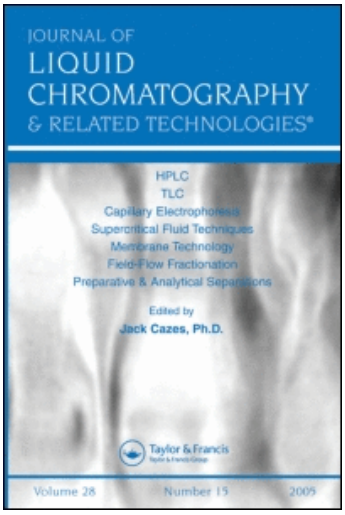

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HPLC OF SOME NUCLEOSIDES AND BASES ON *p-tert*-BUTYL-CALIX[6]ARENE-BONDED SILICA GEL STATIONARY PHASE

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ABSTRACT

The high-performance liquid chromatographic behavior of some nucleosides and bases was studied on a new *p-tert*-butyl-calix[6]arene-bonded silica gel stationary phase. The effect of mobile phase variables, such as ionic strength, methanol content, and pH on their chromatographic behavior was investigated. Some nucleosides and bases were successfully separated on the new stationary phase. Their retention behavior was compared with that on both Zorbax C_{18} phase and-(ethylenediamino)propyl-triethoxylsilane-bonded silica gel. The results indicate that the new stationary phase behaves as a reversed-phase packing, but its hydrophobicity is much weaker than that of Zorbax C_{18} phase. The retention mechanism on the new stationary phase was also discussed.

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INTRODUCTION

Nucleosides and bases are of great importance in many research areas, such as biochemistry, medicine, and pharmacy. Their separation and determination have extensively received attention for many years. Thin-layer chromatography, gas chromatography, column liquid chromatography, capillary electrophoresis, and capillary electrochromatography have been used for their separation and analysis. Reversed-phase high-performance liquid chromatography is one of the most efficient techniques extremely suitable for the analysis of nucleosides and bases. The liquid chromatographic behavior of these compounds was well investigated on the silica-based reversed-phase stationary phases. Brown and his coworkers described the retention of nucleosides and bases on the ODS column(1) and showed the relationship between reversed-phase behavior and the structure and physiochemical properties of these compounds.(2) Galushko et al.(3) studied the chromatographic behavior of some pyrimidine bases and nucleosides on an ODS column applying solvophobic theory, and discussed the mechanism of base and nucleoside interactions with the surface of the hydrocarbonaceous stationary phase.

Many attempts have been made to optimize the separation conditions, such as pH,(4) temperature,(5) organic modifier,(4) ionic strength(4) and packing material(4-6) for the separation of nucleosides and bases. In addition, Fu et al.(7) investigated the effect of mobile phase variables such as methanol content, ionic strength, and pH on the chromatographic behavior of some nucleosides and bases on an alkylphosphonate-modified magnesia-zirconia reversed-phase stationary phase.

Calixarenes(8) are cavity-shaped cyclic molecules comprising phenol units linked via a alkylidene group. Calixarene chemistry is a rapidly developing area of supramolecular chemistry following crown ethers and cyclodextrins, because calixarenes are able to form host-guest inclusion complexes with a variety of molecules selectively. In the field of liquid chromatography, calixarenes as stationary phases have attracted many researchers' attention during the last few years. Glennon and coworkers(9-11) prepared silica-bonded calix[4]arene tetraester and silica-bonded calix[4]arene tetradiethylamide stationary phases to separate metal ions and amino acid esters. Park et al.(12) successfully separated some mono-substituted phenol regioisomers and some other aromatic positional isomers on a calix[6]arene-*p*-sulfonate-bonded silica stationary phase. Gebauer et al.(13-15) reported the chromatographic separation of 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.

In the past few years, by adopting the new method of heterogeneous functionalisation of suspended porous silica, our research group prepared *p-tert*-

butyl-calix[6]arene-bonded silica gel stationary phase with 3-glycidoxypropyltriethoxysilane as a coupling reagent(16) and *p-tert*-butyl-calix[n]arene-bonded (n = 4, 8) silica gel stationary phases with γ-(ethylenediamino)propyltriethoxylsilane as a coupling reagent,(17,18) and investigated the chromatographic separation of some positional isomers, polycyclic aromatic hydrocarbons, nucleosides and bases.

In this paper, we report another new *p-tert*-butyl-calix[6]arene-bonded silica gel stationary phase successfully prepared in our laboratory with γ-(ethylenediamino)propyltriethoxylsilane as a coupling reagent. Its structure was characterized by using elemental analysis and FTIR. The focus of our investigation is on the application of this new calix[6]arene-bonded silica gel stationary phase in the separation of nucleosides and bases. The influence of mobile phase parameters on the chromatographic behavior of nucleosides and bases and the retention mechanism of the solutes on the new stationary phase, were also studied.

