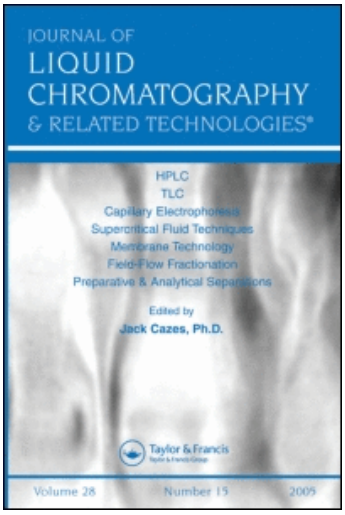

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Application of Humic Acid Bonded-Silica as a Hydrophilic-interaction Chromatographic Stationary Phase in Separation of Polar Compounds

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Abstract: In this paper, humic acid bonded silica (HAAS) stationary phase was prepared through the spacer of aminoalkyl silanes. The high performance liquid chromatographic behavior of several polar compounds, including four alkaloids and clenbuterol, was studied on HAAS. The effect of mobile phase variables such as organic solvent content, buffer pH, and ionic strength on their chromatographic behavior was investigated. The retention mechanism of tested compounds on the stationary phase was elucidated. The results indicate that the HAAS stationary phase behaved as hydrophilic interaction chromatographic packing, using high content organic solvent as mobile phase, and the stationary phase showed good separation selectivity for four alkaloids and clenbuterol.

Keywords: Polar compounds, Separation, Humic acid, Hydrophilic interaction chromatography

INTRODUCTION

Generally, most pharmaceutical analysis was conducted in reversed-phase liquid chromatography (RP-HPLC) mode because of its high selectivity and sensitivity for a large range of compounds.[1] However, the separation of small polar compounds is often very challenging to method development

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due to poor retention on conventional reversed-phase chromatographic packing. Although very effective in separating polar compounds, normal phase liquid chromatographic (NPLC) methods are generally not desirable for routine applications in the pharmaceutical industry because of poor reproducibility. The interfacing with electrospray mass is also a problem with NPLC, since ionization is not easily achieved in totally organic and nonpolar eluents.[2] In addition, if solutes can only be dissolved in aqueous solution, the separation of such polar compounds is impossible in NPLC mode. Although stationary phases containing polar functional groups can enhance the retention of polar compounds with a mostly aqueous mobile phase in RP-HPLC, the approach is unsuitable for compounds with low aqueous solubility.[3]

Hydrophilic interaction chromatography (HILIC) provides an alternative approach to effectively separate small polar compounds on polar stationary phases. Similar to NPLC, polar compounds are more strongly retained in HILIC, but non-aqueous mobile phase in NPLC is replaced with an aqueous–organic mixture with water being the stronger solvent.[4] This feature not only helps to eliminate the problem associated with aqueous solubility, but also makes HILIC more amenable to MS detection and improves the MS sensitivity.[4,5] HILIC separation employs polar stationary phases such as hydroxyl,[6,7] amide,[1] sulfonic acid,[8] and polyacrylamide[9] and partly aqueous mobile phase to separate some polar compounds. Many methods of HILIC have been applied successfully to separate carbohydrates,[10] peptides,[11] amides,[12] nicotinic acid,[13] acetamide,[3] urea,[14] pyrimidines,[1] and some polar compounds in natural product extracts such as aminoglycosides in serum,[15] glutathione in human saliva,[16] and plant somatic embryos.[17]

Humic acids (HAs) are large, complex and organic molecules that are the most widespread ubiquitous components of natural waters and soils as products of decomposition of plant material and animal biomass. Usually the structures of humic acids include alkyl and aromatic units with many functional groups such as carbonyl, amide, hydroxyl, phenolic, and quinine groups.[18] At present, HAs have been immobilized onto the alkyl bonded silica, but these materials are relatively unstable in polar solvent because HAs are only adsorbed onto rather than covalently bonded to C_{18} or C_1 stationary phase.[19,20] Other HAs chemically bonded silica gel was synthesized via an amide linkage between HAs and glutardialdehyde bonded NH_2-silica.[21,22] Recently, Feng and coworkers proposed a new approach to synthesize humic acid bonded silica (HAAS) that can be successfully applied as a solid phase extraction sorbent.[23] The sorbent was prepared via an amide linkage between humyl chloride and the amido terminus of 3-aminopropyltriethoxysilane (APTS) bonded silica. The resulting sorbent was applied to the extraction of benzo[a]pyrene in edible oils.

