Preparation and Characterization of 3-(Aza-18-Crown-6) Propylsilyl Bonded Phase for Reversed-Phase Liquid Chromatography

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Abstract

3-(Aza-18-crown-6) propylsilyl-bonded silica, a new stationary phase for high-performance liquid chromatography, is synthesized for the first time using two different reaction routes. The stationary phase is characterized by means of elemental analysis, infrared spectra, and the measurement of complexing capacity with some metal ions. The chromatographic behavior of the stationary phase is also studied with disubstituted benzenes, amino acids, nucleosides, and nucleobases as solutes. The retention and selectivity of different compounds on the bonded phase depend on the hydrophobicity, dipolarity, hydrogen bonding, and host–guest complexation interaction according to the property and composition of the mobile phases used.

Introduction

With the development of bonded stationary phases, the separation selectivity of reversed-phase high-performance liquid chromatography (RP-HPLC) can be improved by tailoring the molecular structure and property of the bonded column packing material (1). Crown ether, a macrocyclic cycle compound containing several oxygens or hetero atoms separated by ethylene moieties, is able to form host–guest complexes not only with alkali metal, transitional metal, and ammonium cations, but also with some organic compounds with polar functional groups. The stability of the complex depends on the size and hetero atoms of the crown ether and the property of the guest molecules (such as charge, size, shape, polarity, etc.). For the specific selectivity of separation, the crown ether-bonded silica gel has been developed as an attractive stationary phase for host–guest complexes, ligand exchange, and multiple interactions in liquid chromatography. Alkali, alkaline earth metal ions, and various organic compounds (e.g., positional isomers and enantiomers) have been successfully separated by the crown ether bonded phases (2–4).

Since the 1980s, the syntheses of the crown ether-bonded phases have usually been accomplished using two different methods. The first approach is to graft the crown ether to the silica gel activated with a silane that has an active functional group (5). The second approach is to prepare the organic silane monomer containing crown ether before grafting (6). More recently, a new method for the preparation of an azacrown ether-bonded silica gel stationary phase has been developed by the authors on the basis of a successive reaction pathway to form the crown ether cycle on the surface of the silica gel (7).

In this paper, the preparation of a new azacrown ether-bonded silica gel stationary phase, 3-(Aza-18-crown-6) propylsilyl-bonded silica gel (ACPS), has been achieved using two methods. The first method, as proposed by our laboratory (7), is the formation of the crown ether cycle on the surface of silica gel, accomplished through a successive liquid–solid phase reaction. The second method, similar to the method reported in the literature (5), is grafting the crown ether to silica gel activated with a silane linkage agent. The azacrown ether-bonded silica gel stationary phases prepared by the two different methods were characterized with chemical and instrumental analyses and a complexation with metal ions. The chromatographic performance of the azacrown ether-bonded silica gel stationary phases has been evaluated by the separation of disubstituted benzene isomers, nucleosides, nucleobases, and amino acids in the reversed-phase chromatographic mode with aqueous solutions as mobile phases.

Experimental

Chemicals

The chemicals used in preparing the bonded phases, the solvents, and the reagents used in the chromatographic experiments were high purity, commercially available, or repuri-

fied. A narrow-pore microparticle silica gel (mean particle diameter, 10 µm; specific surface area, approximately 200 m^2/g) was obtained from the Qingdao Sea Chemical Plant (Qingdao, China). γ-Chloropropyl triethoxy silane, purchased from the Chemical plant of Wuhan University, was redistilled under vacuum before use. 2,2-Dihydrodiethyl amine, tetraethylene glycol, *p*-toluene sulphonyl chloride, sodium hydride, disubstituted benzene derivatives, nucleoside, nucleobase, and amino acids were purchased from Shanghai General Chemical Reagent Factory (Shanghai, China) and Sigma (St. Louis, MO). 3-(Aza-18-crown-6) ether was synthesized in our laboratory according to the method reported in the literature (8); the melting point (48.5–51°C) and the Fourier transform infrared (FTIR) spectrum agreed with those reported in the literature.

Instrumentation

Elemental analysis was performed with a MOD1106 elemental analyzer (Carlo Erba, Strumentazione, Italy). A model 710 instrument (Nicolet Analytical Instruments, Beijing, China) was used for FTIR spectroanalysis. A Shimadzu (Shanghai, People's Republic of China) UV 256 ultraviolet (UV)-visible spectrophotometer and a Hitachi (Shanghai, China) 180-80 atomic adsorption spectrometer were used for the determination of complexing capacity. The HPLC system consisted of a Shimadzu LC-6A or LC-10A pump, a Rheodyne (Rohnert Park, CA) model 7125 injector with a 20-µL loop, a 150 × 4.6-mm or 250 × 4.6-mm-i.d. stainless steel column, an LC-10AD variable-wavelength UV detector, a Sichuan Instrumental Factory (Sichuan, People's Republic of China) model 3066 recorder, and a Shimadzu C-R3A integrator. The wavelength was set at 200 nm for the detection of amino acids and 254 nm for the other solutes.