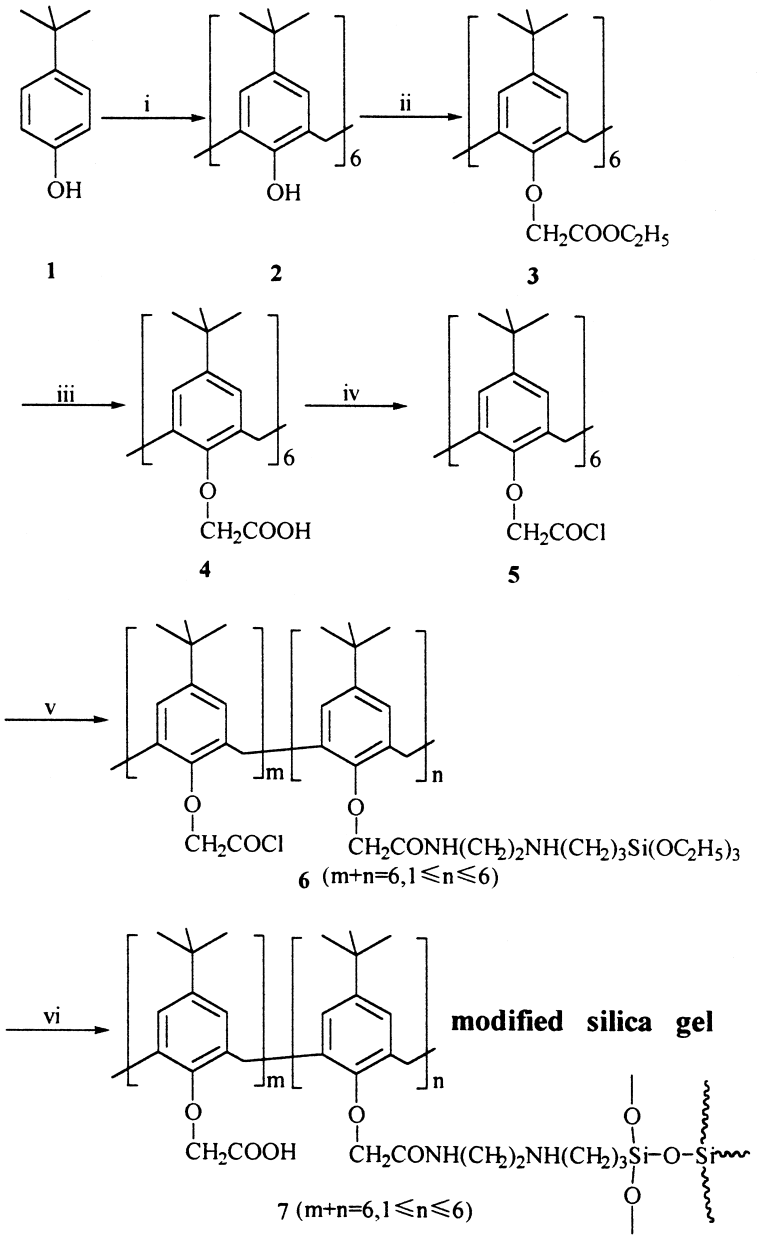
EXPERIMENTAL

Chemicals and Reagents

Spherical silica was home-made,(19) with 5~7 μm particle size and 230 m^2 g^{-1} specific surface area. γ-(ethylenediamino)propyltriethoxylsilane (content ≥90%) was purchased from Wuhan University Chemical Plant (Wuhan, China). Other reagents were obtained from various commercial sources and were analytical grade, unless otherwise indicated. Adenosine (Ado), cytidine (Cyd), cytosine (Cyt), 4,6-diaminopyrimidine (4,6-DAP), and caffeine (Caf) were purchased from the No.2 Chemical Reagent Plant of Shanghai (Shanghai, China). Guanosine (Guo), guanine (Gua), uridine (Urd), thymine (Thy), uracil (Ura), adenine (Ade), 5-flurouracil (5-FU), and 5-iodouracil (5-IU) were purchased from Sigma. Thymidine (Thd), 6-mercaptopurine (6-Mp), and hypoxanthine (Hyp) were purchased from Fluka.

Preparation and Characterization of *p-tert*-Butyl-calix[6]arene-Bonded Silica Gel Stationary Phase

The new *p-tert*-butyl-calix[6]arene-bonded silica gel stationary phase was prepared according to scheme 1. Parent calixarene (**2**) was synthesized according to the procedure.(20) Compound (**3**), *p-tert*-butyl-calix[6]arene hexaester was obtained by refluxing parent calixarene (**2**) with excessive ethyl bromoacetate in dry acetone for 7 days under an atmosphere of nitrogen gas, using anhydrous



Scheme 1. Synthesis of the *p-tert*-butyl-calix[6]arene-bonded silica gel stationary phase. (i) HCHO, KOH, xylene, reflux for 3h; (ii) $BrCH_2COOC_2H_5$, K_2CO_3, acetone, reflux for 7 days; (iii) ethanol, NaOH, reflux for 24h; (iv) $SOCl_2$, reflux for 4h; (v) $N(C_2H_5)_3$, $NH_2(CH_2)_2NH(CH_2)_3Si(OC_2H_5)_3$, toluene, at room temperature for 16h; (vi) $N(C_2H_5)_3$, silica gel, toluene, reflux for 24h.

potassium carbonate as the catalyst.(21m22) Then, compound (**3**), in a mixture solution of sodium hydroxide aqueous solution and ethanol, was heated to produce *p-tert*-butyl-calix[6]arene hexaacid, compound (**4**), under reflux for 24 hr.(22) Following that, compound (**5**), hexa(chloroformylmethoxy)calix[6]arene, was quantitatively prepared by refluxing compound (**4**) with thionyl chloride for 4h and the excessive thionyl chloride was removed in vacuum.(23) Compound (**5**) was used in subsequent preparations without further purification.

Calix[6]arene-bonded silica gel stationary phase was prepared as follow: a mixture of Compound (**5**), γ-(ethylenediamino)propyltriethoxylsilane and triethylamine in freshly distilled dry toluene, was stirred for 16 h at room temperature in an inert atmosphere of dry nitrogen gas,(24,25) after which activated silica gel was added. The mixture was stirred and boiled at reflex under streaming dry nitrogen gas for 24 h. Finally, the solid product was filtered and washed in sequence with warm toluene, acetone, methanol, and double-distilled water three times. The product was dried at 120°C, under vacuum, for 3 h and kept in a desiccator before use.

