS, 5 μm particle size, the bonded amount 0.071 mmol g^{-1}) by using vitamin standards as probes for HPLC. The comparative study of the separation of these compounds was done by using CABS and ODS as stationary phases under the same chromatographic conditions. The better separation of six vitamins including: B_1, B_2, B_6, B_{12}, C, and nicotinic acid (B_5), on CABS can be achieved by using isocratic mode with methanol–phosphate buffer (25:75, (v/v)) as mobile phase within 20 min. The results show that the calix[8]arene-bonded phase exhibits high selectivity for water-soluble vitamins. We found that the elution order of B_2 (12.08 min) and B_{12} (16.42 min) on CABS was very different from that of B_{12} (7.76 min) and B_2 (18.47 min) on ODS, which indicate that different retention mechanisms exist in the chromatographic processes of the two stationary phases. According to the chromatographic data, it can be concluded that various chromatographic retention mechanisms are responsible for the separation of above compounds on CABS, such as hydrophobic interaction, hydrogen bonding interaction, and π–π interaction. The new packing has two advantages over ODS. On one hand, the polar and ionized analytes, such as C and B_5, exhibited stronger affinities to CABS because of hydrogen bonding interaction. On the other hand, the retention of B_2 and B_{12} became shorter on CABS with weaker hydrophobicity in comparison with ODS. The new material exhibits the promising application in the separation of water-soluble vitamins.

Keywords: Column liquid chromatography; *p-tert*-Butylcalix[8]arene-bonded silica gel stationary phase; Retention mechanism; Water-soluble vitamins

1. Introduction

Vitamins are essential for the normal growth and function of human and animal bodies. These compounds can be classified in two main groups: water-soluble and fat-soluble vitamins. Water-soluble vitamins include B_1, B_2, B_6, B_{12}, C, B_5 and so on. They play different specific and vital functions in metabolism, and their lack or excess produces specific diseases. Food is the main resource of vitamins for human and animals. However, loss of vitamins, especially, water-soluble vitamins often occurs in the inappropriate processing and storage of food. Therefore, it is necessary to develop efficient analytical methods for determination of vitamins for the quality control of food and relative products.

In the past decades, traditional analytical methods including different physical, chemical and biological methods were used to analyze each vitamin, which were sometimes tedious and time-consuming. These methods are mainly microbiological procedures, spectrophotometric, fluorimetric, electrochemical methods and thin-layer [1–3]. In recent years, great progress has been achieved in rapid and specific methods for vitamin analysis. A lot of papers have been published concerning the separation and quantification of vitamins in a wide range of products, such as rice, milk, eggs, oral liquid tonics, and multi-vitamin formulation by more simple methodologies. Among them the techniques most widely used are high-performance liquid chromatography (HPLC) [4–11] and capillary electrophoresis (CE) [12–14]. Though

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CE exhibits higher column efficiency, HPLC is still an important method for vitamins analysis because of its relative simplicity, better repeatability, and suitability for various sample matrices.

In 1979, Toma and Tabckhia [15] obtained good resolution in the analysis of thiamine, riboflavin, and niacin by using a reversed-phase column with adding an ion-pairing reagent to the mobile phase. Since then, HPLC was widely used for the determination of water-soluble vitamins. A choice of HPLC methods for water-soluble vitamins can be made: reversed-phase chromatography (RPC) [4–7], ion-exchange chromatography (IEC) [8], ion-pairing chromatography (IPC) [9,10], and normal-phase chromatography (NPC) [11]. Because of its simplicity and better column performance, RPC with RP-C_{18} as stationary phase [14–16] is usually the best starting point. Among available RPC methods for water-soluble vitamins, individual vitamins, two or three vitamins can be chromatographed isocratically; the simultaneous chromatography of more complicate mixtures, in general, requires a gradient elution program involving complex buffer mobile phases or ion-pairing reagents. Several detection methods can be applied, such as UV-Vis absorbance with variable wavelength, photodiode array, fluorimetric, or electrochemical [16,17]. In contrast, the stationary phases used in the separation of water-soluble vitamins were almost uniform RP-C_{18} besides a few cyclodextrin packings [18]. Though a lot of work has been done for quantitative analysis of the vitamins, the retention mechanism of these compounds still require further to be understood, especially, on different stationary phases.