Preparation of bonded stationary phase

ACPS was synthesized using two procedures; the chemical pathways are presented in Figure 1. Procedure I, similar to the procedure reported in our previous paper (7), is a successive reaction pathway with three steps on the surface of silica gel.

Silanization reaction

Ten grams of silica gel were treated with 100 mL of 6 mol/L HCl at room temperature for 24 h. After filtration, the silica gel was washed with distilled water to neutrality and then dried at 120°C under reduced pressure for 12 h. The silica gel (10 g) was suspended in 120 mL anhydrous toluene containing 10 mL of γ-chloropropyltriethoxy silane, and three drops of triethylamine were added into the mixture. The mixture was heated under reflux and stirred under a stream of nitrogen for 24 h. The γ-chloropropylsilyl-bonded silica gel (CPS) was filtered and washed in sequence with distilled water, methanol, acetone, and ether. Subsequently, CPS was dried under a vacuum over P_2O_5 at 100°C overnight.

Condensation reaction

3-Dihydroxydiethyl aminopropylsilyl-bonded silica gel (DEAP) was prepared by the condensation of CPS with 2,2'-dihydroxydiethylamine in a dimethylformamide (DMF) medium containing bases such as pyridine, triethylamine, potassium, or sodium hydroxide.

Closing ring reaction

The tetraethylene glycol ditosylate was prepared according to a procedure reported in the literature (9). ACPS(I) was synthesized according to a closing ring reaction of DEAP with tetraethylene glycol ditosylate. Under a nitrogen atmosphere, DEAP and tetraethylene glycol ditosylate reacted in tetrahydrofuran reflux for 12 h in the presence of NaH. After filtration, the product was washed in sequence with chloroform, methanol, distilled water, and acetone, and then dried at 120°C under a vacuum. To minimize the effect of residual silanol groups, ACPS should be endcapped by a silanization reaction with trimethylchlorosilane in anhydrous toluene.

Procedure II is a direct condensation of CPS with 3-(Aza-18-crown-6) ether. 3-(Aza-18-crown-6) ether was allowed to react with NaH in DMF until a complete reaction was attained. After the reaction, the mixture was filtered, and the CPS and pyridine were added to the filtrate. The mixture was allowed to react while heating and stirring under a nitrogen atmosphere for 12 h. The ACPS(II) was also endcapped before use.

Determination of complexing capacity

The complexing capacities of the crown ether-bonded silica gel stationary phases were determined according to the procedure described in the literature (7,10). The concentration of metal ions before and after complexation was determined with UV spectrophotometry for copper ions and with atomic absorption spectroscopy for the other ions.

Chromatographic procedure

The bonded packings CPS, DEAP, ACPS (Figure 1), and Zorbax ODS (5 µm, DuPont, Wilmington, NC) were packed into the stainless steel columns by a modified balance-density slurry technique (11). The mobile phases were methanol–water, methanol–buffer solution, and buffer solution. The disubstituted benzene derivatives, amino acids, nuclosides, and nucleobases were used as solutes. The sample concentration in methanol or methanol–water was approximately 0.5–5 mmol/L, and the volume injected was 5–10 µL. Chromatography was carried out at ambient temperature. All the reported retention data were based at least on duplicate determinations.

Results and Discussion

Preparation of bonded stationary phase

The 3-(Aza-18-crown-6) propylsilyl bonded phase has been successfully prepared for the first time using a successive reaction pathway to produce macrocycle on the surface of the silica gel (Figure 1) according to the results of chemical and physical analyses for the bonded product of every step of the reaction. The change in concentration of the hydroxyl group on the DEAP surface during the reaction was monitored by a microtitrimetric method based on acetylation of hydroxyl groups with acetic anhydride-perchloric acid in anhydrous acetic acid.