γ-(Ethylenediamino)propyltriethoxylsilane-bonded silica gel (Diamino-bonded phase) was also prepared and used as a reference stationary phase.

Elemental analysis was performed with a MOD-1106 elemental analyzer (Italy). FTIR measurements referred to KBr were carried out on an FTIR-8201PC spectrophotometer (Shimadzu, Japan).

Chromatographic Procedure

The liquid chromatographic system was composed of a Model LC-10AT pump (Shimadzu, Japan), a Reodyne 7725 injector with 20 μL sample loop, a Model SPD-10A UV-Vis spectrophotometric detector (Shimadzu, Japan), and a C-R6A Chromatopac (Shimadzu, Japan) or a Type 3066 pen recorder (Sichuan the Fourth Instrument Plant, Sichuan, China). The *p-tert*-butyl-calix[6]arene-bonded silica gel stationary phase was slurry-packed into a 15 cm × 4.6 mm (i.d.) stainless-steel column with isopropanol (17 mL), and methanol was used as eluent.

Buffer solution or a mixture of methanol and buffer solution was used as mobile phases. Before use, the mobile phases were generally filtered through a G-4 fritted glass funnel and degassed in an ultrasonic bath for about 5 min under reduced pressure. The flow rate was set at 0.5 or 1.0 mL min^{-1}. The samples were dissolved in methanol.

The wavelength used for detection was 254 nm. The retention time of the solvent peak was used as void time for the calculation of capacity factor. All measurements were carried out at ambient temperature (27±2°C) and repeated at least twice.

RESULTS AND DISCUSSION

Characterization of the Structure of the New Calix[6]arene-Bonded Phase

The FTIR spectrums (KBr) of the new calix[6]arene-bonded phase and Diamino-bonded phase, after subtraction of the bare silica spectrum, are shown in Figure 1 and Figure 2, respectively. The spectrums show the disappearance of a strong absorption band at 3600-3700 cm^{-1}, which is the characteristic of the Si-OH stretching frequency of bare silica. The wide absorption band at 3419.6 cm^{-1} indicates the presence of amino groups. Peaks at 2964.4 cm^{-1}, 2962.4 cm^{-1}, and 2881.4 cm^{-1} are assigned to C-H stretching frequency. The characteristic absorption band of the benzene ring should appear at 1600-1800 cm^{-1},(8) which is overlapped by the strong absorption band of carbonyl groups at 1600-1800 cm^{-1}. Therefore, the peak at 1627.8 cm^{-1} is a mixed absorption, which does not appear in the spectrum of Diamino-bonded phase. In the fingerprint area, the characteristic absorption of a tetra-substituted benzene ring is too weak to be discerned. Table 1 gives the results of elemental analysis. The above FTIR spectrums and elemental analysis demonstrate that both the calix[6]arene and γ-(ethylenediamino)propyltriethoxylsilane have successfully been immobilized to the silica

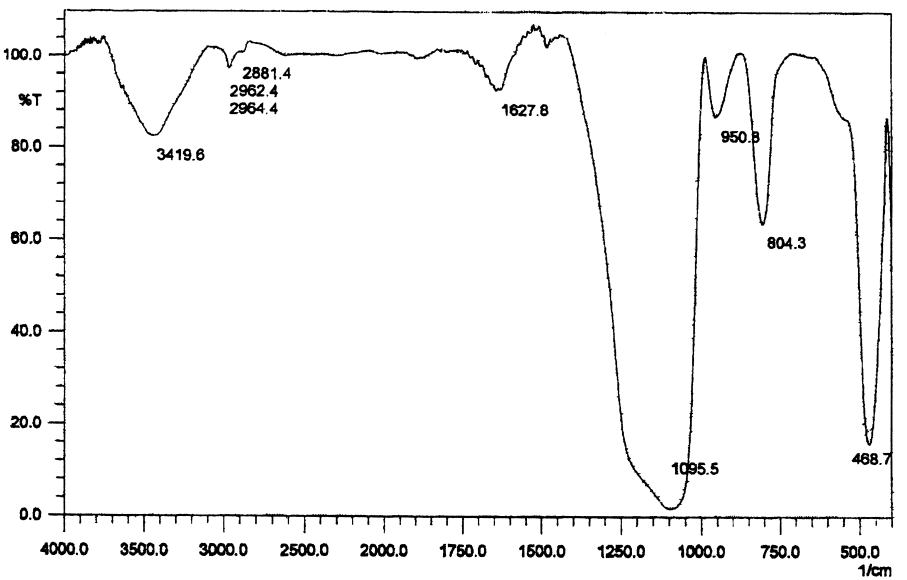


Figure 1. FTIR spectrum (KBr) of the new *p-tert*-butyl-calix[6]arene-bonded silica gel stationary phase.