In this study, we followed the same synthetic method to immobilize humic acids onto spherical silica. Since humic acids ligands contain some hydrophilic groups such as carbonyl, hydroxyl, amide, phenolic groups, the

resulting packing can be used for HILIC separation using four alkaloids and clenbuterol as probes, which are an important class of biological and pharmacologically active compounds. Hence, it is crucial to develop an efficient analytical method to facilitate the study of these products. The chemical structures of four alkaloids and clenbuterol[24,25] are presented in Figure 1. In this study, we investigated the separation of four alkaloids and clenbuterol on HAAS stationary phase. The influence of the mobile phase parameters with respect to the acetonitrile content, buffer pH, ionic strength, and salt type in the mobile phase on the chromatographic behavior of four alkaloids and clenbuterol was studied in detail. The retention mechanism of the solutes on the HAAS stationary phase was also discussed.

EXPERIMENTAL

Reagents and Materials

All reagents were obtained from commercial sources and were of analytical reagent grade. Acetonitrile, ammonium acetate, and disodium phosphate were purchased from the Beijing Chemical Plant (Beijing, China). Double distilled water was used for all experiments. Clenbuterol, ephedrine, atropine, theophylline, and nicotine were obtained from the National

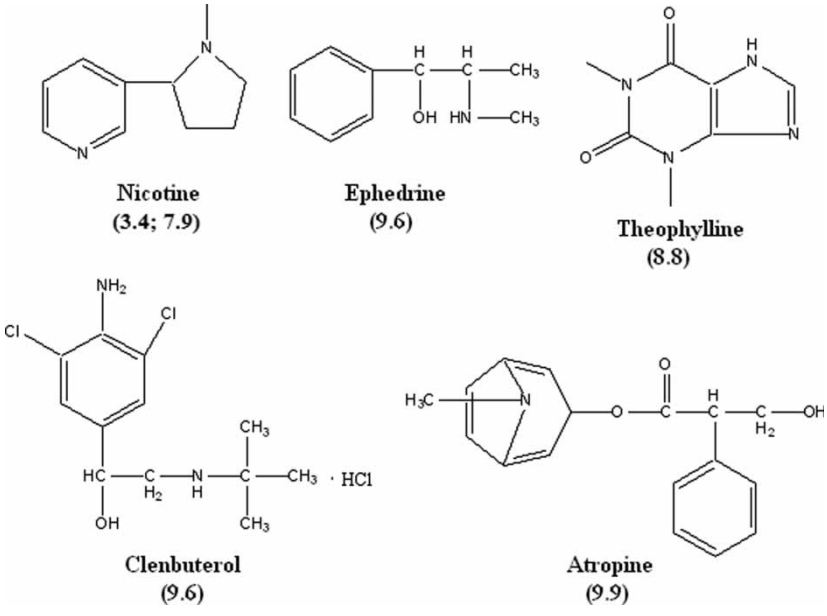

Figure 1. Molecular structures and *pK*as of five polar compounds.