CPS was used as a blank to eliminate the interference of silanols in the determination. The completion of condensation and closing ring reactions in Procedure I can be obtained using the more excessive of 2,2'-dihydrodiethylamine and ditosylate of tetraethylene glycol. FTIR spectra can also be used for optimization of the reaction conditions for condensation and closing rings. The base is required for the condensation reaction in Procedure I. Experiments with various bases show that the best conversion and the highest column efficiency of ACPS(I) can be obtained with pyridine; triethyamine is also good, but the column efficiency and permeability are very poor if KOH is utilized as a catalyst. The other ACPS, referred to as ACPS(II) to distinguish it from ACPS(I) prepared using Procedure I, was also synthesized by the condensation reaction of azacrown ether and CPS. According to the physical and chemical analysis and chromatographic tests, the ACPSs prepared using the two different procedures exhibited similar structure and properties (performances). This is further support for

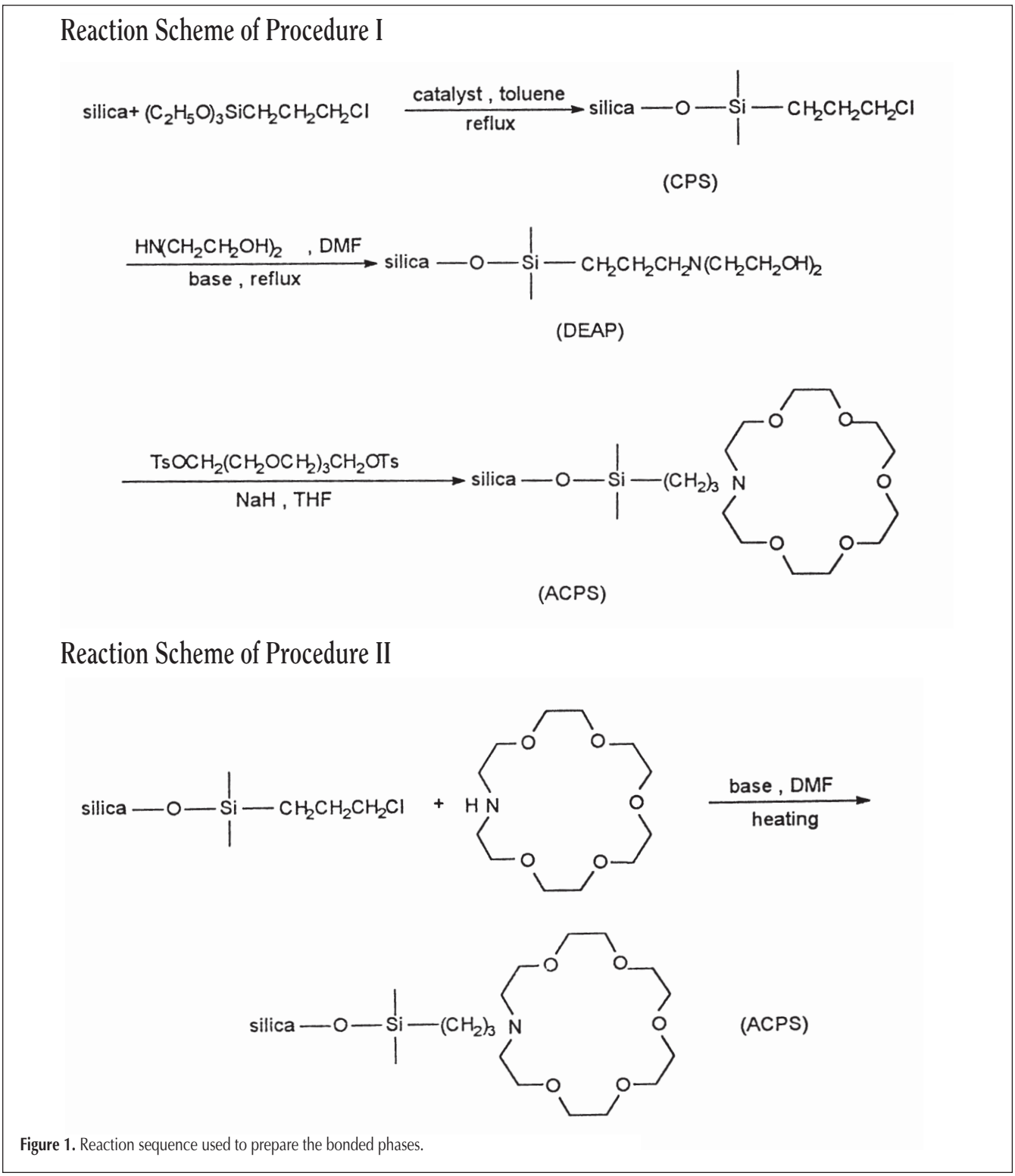

Figure 1. Reaction sequence used to prepare the bonded phases.

Table I. Elemental and Group Analyses of the Bonded Phases

Sample	Element (%) C	H	N	Hydroxy group (mmol/g)	C/H ratio cal.	det.	C/N ratio cal.	det.	Concentration bonded* (mmol/g) C	N
CPS(I)	4.37	0.98			0.45	0.41			0.75	
CPS(II)	5.25	1.03			0.45	0.43			0.83	
DEAP	5.62	1.18	0.72	10.5	0.44	0.40	7.0	8.10	0.67	0.55
ACPS(I)	7.76	1.13	0.59		0.50	0.57	14.94	15.38	0.45	0.43
ACPS(II)	8.18	1.33	0.68		0.50	0.51	14.94	14.16	0.47	0.49

* Concentration bonded was calculated according to carbon and nitrogen content.