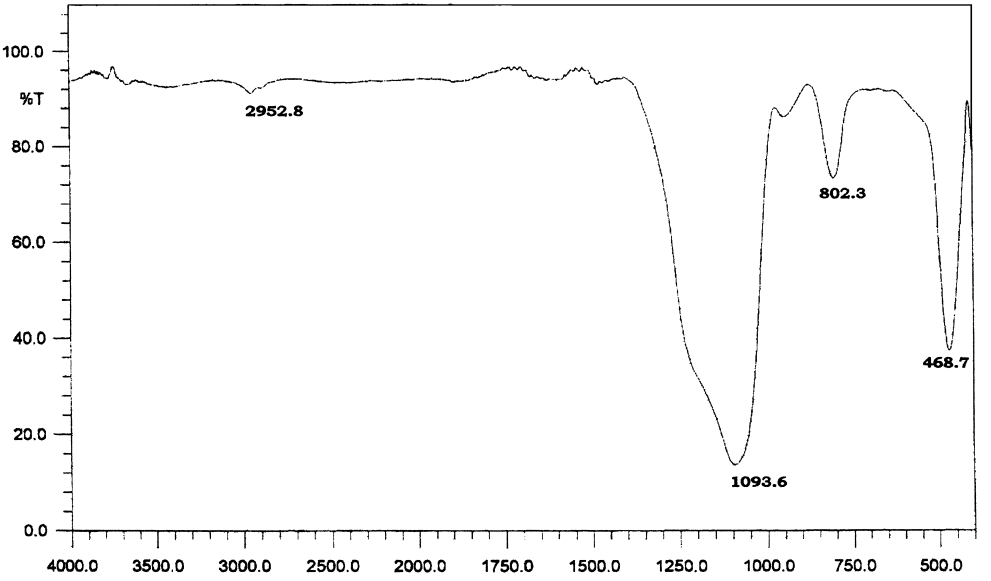


Figure 2. FTIR spectrum (KBr) of diamino-bonded phase.

gel. The surface coverage of the new calix[6]arene-bonded phase calculated according to its carbon and nitrogen content, after subtracting that of Diamino-bonded phase, is 0.11 mmol g^{-1}, which is much higher than that (0.06 mmol g^{-1}) of the calix[6]arene-bonded phase with 3-glycidoxypropyltriethoxysilane as a coupling reagent.(16)

Efficiency

The column efficiency of the new calix[6]arene-bonded phase was determined by using methanol as mobile phase and biphenyl as solute. The number of theoretical plates was found to be 28784 m^{-1}, which is much higher than that

Table 1. Elemental Analysis of the New Calix[6]arene-Bonded Phase and Diamino-Bonded Phase

Stationary Phase	Element (%)			Surface Coverage (mmol g^{-1} Silica)
	C	H	N	
Calix[6]arene-bonded phase	11.48	1.93	1.22	0.11
Diamino-bonded phase	6.13	1.49	1.18	—

(19000 m^{-1}) of the calix[6]arene-bonded phase with 3-glycidoxypropyltriethoxysilane as coupling reagent(16) under the same condition.

Chromatographic Behavior of Nucleosides and Bases

Effect of Ionic Strength

The ionic strength of aqueous mobile phase at a constant pH of 5.50 was increased by increasing the KH_2PO_4 buffer concentration (from 0.005 mol L^{-1} to 0.03 mol L^{-1}). The effect of ionic strength on capacity factor is shown in Figure 3 and Figure 4, respectively. As can be observed, the ionic strength affects the retention behavior of the solutes only minimally, suggesting that ion-exchange interaction is hardly operative to the retention of nucleosides and bases.

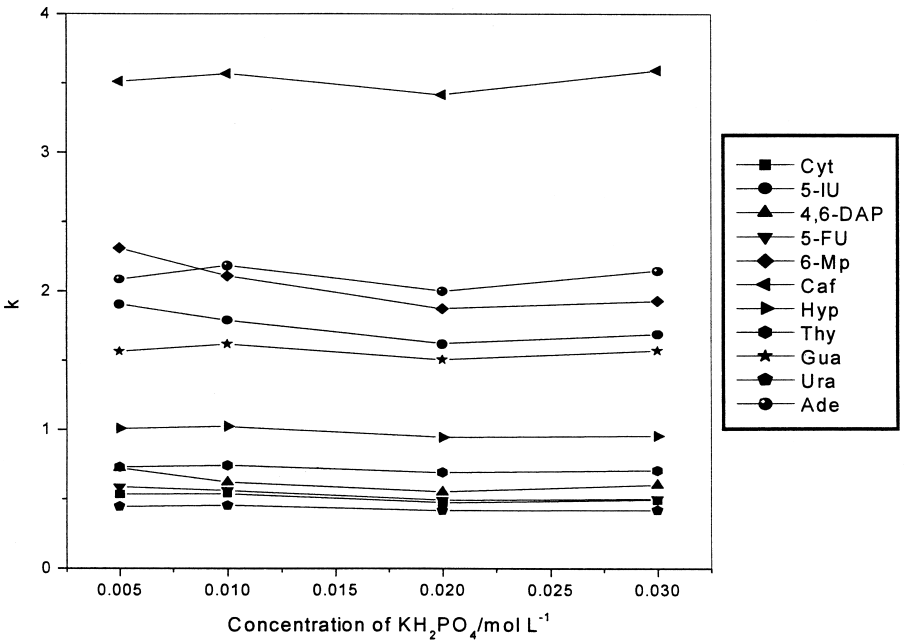


Figure 3. Influence of the concentration of KH_2PO_4 in mobile phase on the capacity factor of some bases on the new calix[6]arene-bonded stationary phase. Mobile phase: KH_2PO_4 buffer solution at pH 5.50. Flow rate: 0.5 mL min^{-1}.