Column packing material is always a key factor in the development of HPLC methods. Recently, calixarenes as separation material have attracted many researcher's attention because it is able to form reversible complexes with many ions and molecules like cyclodextrins and crown ethers. In 1993, Glennon et al. [19,20] prepared silica-bonded calix[4]arene tetraester and silica-bonded calix[4]arene tetradiethylamide stationary phases to separate metal ions and amino acid esters for the first time. Lee et al. [21] reported the separation of some substituted phenol regioisomers and some other aromatic positional isomers on a calix[6]arene-*p*-sulfonate-bonded silica stationary phase. The disubstituted aromatics, nucleosides, uracil derivatives, estradiol epimers and *cis*/*trans* isomers of proline-containing peptides on calix[*n*]arene-bonded (n = 4, 5, 6, 8) silica gel were successfully separated by Gebauer et al. [22–24]. Menyes et al. reported that a hexapropylether of *p-tert*-butyl-calix[6]arene covalently linked to silica, was used for the separation of polycyclic aromatic hydrocarbons (PAHs) and fullerenes, and showed higher selectivity and lower consumption of solvent than conventional RP-C_{18} [25]. Healy et al. [26] prepared an *L*(−)-ephedrinyl-calix[4]arene-bonded phase and used it for the separation of *R*(−) and *S*(+)-1-phenyl-2,2,2-trifluorethanol. In the past few years, our research group prepared *p-tert*-butyl-calix[6]arene-bonded silica gel stationary phase and *p-tert*-butyl-calix[*n*]arene-bonded (n = 4, 6, 8) silica gel stationary phases with different coupling reagents in one-pot method [27,28] and investigated the chromatographic separation of some positional isomers, polycyclic aromatic hydrocarbons, nucleosides, and sulfonamide drugs [29]. The results show that calixarene-bonded stationary phases are excellent reverse-phase packings with inclusion capability.

Recently, we have successfully prepared another new *p-tert*-butyl-calix[8]arene-bonded silica gel stationary phase (CABS) with 3-glycidoxypropyltrimethoxysilane as coupling reagent in the presence of NaH and catalyst. The structure of the new bonded phase has been characterized by using elemental analysis and diffuse reflectance infrared Fourier transform spectroscopy (DRIFT) [30].

In this paper, we reported that six water-soluble vitamins including: B_1, B_2, B_6, B_{12}, C and B_5 were successfully separated on CABS by using isocratic elution with methanol–phosphate buffer as mobile phase. Our work focus on the retention behavior of the vitamins on CABS in comparison with that on ODS by using the vitamin standards as probes, which will contribute to the applications of CABS in the separation of different vitamin samples. The influence of mobile parameters, such as methanol content, pH and ionic strength on retention behavior of the analytes on CABS was also investigated. The separation mechanism was proposed. The results show that the calix[8]arene-bonded phase exhibits high selectivity for above analytes, which was ascribed to moderate hydrophobicity and various action sites providing for the analytes. Six vitamins on CABS can be successfully separated by using isocratic elution within 20 min, which is the obvious advantage over ODS.

2. Experimental

2.1. Chemicals

Silica (Kromasil, 5 μm particle size, 100 Å) and C_{18} (Kromasil, 5 μm particle size) were purchased from Akzo Nobel (Sweden). 3-Glycidoxypropyltrimethoxysilane was purchased from Wuhan University Chemical Plant (Wuhan, China). Thiamin hydroxychloride (B_1), pyridoxine (B_6), riboflavin (B_2), cyanocobalamine (B_{12}), nicotinic acid (B_5) and ascorbic acid (C) were purchased from Sigma. Other chemicals were of analytical reagent grade and were not further purified. Water was double-distilled water.

3-Glycidoxypropyl-bonded stationary phase (GBS, the spacer silica gel, 5 μm, the bonded amount 0.875 mmol g^{-1}) and the *p-tert*-butyl-calix[8]arene-bonded silica gel stationery phase (CABS, 5 μm, the bonded amount 0.071 mmol g^{-1}) were prepared according to a previously reported procedure [30].

2.2. Apparatus

The liquid chromatographic system consisted of a P200 II pump, a UV200 II variable wavelength UV-detector attached Echrom 98 chromatographic data system (Dalian Elite company, China), and a Rheodyne model 7125 injector with 20 μl loop.