Table II. Complexing Capacities of the Bonded Phases for Metal Ions (µmol/g)

Stationary Phases	Metal ions Na^+	K^+	Cu^{2+}	Ag^+	Ni^{2+}
SiO_2	1.4	0.8	30.6	21.5	36.5
CPS	1.0	0.4	31.1	37.6	35.5
DEAP	3.8	6.2	48.4	44.5	56.7
ACPS(I)	58.6	78.2	52.8	43.5	44.8
ACPS(II)	64.6	79.1	53.5	41.9	49.6

forming of the macrocycle of crown ether on the surface of silica gel using the successive reaction pathway.

In general, Procedure I is more practical than Procedure II for the preparation of ACPS, because it is simple to operate, costs less, and easily meets the needs of the reagents used (although the column efficiency is slightly lower).

Characterization of the bonded stationary phase

The elemental analysis, FTIR spectra, and complexing measurements of the bonded phases were carried out in order to achieve a better understanding of the characteristics of the bonded packings. Table I lists the typical carbon, hydrogen, and nitrogen contents and the concentration of bonded groups for the bonded material. The C/H and C/N ratios determined are almost identical with the calculated values according to 3-(Aza-18-crown-6) propyl group bonded.

FTIR spectra of the bonded phases, after the subtraction of the native silica, show the weakness or disappearance of a broad and strong absorption band around 3400–3600/cm, which is characteristic of the silanol stretching frequency for bare silica. All of the bonded materials exhibit peaks at 2850 and 2925/cm as C–H aliphatic CH_2 stretching frequencies. In addition, the spectra of CPS were identified at 800/cm as C–Cl and at 1100/cm as Si–O stretching absorbances. DEAP offers a strong absorption at 1100–1200/cm as C–O,C–N stretching and absorption around 3500–3600/cm as O–H,C–N stretching. In spectra of ACPS(I) and ACPS(II), the same characteristic absorption was observed, having strong absorbances at 1150–1070/cm as asymmetric stretches of ether linkage and 1250/cm and 950–500/cm as characteristic stretching frequencies of macrocyclic polyether (12) similar to the successive absorption peaks of BCN 18-C-6 (7).

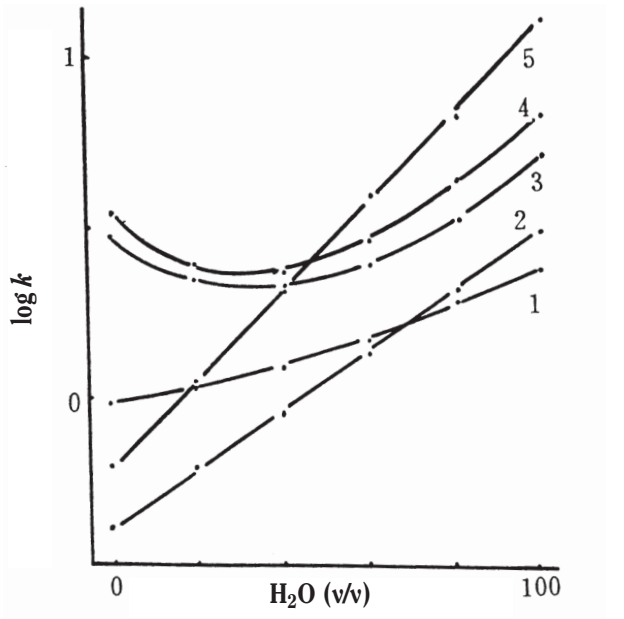

Figure 2. Plot of logarithms of capacity factors on the bonded phases versus the content of methanol in the mobile phase. The solute was *p*-nitrophenol. Stationary phases: 1, DEAP; 2, CPS; 3, ACPS(I); 4, ACPS(II); 5, ODS.

The cation complexing abilities, particularly for alkali and alkaline earth metal cations, are the most important characteristic of the crown ethers and crown ether immobilized silica. The complexing capacities of various bonded materials for some metal cations are given in Table II. The adsorption quantity of metal ions on silica is also given to illustrate the difference in adsorption of different metal ions on silica. It can be seen from Table II that ACPS exhibits the highest complexing capacities for Na^+ and K^+, and no distinct difference in the properties between ACPS(I) and ACPS(II) was observed. However, ACPS is remarkably different from other bonded phases such as CPS and DEAP. On the basis of the hard–soft acids–bases principle (13), the nitrogen of azacrown ether is a soft base that can form stable complexes (to a certain extent) with soft acids such as Cu_2^+, Ag^+, and Ni_2^+ (14). Therefore, the ACPS exhibits considerable complexing capacities for Cu_2^+, Ag^+, and Ni_2^+, even though it subtracts from the adsorption remnant on surface of silica. This is also evidence of success for the preparation of the azacrown ether bonded silica using Procedure I.