Institute for the Control of Pharmaceutical and Biological Products of China (NICPBP, Beijing, China). Spherical silica gel was made by the oil emulsion method[26] in our laboratory. The mean particle sizes of silica gel were 5–7 μm. Humic acids were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).[27]

Preparation and Characterization of Humic Acid Bonded Silica (HAAS)

The silica gel with chemically bonded humic acids (HAAS) was prepared according to the method we proposed.[23] First, 3-aminopropyltrimethoxylsilane bonded silica (AS) was obtained via the reaction between silica and 3-aminopropyltrimethoxylsilane. Then, the humic acid bonded silica (HAAS) was prepared through an amide linkage between humyl chloride and the amido terminus of 3-aminopropyltriethoxysilane (APTS) bonded silica. Humyl chloride (HAC) was synthesized via humic acids reacting with thionyl chloride.

The resulting material was characterized by FT-IR, elemental analysis, and nitrogen adsorption analysis. FT-IR spectra were determined by using a Thermo Nicolet 670FT-IR (USA) equipped with a diffuse reflectance accessory. The elemental analysis was performed with an Elementar VarioEL β elemental analyzer (Hanau, Germany). The surface areas, average pore size, and the pore volume were obtained by the results of nitrogen adsorption analysis with Beckman SA3100 specific surface area analyzer (Fullerton, CA, USA).

Instrumentation and Chromatography

The HPLC system consisted of a FL 2200 pump, an UV 2200 detector (Fuli Company, Hangzhou, China) and a Rheodyne 7725 injector (USA) with 20 μL loop. The data were processed by Echrom 2000 ChemStation software (Elite Company, Dalian, China). Approximately 2.0 g HAAS were packed into stainless steel columns (150 mm × 4.6 mm i.d.) using the slurry technique. The mobile phases were filtered through a G-4 fritted glass funnel and degassed in an ultrasonic bath for 5 min before use.

The four alkaloids and clenbuterol were dissolved in methanol and the concentration was $0.1\ mg \cdot mL^{-1}$. All measurements were carried out at ambient temperature and tested at least twice. The flow rate of the mobile phase was $0.7\ mL \cdot min^{-1}$. The wavelength used for detection was 214 nm. The void volume was determined using cyclohexane for the calculation of the capacity factor.

RESULTS AND DISCUSSION

Physicochemical Properties

For evaluation of the pore structures of the stationary phase, the nitrogen adsorption analysis was performed. The results are presented in Table 1. The surface area, the pore volume, and the average pore diameters of the obtained HAAS are smaller than those of their original bare substrate. The results of elemental analysis, as seen in Table 1, provide evidence of the successful immobilization of the organic groups onto the silica.

Figure 2 shows the FT-IR spectra of the humic acids and humic acid bonded silica. It can be found that the spectra exhibit typical broad bands and shoulders found in the IR spectra of many soil HA.[28] The region between 3800 and 2200 cm^{-1} exhibits very broad bands with several distinct frequency ranges. In the spectrum of humic acid, the high frequency modes above 3500 cm^{-1} are assigned to nonbonded OH stretches. Most of these occur as broad shoulders of poorly resolved aromatic C-H stretches in the 3400–3000 cm^{-1} region. The 3000–2800 cm^{-1} region exhibits high intensity bands characteristic of symmetric and asymmetric aliphatic CH_2 and CH_3 stretches. In the 1800–1300 cm^{-1} region, we observe several peaks as follows: (i) C═O stretches from COOH, CONH groups, (ii) aromatic C═C stretches, (iii) CH deformation of CH_3 groups, and (iv) C-H bending of CH_2 groups. In the 1270–760 cm^{-1} region, we observe several peaks as follows: (i) aromatic C-H and C-OH stretches and (ii) out-of-plane C-H bends. These are assigned to mono, di, and tri hydroxyl substituted aromatics. Characteristic aliphatic C-O and C-O stretches of carbohydrates are also observed in the 1270–760 cm^{-1} region. These results provide evidence of the successful modification of silica gel with humic acids.