2.3. Chromatographic procedure

The bonded phases (GBS, CABS and ODS) were packed into stainless-steel columns (150 mm × 4.6 mm i.d.) by using slurry technique. The mobile phases were methanol–phosphate buffers (different volume ratio). The flow rates were generally set at 0.8 ml min^{-1}. The samples to be tested are the mixture of standards dissolved in methanol–water (30:70, (v/v)), and kept in a refrigerator (in the dark). The samples were centrifuged at 4000 rps min^{-1} for 5 min before use. The wavelength of detection was generally at 254 nm. The concentration of samples were from 20 to 200 μg ml^{-1}. Typically, 5 μl of sample solutions were injected. The aqueous solution of sodium nitrate (0.05 mol l^{-1}) was used as probe to determine dead time. All measurements were carried out at ambient temperature (30 ± 2 °C) and repeated at least twice.

3. Results and discussion

3.1. The preparation of CABS

p-tert-Butyl-calix[8]arene-bonded silica gel stationary phase was prepared according to a previously reported procedure, as scheme in Fig. 1, the structure of CABS has been characterized by elemental analysis, FTIR, and chromatographic performance [30]. The process was described briefly as follows.

Table 1
The results of elemental analysis of the bonded phases

Bonded phases	C (%)	H (%)	Bonded amounts (mmol g^{-1})
CABS	15.93	2.85	0.071
GBS (the spacer)	8.40	1.42	0.875
ODS (Kromasil C_{18})	19.0	3.16	1.054

Table 2
The retention factors (*k*) of PAHs on GBS and CABS (methanol–water, 60:40 (v/v))

Solutes	GBS	CABS	ODS
Benzene	0.68	5.41	8.35
Toluene	0.80	8.47	18.37
Xylene	0.95	14.20	47.16
Naphthalene	1.62	27.35	–[a]
Biphenyl	2.06	36.78	–[a]
Fluorene	2.75	–[a]	–[a]

[a] Indicates that the solutes can not be eluted within 100 min.

A mixture of 3-glycidoxypropyltrimethoxysilane and activated silica gel in dry toluene was stirred and heated at reflux under dry nitrogen gas with triethylamine as catalyst for 6 h. At first the 3-glycidoxypropyl-bonded stationary phase can be obtained. Subsequently, GBS and a little amount of catalyst were added to the mixture of *p-tert*-butyl-calix[8]arene and NaH in toluene and refluxed under an inert atmosphere for 24 h. The mixture was filtered, washed and dried. The stationary phase was obtained. The bonded amount was found to be 0.071 mmol g^{-1} according to the carbon content shown in Table 1. The characteristic IR absorption band of the benzene ring has been found at 1659, 1608, 1536, and 1470 cm^{-1}. The retention factors (*k*) of PAHs on GBS, CABS and ODS were shown in Table 2. The retention values of polycyclic aromatic hydrocarbons (PAHs) on CABS are much higher than those on GBS, but less than those on ODS. The results indicate that *p-tert*-butyl-calix[8]arene