Chromatographic characterization of the azacrown ether bonded phases

The chromatographic characterization of the 3-(aza-18-crown-6) propylisilyl bonded phases, ACPS(I), and ACPS(II), was performed using methanol and methanol–buffer solution as mobile phases and disubstituted benzene isomers such as nitrophenol, nitroaniline, and methylaniline as solutes. The column efficiency of ACPS was determined using diphenyl and phenylaniline as solutes with methanol as the mobile phase. The average values of theoretical plate numbers were found to be 14850 plates/m for ACPS(I) and 20806 plates/m for ACPS(II), which are similar to the column efficiency (approximately 15000 plates/m) of another azacrown ether bonded stationary phase with a spacer of six carbons (7). The results show that the column efficiency of ACPS(II) is higher than that of ACPS(I), which can be explicated by increasing the probability of destruction of porous surface structure and uniform particles of silica in Procedure I because of stirring and the presence of base.

The influence of methanol content of the mobile phases on the retention of solutes has been studied. The typical dependence of retention of *p*-nitrophenol using ACPS(I) and ACPS(II) bonded phases on the methanol content in the mobile phase is shown in Figure 2. For comparison, the dependence of retention on the mobile phase composition examined on ODS, CPS, and DEAP are also given in Figure 2. It can be seen from Figure 2 that the variations in the retention of solute on ACPS(I) and ACPS(II) show similar dependence of hyperbolic curves that exhibit the higher retention of the solute in conditions of 100% methanol and 100% water as mobile phases and differ greatly from the typical hydrophobic stationary phase, ODS.

This phenomenon implies that the retention mechanism of solutes on the ACPS is complicated. A variety of interactions may exist, such as polar, hydrophobic, hydrogen bonding, and complexing on the bonded phase with solutes. It can be expected that the hydrophobic effect is the dominant contribution to the retention when the water-rich mobile phase is used; however, when a methanol-rich mobile phase is employed, the polar and hydrogen bonding interactions become the main effect on retention. Comparing the slopes of the straight lines of the linear increase region for the dependence of logarithmic capacity factor on the composition of mobile phase as shown in Figure 2, the hydrophobicity decreases roughly in the order of ODS > CPS > ACPS > DEAP,

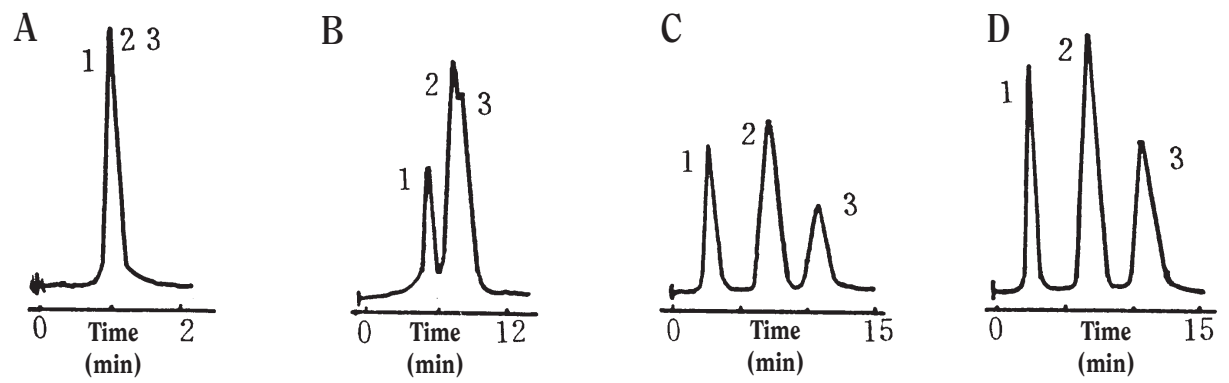

Figure 3. The separation of nitrophenol isomers using methanol as a mobile phase. Stationary phases: CPS (A); DEAP (B); ACPS(I) (C); ACPS(II) (D). Solutes: 1, *m*-nitrophenol; 2, *o*-nitrophenol; 3, *p*-nitrophenol.