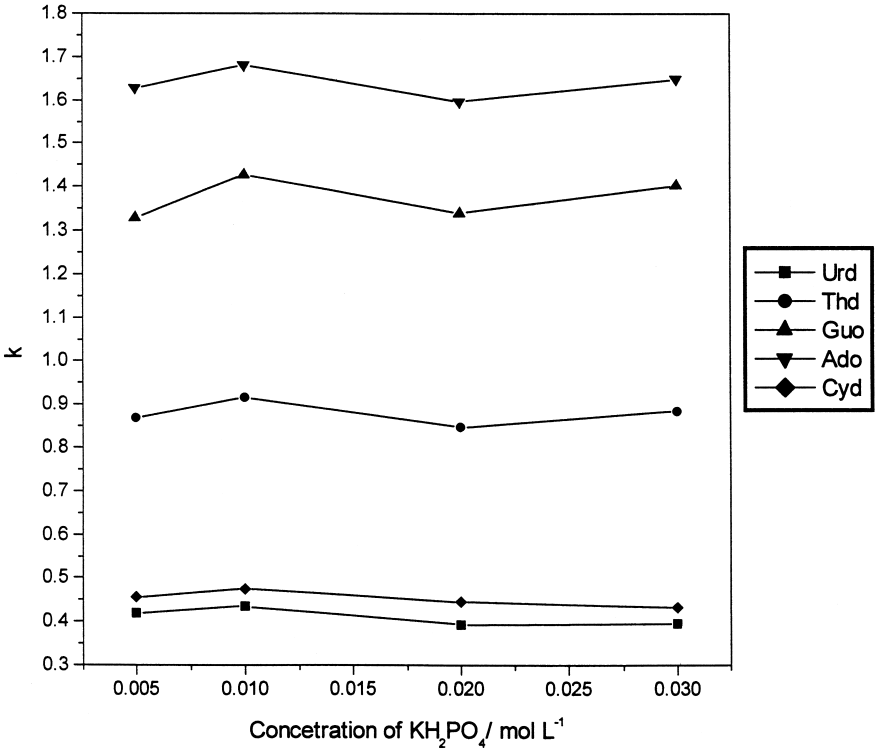


Figure 4. Influence of the concentration of KH_2PO_4 in mobile phase on the capacity factor of some nucleosides on the new calix[6]arene-bonded stationary phase. Mobile phase: KH_2PO_4 buffer solution at pH 5.50. Flow rate: 0.5 mL min^{-1}.

Effect of Organic Modifier

Figure 5 and Figure 6 illustrate the plots of logarithmic capacity factor of the solutes against the volume percentage of methanol in mobile phase at constant pH (5.50) and constant buffer concentration (0.01 mol L^{-1} KH_2PO_4). As the methanol content of mobile phase is increased, the retention values of the solutes decrease, which is similar to the retention behavior on an ODS.(4) This result indicates that the new stationary phase can behave as a reversed-phase packing and the hydrophobic interaction is probably one of the predominant factors for the retention of nucleosides and bases.

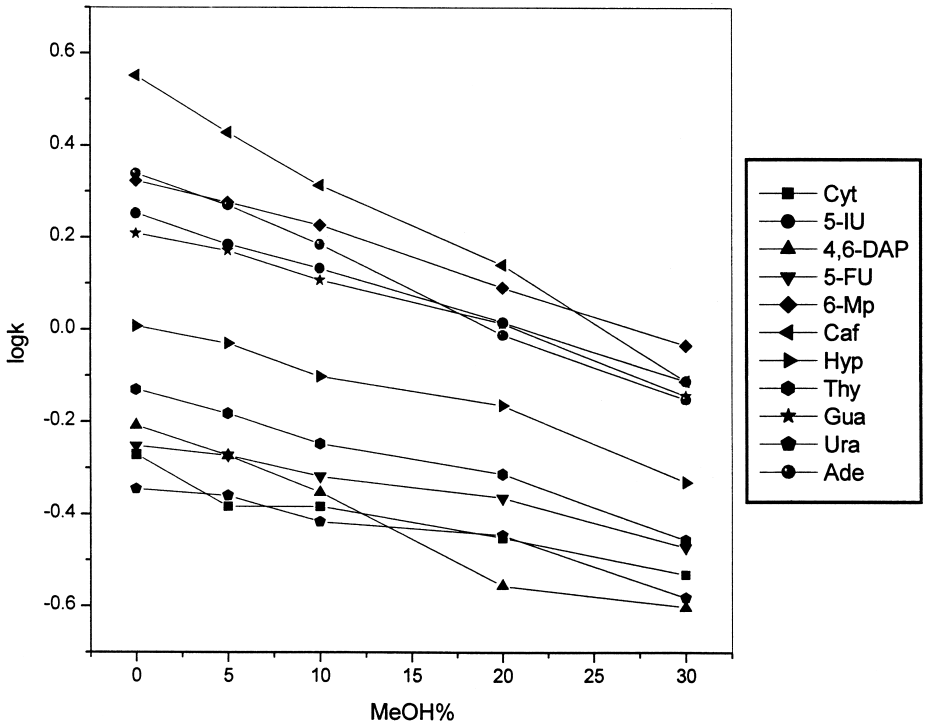


Figure 5. Influence of the methanol content of mobile phase on the logarithmic capacity factor of some bases on the new calix[6]arene-bonded stationary phase. Mobile phase: 0.01 mol L^{-1} KH_2PO_4 buffer solution at pH 5.50 with methanol. Flow rate: 0.5 mL min^{-1}.

Effect of pH

In general, the retention behavior of nucleosides and bases is typical of the classical concept of reversed-phase chromatography, in which charged species are rapidly eluted, whereas the retention of neutral molecules increases on the hydrophobic packing.