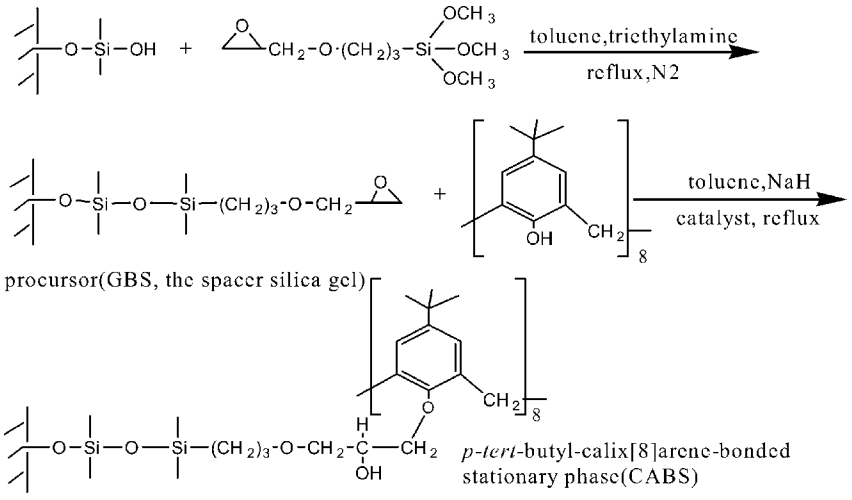
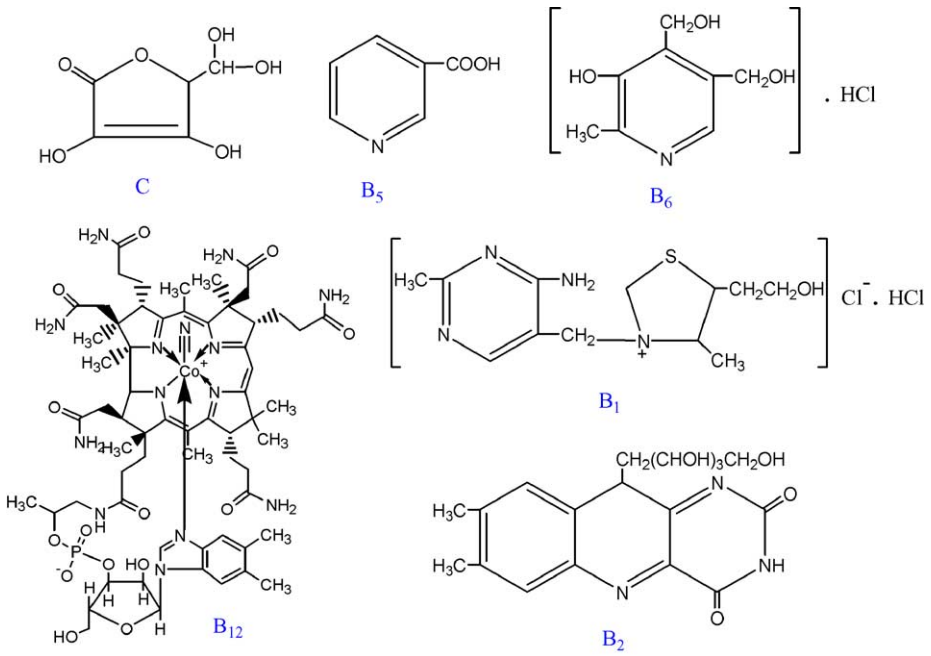


Fig. 1. The preparation scheme of *p-tert*-butyl-calix[8]arene-bonded silica gel stationary phase (CABS).



Solutes	C	B_1	B_2	B_5	B_6	B_{12}
pKa_1	4.19	4.80	-0.2	4.82	5.0	7.64
pKa_2	11.57	9.20	9.69	12.0	8.96	/

Fig. 2. The chemical structures and dissociation constants of water-soluble vitamins.

was successfully immobilized to silica gel. The new packing exhibits an excellent reversed-phase performance and its hydrophobicity is weaker than that of ODS.

3.2. The separation of water-soluble vitamins on CABS

The chemical structures and dissociation constants of several vitamins were given in Fig. 2. The chromatograms of six water-soluble vitamins on CABS and on ODS are shown in Fig. 3(a) and (b), respectively. The influence of the methanol content and the pH of mobile phases on the retention factors (k) of the vitamins were shown in Fig. 4(a) and (b). The plot of the retention factors (k) of the vitamins versus the ionic strength of mobile phases was given in Fig. 5.

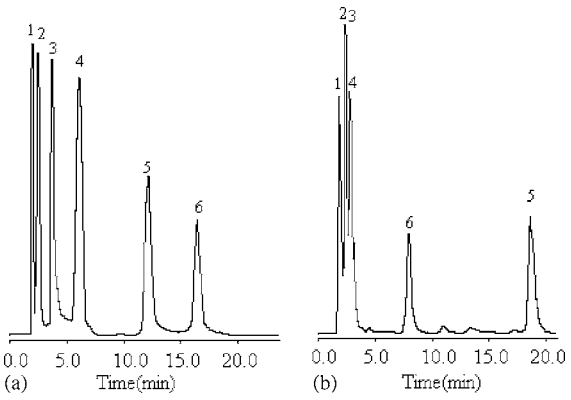


Fig. 3. The chromatograms of water-soluble vitamins on CABS (a) and ODS (b) mobile phases: methanol and 0.01 mol l^{-1} KH_2PO_4 (25:75, (v/v), pH 4.0); flow rates: 0.8 ml min^{-1}; UV: 254 nm. (1) B_1; (2) B_6; (3) C; (4) B_5; (5) B_2; and (6) B_{12}.

The limited capability to separate the water-soluble vitamins on GBS was observed. Moreover, the retention values of the analytes on this packing fluctuated in acidic mobile phases, which might result from the hydrolysis of the epoxy group partially.