Table III. Retention Data (*k*) of the Solutes on the Bonded Phases

	CPS		DEAP		ACPS(I)		ACPS(II)	
Solutes	A*	B	A	B	A	B	A	B
o-methylaniline	0.62	0.81	0.84	0.82	0.94	0.77	1.01	0.83
m-methylaniline	0.71	0.98	1.11	1.01	1.28	0.98	1.46	1.07
p-methylaniline	0.86	1.22	1.24	1.16	2.43	1.20	2.57	1.36
o-nitroaniline	1.64	1.67	1.59	1.65	2.28	2.35	2.62	2.54
m-nitroaniline	1.22	1.22	1.36	1.39	1.89	1.80	2.09	2.18
p-nitroaniline	1.37	1.34	1.12	1.15	1.75	1.69	1.86	1.94
o-nitrophenol	1.89	1.08	1.40	2.05	2.38	4.68	2.52	4.92
m-nitrophenol	1.51	1.00	1.38	1.16	1.79	2.88	1.97	2.76
p-nitrophenol	1.26	0.84	1.11	1.54	3.13	3.63	3.62	3.59

* Mobile phase A consisted of MeOH–70mM triethylamine–HCl buffer solution (ph 3.5, 50:50, v/v).
† Mobile phase B consisted of MeOH–70mM triethylamine–HCl buffer solution (ph 7.5, 50:50, v/v).

which is in agreement with the hydrophobicity of the organic moieties on the surfaces of the bonded phases. The separation of nitrophenol isomers on these bonded phases was also investigated using methanol as the mobile phase. As shown in Figure 3, the baseline separation of nitrophenol isomers is achieved on ACPS, partially achieved on DEAP, but not observed on ODS and CPS.

The influence of mobile phase pH on the retention of ionization solutes, methylaniline (MA), nitroaniline (NA), and nitrophenol (NP), has been investigated. Table III lists the capacity factors of MA, NA, and NP on the bonded phases using methanol–buffer solutions at pH 3.5 and 7.5 as the mobile phases.

The retention data in Table III show the remarkable effect of pH in the mobile phases on the retention of ionizing MA (pK_a 4~5) and NP (pK_a 7~8) on ACPS(I) and ACPS(II). MA cation in the mobile phase with low pH and NP anion in the mobile phase with high pH exhibit much higher retention on ACPS than MA and NP molecules undissociated. These phenomena show that host–guest complexation between crown ether bonded and protonated amine cation, dipole, hydrogen bonding, and electrostatic interaction enhances the retention. The effect of pH on the retention of MA and NP on DEAP is similar to that on ACPS (however, it is less). The changes in retention of MA and NP on CPS with changing pH in the mobile phases are less; however, they are opposite to that on ACPS. It may be the weaker hydrophobicity of CPS which has an influence on the retention of solutes.

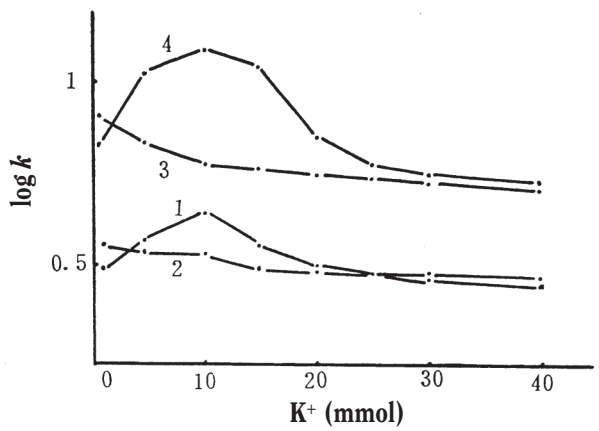

Figure 4. The influence of K^+ concentration in the mobile phase on the retention of solutes. The stationary phase was ACPS. Mobile phases: Tris–HCl buffer solutions at pH 3 (1 and 3) and pH 7.0 (2 and 4). Solutes: *p*-methylaniline (1 and 2) and *p*-nitrophenol (3 and 4).

Nitroaniline is close to neutrality (pK_a < 2.5) and always exists as a neutral unprotonated base under the examined conditions of pH, so that the influence of pH on the retention of NA was not obvious.

The powerful effect of metal ions on the retention and separation selectivity of solutes on ACPS was observed. The retention dependence of MA and NP on K^+ ion concentration is given in Figure 4. Upon adding a certain concentration of KCl to the aqueous mobile phase at pH 3, the retention of MA cation on ACPS increased with the increasing concentration of K^+ ion in the range of 0–15mM. The phenomenon can be attributed to the electrostatic repulsive force between the MA cation and K^+ in the mobile phase, leading to the increase in the complexing ability of ACPS to MA cation; this results in the retention enhancement. The decrease in the retention of MA cation with further increasing KCl concentration can be attributed to the increase in eluent ability of the mobile phase, because crown ether bonded phases have similar properties of ion exchange chromatography (15). The exhibition of maximum retention may be due to the competition complexing equilibrium between MA cation and K^+ on ACPS. The change in the retention of MA molecules at high pH with K^+ concentration is less than that of MA cation at low pH.