Comparison of Retentions of Polar Compounds on Silica and HAAS

In recently published work, conventional bare silica was used as the HILIC material based on the hydrophilic silanols such as Atlantis[29] and Kromasil.[30] Therefore, in order to investigate the hydrophilicity, it is

Table 1. Physicochemical properties of silica and HAAS

Stationary phase	Specific surface area ($m^2 \cdot g^{-1}$)	Average pore size (nm)	Specific pore volume ($cm^3 \cdot g^{-1}$)	Carbon content (%)
Silica	254	24	0.99	0
HAAS	139	21	0.66	10.5

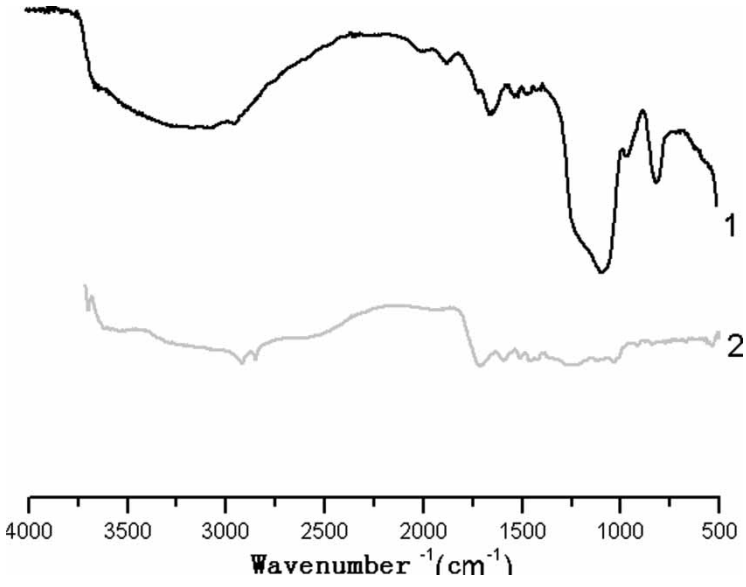

Figure 2. Diffused reflectance FT-IR spectra of (1) HAAS, (2) humic acid.

important to compare the retentions of polar compounds on bare silica and HAAS. In this study, we used some small polar molecules such as atropine, ephedrine, clenbuterol and thymine, uracil, 6-hydropurine, adenine hydrochloride, as probes to investigate the hydrophilicity of HAAS by comparing their retention on silica and HAAS as seen in Table 2. The concentration of disodium phosphate in the mobile phase is kept at 5 mmol $\cdot$ L^{-1} and buffer pH constant at 3.5. The capacity factors of all the tested solutes on HAAS columns are much higher than those on silica columns, which indicate that

Table 2. Comparison of capacity factors (k′) of polar compounds between on HAAS and on bared silica

Column	Silica			HAAS		
Acetonitrile-buffer solution (V/V)	65:35	80:20	90:10	65:35	80:20	90:10
Clenbuterol	0.54	0.97	5.03	1.96	4.56	18.3
Ephedrine	0.56	0.8	6.77	2.8	4.67	15.3
Atropine	0.86	1.77	9.6	2.27	4.87	10.8
Thymine	0.36	0.39	0.45	0.38	0.46	0.76
Uracail	0.34	0.39	0.46	0.38	0.49	0.92
6-Hydropurine	0.45	0.54	1.05	0.59	1.36	3.76
Adenine hydrochloride	0.55	0.81	1.72	1.01	2.18	5.84

HAAS has stronger hydrophilicity than silica. Furthermore, the retentions of small compounds followed the same order as their hydrophilicity. These indicate that small polar compounds are retained on HAAS mainly through the hydrophilic interactions mechanism.