As can be seen in Fig. 3, six water-soluble vitamins on CABS were successfully separated by using an isocratic mode, instead of ODS. Further, optimizing chromatographic condition, we found that it was very difficult to achieve the excellent separation of above solutes on ODS in isocratic mode. This may mainly be dependent on the structures of these compounds and the retention mechanism of ODS only based the hydrophobicity. On one hand, B_1, B_5, etc. often exist in ionic forms in the mobile phases, and these solutes exhibit weak retention on ODS. On the other hand, B_2 with the similar anthrquinone structure has a strong affinity to ODS, because of high hydrophobicity. So, a gradient mode is often considered as a good choice for the separation of these solutes. It is worth noticing that the elution order of B_{12} and B_2 on CABS was different from that on ODS. Obviously, different the retention mechanisms of the analytes existed on the two packings. According to chromatographic data, the possible mechanisms on CABS are proposed as follows.

(i) The hyhrophobic interaction plays an important role in the separation of the water-soluble vitamins. Fig. 4(a)

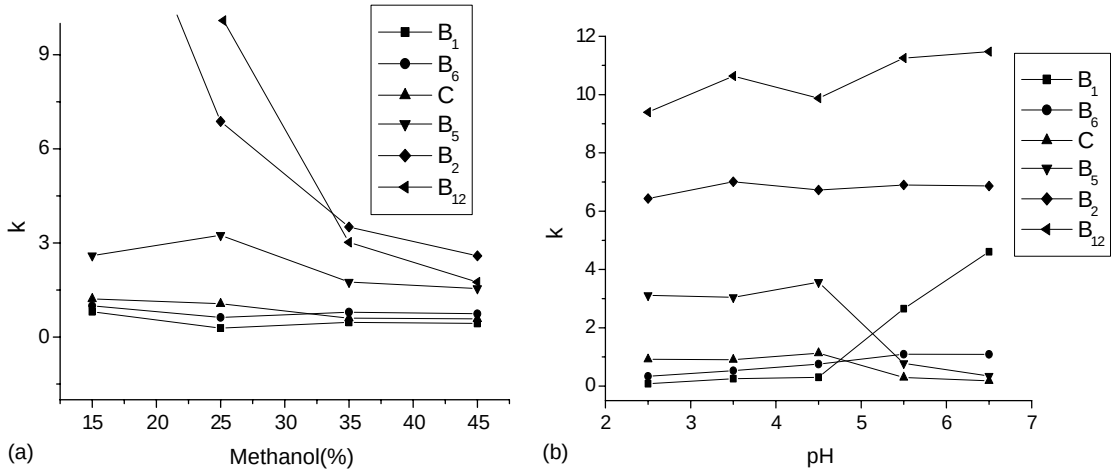


Fig. 4. The influence of the methanol contents (a) and the pH (b) of mobile phases on the retention factors (k) of water-soluble vitamins on CABS. Mobile phases: (a) methanol and 0.01 mol l^{-1} KH_2PO_4 (pH 4.0); (b) methanol and 0.01 mol l^{-1} KH_2PO_4 (25:75, (v/v)); flow rates: 0.8 ml min^{-1}; UV: 254 nm.

shows that the retention of the analytes decreased with increasing methanol content in mobile phases. The solutes can roughly be divided into two groups by their hydrophobicities. The retention values of B_1, B_6, C and B_5 with polar or ionized groups on CABS were small, inversely, for B_2 and B_{12} containing hydrophobic or large groups (see Fig. 3(a)). The phenomenon correlates with the natural reversed-phase property of CABS. Further, B_1 (pK_a 4.80) and B_6 (pK_a 4.82) were eluted firstly because of protonation of their amino ($–NH^+$) in the mobile phases (pH 4.0). C and B_5 were eluted subsequently, which ascribed to the partial suppression of their ionization in the weaker acidic medium. Therefore, the suppressive ionization of the analytes and adjusting proper elution strength of mobile phases are very important in the separation of these solutes on CABS. However, the retention values of most solutes with hydrogen-donor groups (–OH, $–NH_2$, –COOH) on CABS are higher than those on ODS, though the latter has the higher bonded amount (see Table 1). Moreover, the resolution for these solutes on CABS is superior to that on ODS. Hence, it is rather reliable that other interactions exist between the solutes and CABS.