In order to study the effect of pH changes on the retention of nucleosides and bases, KH_2PO_4 buffer solution was used as mobile phase with nearly constant ionic strength at 0.02 mol L^{-1}. The results are shown in Figure 7 and Figure 8, respectively. As can be seen, the retention of the solutes is related to their pK_a values (Table 2). The solutes, whose pK_a values are ether above or below the pH range of mobile phase, do not undergo a significant change in retention and generally remain in the same elution order with increasing pH because they are largely neutral in the pH range covered. However, for Cyt, Gua, Ade, Ado, and

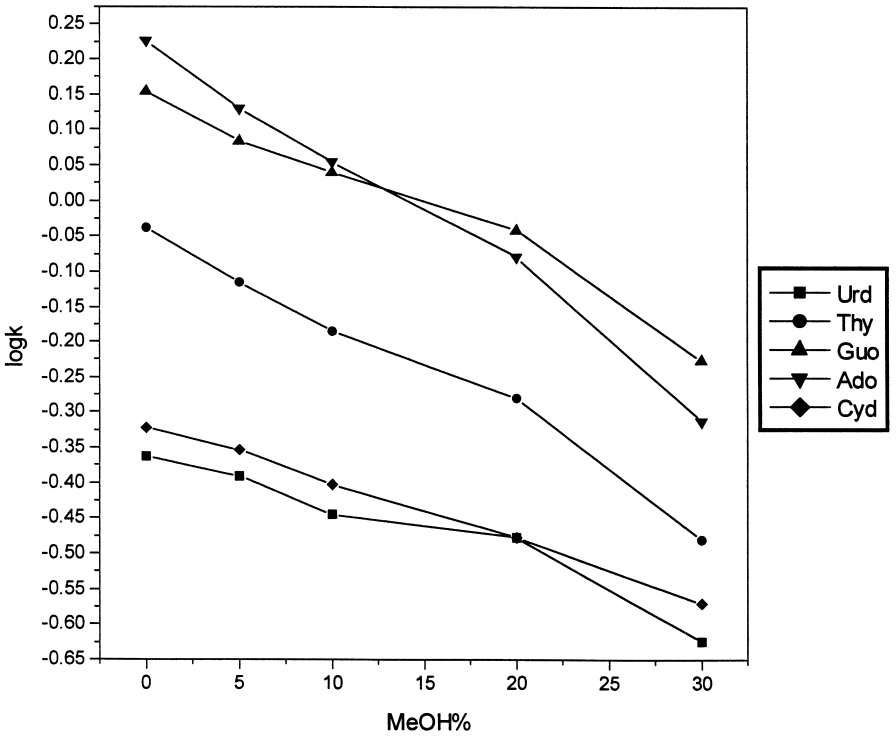


Figure 6. Influence of the methanol content of mobile phase on the logarithmic capacity factor of some nucleosides on the new calix[6]arene-bonded stationary phase. Mobile phase: 0.01 mol L^{-1} KH_2PO_4 buffer solution at pH 5.50 with methanol. Flow rate: 0.5 mL min^{-1}.

Cyd with pK_a values within the pH range being examined, the retention increases with the increasing pH of mobile phase and reaches maximum at moderate pH. This can be attributed to the fact that the protonated solutes at lower pH deprotonate and change to the neutral form gradually with increasing pH, leading to the stronger hydrophobic interaction between the solutes and the new stationary phase.

When the pH of mobile phase is over the pH at which the solutes release proton and form anions, the retention of the solutes decreases owing to weak hydrophobic interaction with the new stationary phase. It was also found that Gua and Ado reach the maximum retention prior to Ade, Cyt, and Cyd, which is ascribed to earlier attainment of complete neutrality due to lower pK_a values of Gua and Ado.

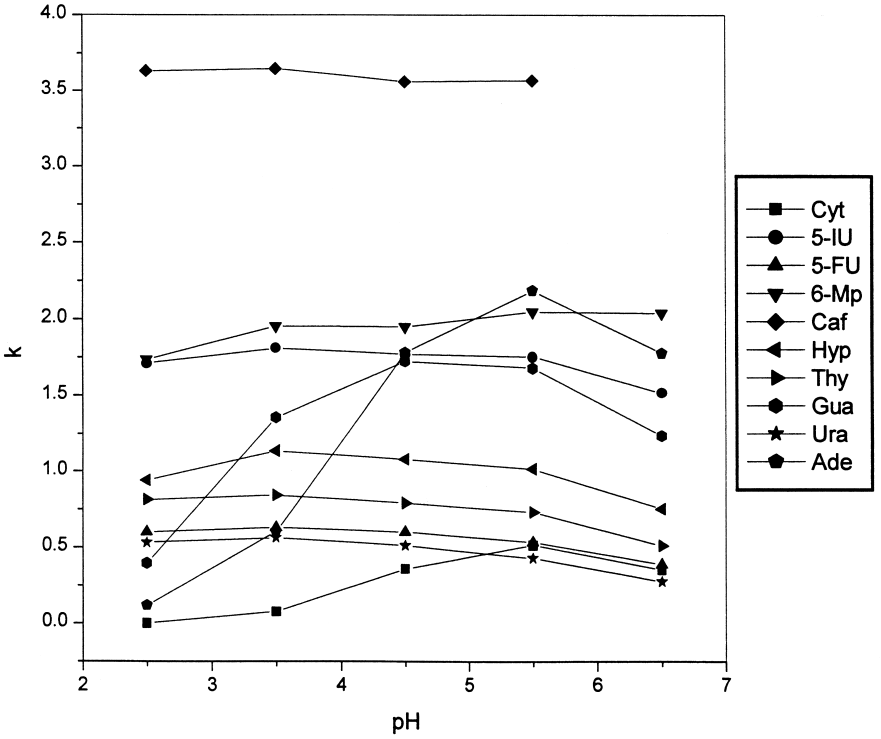


Figure 7. Influence of the pH of mobile phase on the capacity factor of some bases on the new calix[6]arene-bonded stationary phase. Mobile phase: 0.02 mol L^{-1} KH_2PO_4 buffer solution. Flow rate: 0.5 mL min^{-1}.