The retention of NP on ACPS is increased sharply by adding KCl to the mobile phase at pH 7.5 as shown in Figure 4. It is shown that an electrostatic attraction between the positive charge of K^+ (to be complexed by ACPS) and the negative ion of NP is generated, which in turn enhances the retention of NP (6). Much improvement has been made in the separation of NP isomers by adding KCl to aqueous mobile phase, as shown in Figure 5.

The separation of nucleosides, nucleobase, and amino acids is an interesting subject in the biomedical field. The separations are mostly achieved by ion-exchange, ligand-exchange, and reversed-phase ion-pair chromatography (16, 17). The retention behavior and separation selectivity of nucleosides, nucleo-acid bases, and amino acids on ACPS have been preliminarily studied using methanol–buffer solutions as mobile phases. The influence of buffer solution property and composition in mobile phases on the retention of these solutes and changes in retention order were observed.

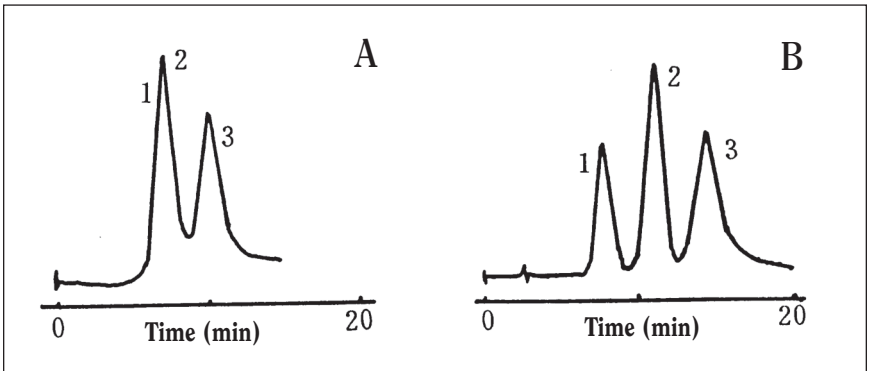

Figure 5. The influence of potassium chloride on the separation of nitrophenol isomers on ACPS. Mobile phase: Tris–HCl buffer solution at pH 7.5 in the absence (A) and presence (B) of KCl. Solutes: 1, *m*-nitrophenol; 2, *p*-nitrophenol; 3, *o*-nitrophenol.

ACPS exhibits more complex retention mechanisms for these compounds, such as complexing ion-exchange and ligand-exchange chromatographic processes (in the presence of metal ions), hydrophobic,

dipolar, and hydrogen bonding interaction. After the optimization of the separation conditions, the typical chromatograms of base and base analogues, nucleosides, and amino acids are shown in Figures 6–9. It can be seen that the rapid separations of nucleobase are achieved using a buffer or aqueous solution as the mobile phase. In a comparison of the two methods mentioned previously, ACPS has merits in efficiency, selectivity, separation speed, and utilization of simple aqueous mobile phase, and it exhibits a potential application to the separation of nucleobase, nucleosides, and amino acids.

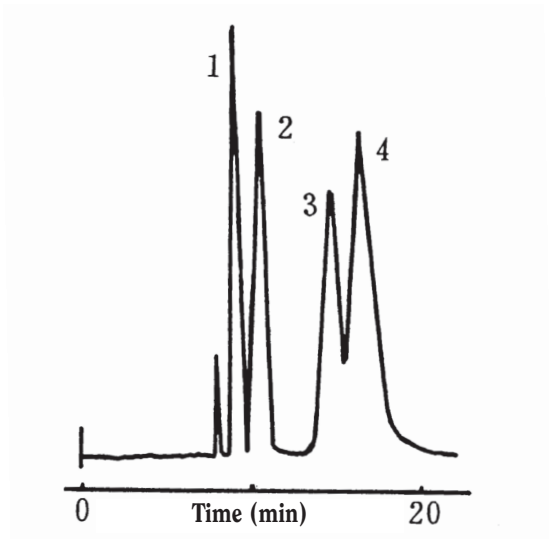

Figure 6. Separation of uracil and 5-halogenated uracils on ACPS. The mobile phase was methanol–0.01M phosphate buffer solution (5:95, v/v) at pH 7.5. Solutes: 1, uracil; 2, 5-fluorouracil; 3, 5-bromouracil; 4, 5-iodouracil.