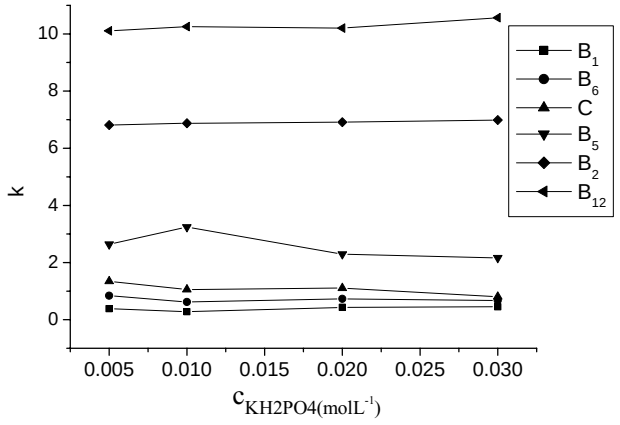


Fig. 5. The influence of the ionic strength of mobile phases on the retention factors (k) of water-soluble vitamins on CABS. Mobile phases: methanol and 0.01 mol l^{-1} KH_2PO_4 (25:75, (v/v), pH 4.0); flow rate: 0.8 ml min^{-1}; UV: 254 nm.

(ii) The hydrogen bonding interaction contributes to the high selectivity of CABS for above solutes. Obviously, making a comparison between the chromatograms 3(a) and 3(b), we can observe that under the same condition, the retentions of B_1 and the other three compounds on ODS were too weak to separate, inversely, these solutes exhibited relative strong retention on CABS and were separated completely. To these polar solutes, it can be assumed that the hydrogen bonding interaction was responsible for the behavior. The above four compounds with hydrogen-donor groups (–OH, –COOH, $–NH_2$) can form hydrogen bonding with the phenolic hydroxyls of CABS is conceivable.

For another example, as can be seen in Fig. 3, the retention time of B_{12} on CABS (16.42 min) was greater than that on ODS (7.76 min). The explanation is that the structure of B_{12} contains six free amido groups, which arranged in the out of its molecule, could easily form hydrogen bond with CABS and thus a strong retention of B_{12} on CABS can be observed.

As you can also noticed in Fig. 3, the retention of B_2 on CABS (12.08 min) was less than that on ODS (18.47 min), which indicated that the hydrophobicity of CABS was weaker than ODS. The hydrogen bonding interaction and hydrophobic interaction together led to different elution order of B_{12} and B_2 on the two columns.

Therefore, it is obvious evidence of hydrogen bonding interaction in the separation of vitamins on CABS.

(iii) Additional π–π interaction might be used for the explanation of the behavior of nicotinic acid (B_5) on CABS.

In comparison with other solutes (Fig. 3(a)), such as B_1, B_2, and C, B_5 exhibited the stronger retention and better resolution. The reason is more likely to be due to the formation of π–π interaction between the aromatic ring of B_5 and the moiety of the calix[8]arene.

(iv) Other actions interactions can also be found, such as electrostatic interaction. Fig. 4(a) illustrates that as the pH of mobile phases was over 4.5 the retention of the acids, such as B_5 and C, slumped. This is due to the ionization of residual phenolic hydroxyl groups of the calix[8]arene with increasing pH of mobile phases [31]. The anions of the analytes were repulsed by the anions on the stationary phase, which led easily to decreasing retention of the acids. Therefore, the separations also deal with electrostatic interaction.

The inclusion complexation is also one of the chromatographic properties of the calixarene-bonded columns. Nevertheless, in this experiment, the typical inclusion interaction can not be confirmed in the separation of the above compounds in this case. Hence, the inclusion interaction is not important in the separation of the above solutes. Of course, it has to be further studied.

3.3. The investigation of reproducibility and separation factors of vitamins on CABS

The retention time of vitamins were repeatedly determined by using methanol and 0.01 mol l^{-1} KH_2PO_4 (25:75, (v/v), pH 4.0, 0.8 ml min^{-1}) as mobile phase. The intra-day relative standard deviations (R.S.D., (%) $n = 5$) of vitamin B_1, B_6, C, B_5, B_2, B_{12} were 0.41, 0.65, 1.16, 1.31, 1.18, 1.83, respectively.