As indicated in the literature,(18) the new calix[6]arene-bonded phase also exhibits double-layer structure, in which the out layer is hydrophobic and the inner layer hydrophilic. Because two amino groups of the inner layer are easy to be protonated at lower pH, which leads to weaker hydrophobicity of the new stationary phase, it should be observed that the retention of all the solutes decreases with decreasing pH. However, the retention behavior of some solutes such as Urd, Thd, Caf, Thy, etc. could not demonstrate the above phenomenon. This seems to imply that the hydrophobic out-layer plays an important role in the interactions between the new stationary phase and nucleosides and bases, while the hydrophilic inner-layer has little influence on the retention of the solutes. It can be further concluded, that the out layer probably has shielding effect on the inner layer to a certain extent.

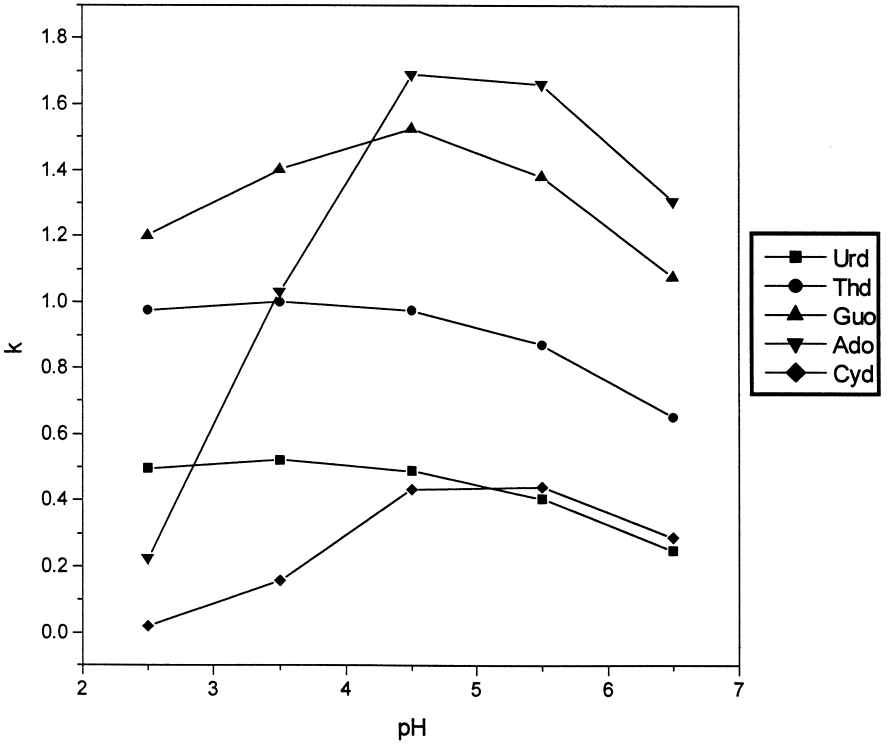


Figure 8. Influence of the pH of mobile phase on the capacity factor of some nucleosides on the new calix[6]arene-bonded stationary phase. Mobile phase: 0.02 mol L^{-1} KH_2PO_4 buffer solution. Flow rate: 0.5 mL min^{-1}.

Table 2. pK_a Values of Nucleosides and Bases (26)

	Nucleosides					Bases						
pK_a	Cyd	Guo	Thd	Ado	Urd	Cyt	Thy	Ade	Gua	Ura	Hyp	Caf
pK_{a1}	4.15	1.5	9.8	3.5	9.2	4.45	9.9	4.15	3.2	9.5	2.0	>8.8
pK_{a2}	12.5	9.2		12.5		12.2		9.8	9.6		8.9	

Separation of Nucleosides and Bases

Four nucleosides and seven bases can be separated by using 0.02 mol L^{-1} KH_2PO_4 buffer solution (pH 3.50) as mobile phase at flow rate of 0.5 mL min^{-1}. Their separation chromatograms are shown in Figure 9 and Figure 10, respectively. It can be seen that the solutes show sharp and symmetrical peaks with reasonable retention times.

Comparison of the New Calix[6]arene, Zorbax C_{18}, and Diamino-Bonded Silica Gel Stationary Phase

A comparison study of the above three stationary phases was carried out under the same chromatographic conditions and the results are shown in Table 3.

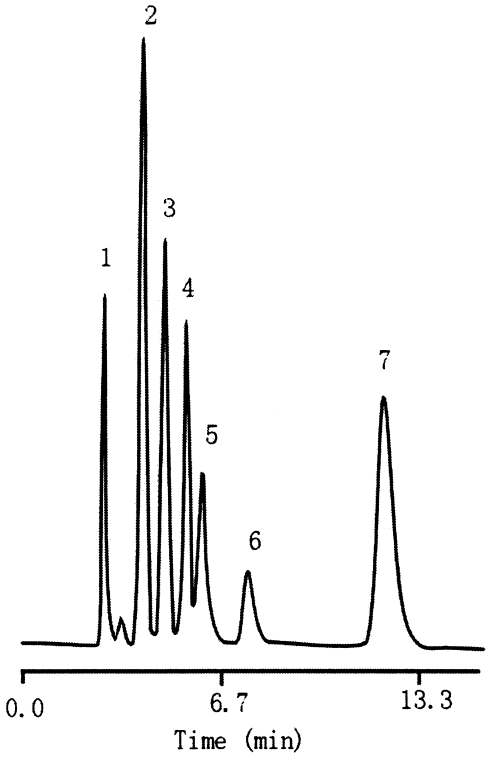


Figure 9. Separation of some bases on the new calix[6]arene-bonded stationary phase. 1: Cyt; 2: Ura; 3: Thy; 4: Hyp; 5: Gua; 6: 6-Mp; 7: Caf.