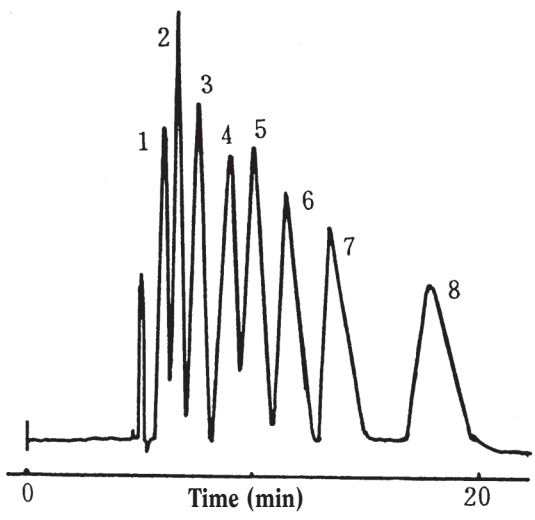

Figure 7. The separation of nuclobase and analogues on ACPS. The mobile phase was methanol–0.01M phosphate buffer solution (5:95, v/v) at pH 7.5. Solutes: 1, cytosine; 2, thymine; 3, hypoxiamthine; 4, guanine; 5, caffine; 6, purine; 7, adenine; 8, uric acid.

Conclusion

ACPS can be synthesized using the three-step reaction method (Procedure I) proposed by our laboratory, in which the crown ether is formed on the surface of silica gel, or the traditional method (Procedure II) that is regarded as the evi-

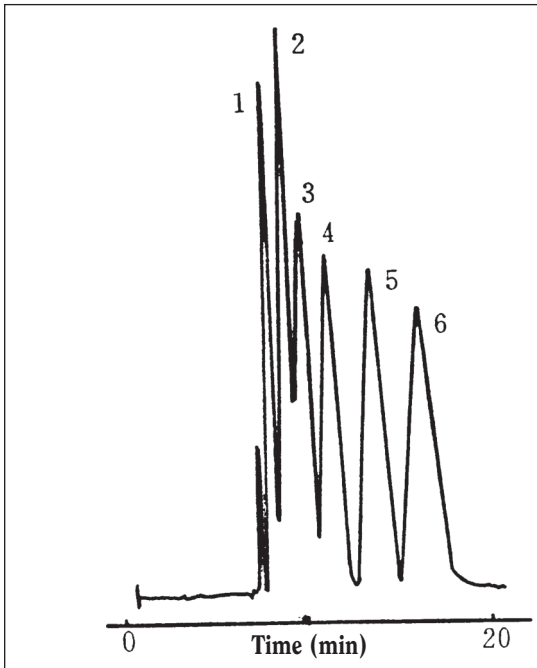

Figure 8. The separation of nucleosides on ACPS. The mobile phase was methanol–0.01M phosphate buffer solution (5:95, v/v) at pH 5.5. Solutes: 1, uridine; 2, cytidine; 3, thymidine; 4, guanosine; 5, adenosine; 6, xanthosine.

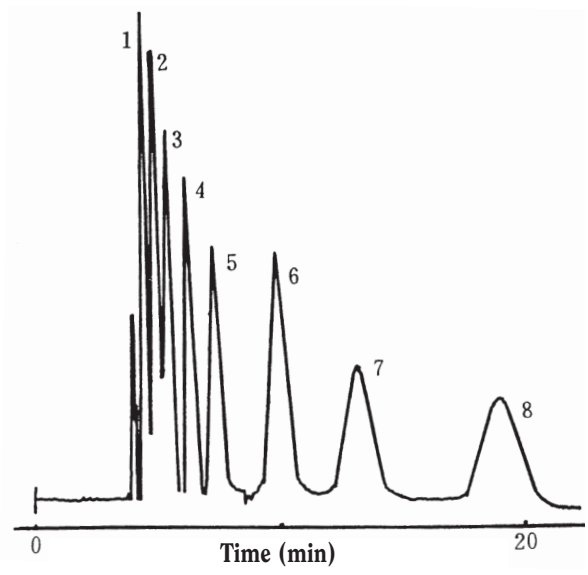

Figure 9. The separation of amino acids on ACPS. The mobile phase was 0.01M $HClO_4$ solution. Solutes: 1, arginine; 2, lysine; 3, cystine; 4, methionine; 5, aspartic acid; 6, tyrosine; 7, benzoyl alanine; 8, *N*-thiohydracrylic glycine.

dence of producing azacrown ether cycle by a successive reaction on the surface of silica gel. No essential difference in the structure or separation efficiency of the ACPS prepared using the two different methods was found by means of chemical and instrumental analyses and chromatography. However, Procedure I is simpler, more rapid, convenient, and cheaper than Procedure II. The aza-18-crown-6 ether bonded phase shows the high complexing capacity with the alkali metal ions Na^+ and K^+. The influence of the metal ion K^+ on the retention and selectivity of solutes, such as disubstituted benzenes, is clearly observed. The change in retention mechanism with the property and composition of mobile phases is exhibited. ACPS also exhibits a potential application to the separation of positional isomers, amino acids, nucleosides, and nucleobases.

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