The retention factors (k) and separation factors ($\alpha_{1,2}$) of vitamins on CABS were shown in Table 3. The results show that the method has better chromatographic reproducibility and CABS exhibits high selectivity for water-soluble vitamins.

3.4. Optimization of chromatographic conditions

In order to optimize the separation conditions, the influence factors on the retentions, such as the methanol content, the pH and the ionic strength of mobile phases have to be investigated. The synergistic effect can be used to understand the chromatographic behavior of the analytes and performance of CABS.

Table 3
The retention factors (k) and separation factors ($\alpha_{1,2}$) of vitamins on CABS

	Water-soluble vitamins					
	B_1	B_6	C	B_5	B_2	B_{12}
k	0.282	0.624	1.059	3.243	6.875	9.704
$\alpha_{1,2}$		2.212	1.697	3.062	2.120	1.411

Mobile phases: methanol and 0.01 mol l^{-1} KH_2PO_4 (25:75, (v/v), pH 4.0); flow rates: 0.8 ml min^{-1}.

Fig. 4(a) shows that the retention values of B_{12} and B_2 were largely dependent on the methanol content in the mobile phases. The retention of B_{12} was stronger than B_2 in the mobile phases with lower methanol contents (<35%). However, when the methanol contents were over 35%, the retention of B_{12} quickly decreased next to B_5 and the elution order of B_{12} and B_2 changed. The results implied that the hydrogen bonding interaction became weak with increasing methanol contents in the mobile phases. In the same way, as the methanol contents were over 35%, the resolutions of the other vitamins, such as B_1, B_6 and C became poor. Therefore, the retention behavior of the water-soluble vitamins on CABS was mainly dependent on the hydrogen-bonding interaction in the mobile phase with lower methanol content. Obviously, better separation on CABS can be obtained in aqueous mobile phases with lower methanol contents (20–30%).

As shown in Fig. 4(b), as the increase of pH in mobile phases the retention values of B_5 and C decreased, the retention of B_1 and B_6 increased, and the retention of B_{12} and B_2 slightly changed, which correspond to their different ionization properties. However, it can be observed that as the pH was over 4.5 the retention values of most analytes changed, apparently, which is related to the stationary phase. This was because that the residual phenolic hydroxyl groups of the calix[8]arene ionized partially and CABS possessed some negative charge [31]. When the pH was over 4.5, the anion of nicotinic acid (B_5) was repelled by the negative charge of CABS, which led to decrease the retention of B_5 dramatically. In contrast to this, the retention value of B_1 largely increased, which can be explained as follow: on one hand, the retention value of B_1 increased for its deprotonation. On the other hand, with deprotonation of B_1 the electron density of its pyrimidine ring increased, which led to stronger π–π interaction between the pyrimidine ring and the moiety of the calix[8]arene. The synergistic effects contribute to the remarkable increase of the retention values of B_1. Therefore, it is necessary that the pH of mobile phases should be controlled in the range from three to four in the above separation process.

As can also be seen in Fig. 5, the ionic strength affects the retention behavior of the solutes only minimally, suggesting that ion-exchange interaction is hardly operative to the retention of the vitamins. The retention value of B_5 has the maximum at 0.01 mol l^{-1} KH_2PO_4, which might be related to the weak ion-exchange interaction of its aromatic carboxylic anions with CABS. Obviously, the best separation of these compounds can be achieved at 0.01 mol l^{-1} KH_2PO_4.

According to above results and stability of the solutes, the better chromatographic condition should be as follows: The range of the methanol content is between 20 and30%, and the pH of mobile phase is from three to four.

4. Conclusions

The chromatographic behavior of some water-soluble vitamins was studied on a *p-tert*-butyl-calix[8]arene-bonded silica gel stationary phase by RP-HPLC. The comparative study of the separation of the solutes was done by using CABS and ODS as stationary phases under the same chromatographic conditions. Better separation of water-soluble vitamins on CABS can be achieved by using an isocratic mode with methanol–phosphate buffer (25:75, (v/v)) as mobile phases. The results show that the calix[8]arene bonded phase exhibits high selectivity for the vitamins and is superior to ODS, especially, strong polar water-soluble vitamins. According to the chromatographic data, it can be concluded that various chromatographic retention mechanisms occur in the separation of water-soluble vitamins on CABS, such as hydrophobic interaction, hydrogen bonding interaction, etc. The new material exhibits the promising application in the separation of water-soluble vitamins. Further application of CABS is in progress.

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