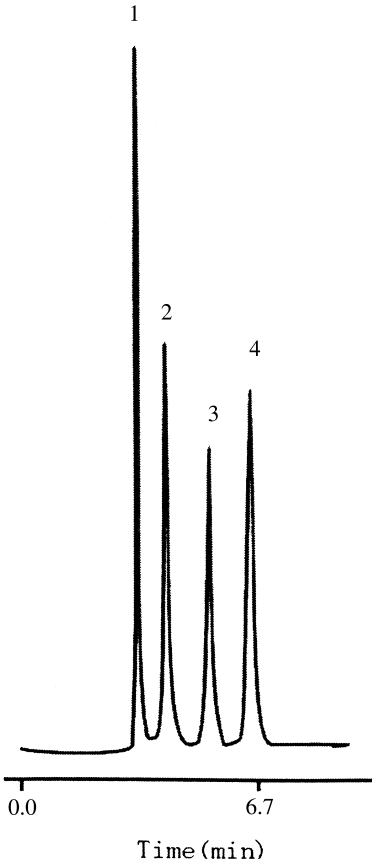


Figure 10. Separation of some nucleosides on the new calix[6]arene-bonded stationary phase. 1: Cyd; 2: Urd; 3: Ado; 4: Guo.

The retention of the solutes on the new calix[6]arene-bonded phase is much less than that on Zorbax C_{18} phase, suggesting that the new calix[6]arene-bonded phase shows significantly weaker hydrophobicity and reasonable analysis speed with aqueous mobile phase, in comparison with Zorbax C_{18} phase. The capacity factors of the solutes obtained on diamino-bonded phase are very small, which indicates that the polar interaction based on two amino groups is weak. Besides, the separation selectivity and its distribution of the new calix[6]arene-bonded phase is similar to that of Zorbax C_{18} phase but greatly superior to that of diamino-bonded phase, which may be caused by hydrophobic interaction and additionally by inclusion complexation of calix[6]arene. Moreover, the retention order on the new calix[6]arene-bonded phase, as shown in Table 3, is different

Table 3. Comparison of the Retention Behavior of Some Nucleosides and Bases on Three Stationary Phases

	The New Calix[6]arene-Bonded Phase			Zorbax C18 Phase			Diamino-Bonded Phase		
Solutes	k	α	α_{12}	k	α	α_{12}	k	α	α_{12}
Bases									
Cyt	0.08	1.00		0.66	1.00		0.32	1.10	1.10
Ura	0.56	7.00	7.00	2.22	3.36	3.36	0.29	1.00	
Thy	0.84	10.50	1.50	8.90	13.48	1.77	0.35	1.21	1.10
Hyp	1.14	14.25	1.36	5.02	7.61	1.16	0.40	1.38	1.08
Gua	1.36	17.00	1.19	4.32	6.55	1.95	0.55	1.90	1.08
6-Mp	1.95	24.38	1.43	10.70	16.21	1.20	0.51	1.76	1.28
Caf	3.65	45.62	1.87	*	*	*	0.37	1.28	1.06
Nucleosides									
Cyd	0.16	1.00		3.34	1.00		0.12	1.00	
Urd	0.52	3.25	3.25	6.88	2.06	2.06	0.24	2.00	2.00
Thd	1.00	6.25	1.92	35.98	10.77	1.66	0.24	2.00	1.00
Ado	1.03	6.44	1.03	46.62	13.96	1.30	0.24	2.00	1.00
Guo	1.40	8.75	1.36	21.70	6.50	3.16	0.38	3.17	1.58

Mobile phase: 0.02 mol L^{-1} KH_2PO_4 buffer solution (pH 3.50) at flow rate of 0.5 mL min^{-1}; k: capacity factor; α: separation factor; α_{12}: the ratio of two neighboring separation factors; *: retention time is more than 2h.

from that on not only Zorbax C_{18} phase but also Diamino-bonded phase, implying that different retention mechanisms take place. The new calix[6]arene-bonded phase may exhibit more complicated retention mechanisms, such as hydrophobic, inclusion complexation, and weak polar interaction.

CONCLUSIONS

The influence of mobile phase variables, such as ionic strength, content of organic modifier, and pH on the retention of nucleosides and bases on a new *p-tert*-butyl-calix[6]arene-bonded silica gel stationary phase has been investigated. The chromatographic behavior of the solutes indicates that the hydrophobic force is a main factor controlling the interaction of nucleosides and bases with the hydrophobic moiety of the new stationary phase.

The effect of pH on the retention relates to pK_a values of the solutes. Little influence of ionic strength on the retention of the solutes was found. The reten-

tion values of most solutes on the new stationary phase are between those on Zorbax C_{18} phase and those on Diamino-bonded phase. The separation selectivity of the new stationary phase is similar to that of Zorbax C_{18} phase but far better than that of Diamino-bonded phase.

The analysis speed is much faster than that of Zorbax C_{18} phase. The retention mechanism of the solutes, which is different from that on both Zorbax C_{18} phase and Diamino-bonded phase, may include hydrophobic, inclusion complexation, and weak polar interaction.

The new stationary phase exhibits a promising application in the separation of nucleosides and bases.

ACKNOWLEDGMENTS

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