Effect of Acetonitrile Content

One of the HILIC characteristics is that the mobile phase consists of an aqueous organic mixture and the hydrophilic interaction retention mechanism is presented when the volume percentage of acetonitrile in the mobile phase is more than 60%.[4] So it is very important to investigate the effect of organic solvent content in the mobile phase on the capacity factor of solutes on the stationary phase in HILIC. In this study, mixtures of acetonitrile and phosphate buffer (5 mmol $\cdot$ L^{-1}) were chosen as mobile phases to investigate the influence of acetonitrile content in the mobile phase on the retention behavior of solutes including four alkaloids and clenbuterol. The buffer pH of the mobile phase was set at 3.5. The capacity factors of five solutes were plotted against the volume percentage of acetonitrile in mobile phase from 35% to 90% as shown in Figure 3. We can learn that the capacity factors of the solutes increased with increasing acetonitrile concentration in the mobile phases above 50%. This result indicates that the hydrophilic interaction is one of the factors influencing the retention of the solutes at high levels of organic solvents, and the hydrophilic interaction roots in carbonyl, hydroxyl, amide, phenolic groups, on HAAS and the polar groups of four alkaloids and clenbuterol. The capacity factors of the

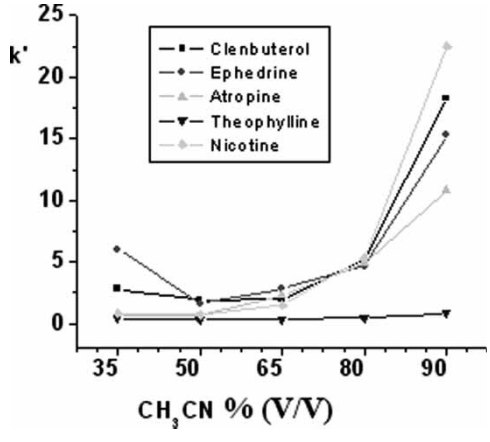

Figure 3. Effect of the acetonitrile content of mobile phase on the capacity factor (k′) of solutes on HAAS stationary phase. Mobile phase: acetonitrile-5 mmol $\cdot$ L^{-1} Na_2HPO_4 buffer solution (pH 3.5). Flow rate: 0.7 mL $\cdot$ min^{-1}.

solutes decreased with increasing acetonitrile content when the acetonitrile concentration was lower than 50%, which indicated that hydrophobic interaction was responsible for the retention of the tested solutes on the HAAS column. However, the hydrophobic interaction is still weak according to the experiment on the retention of the solutes.

Effect of Buffer pH

pH can influence the degree of solute ionization in the mobile phase[31] and the existent condition of polar groups on stationary phase. Therefore, it has a very significant impact on the retention and selectivity of the tested compounds.

Figure 4 shows the effect of buffer pH on retention behavior. Buffer containing 5 mmol $\cdot$ L^{-1} disodium phosphate was prepared and adjusted in the range of 3.5–6.5. Mobile phase containing acetonitrile and buffer was mixed at the ratio of 65:35 (V/V).

As shown in Figure 4, it can be seen that the capacity factors of the tested solutes, except for nicotine, evidently increased as the pH of mobile phase increased from 3.5 to 6.5. In the pH range of 3.5–6.5, four alkaloids and clenbuterol were always protonated. However, humic acid is a very complex molecule, which consists of large numbers of carboxylic acid functional groups. It is reasonable to assume there are still quite a number of carboxylic acid groups left on HAAS. The deprotonated carboxylic acid groups on HAAS can either attract the immobilized water layer to facilitate the HILIC interaction, or is directly involved in ion exchange interactions,[32] which enhances the analyte interaction with the stationary phase and, hence, results in improved retention when the buffer pH was above pKa of carboxylic

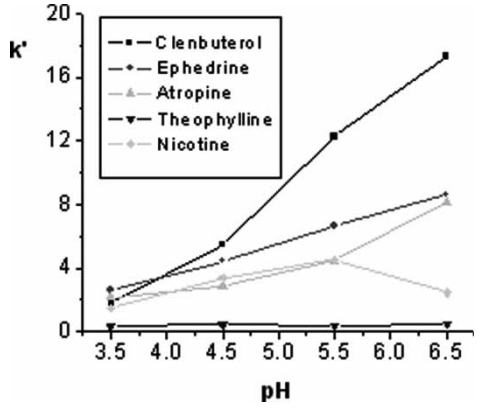

Figure 4. Effect of buffer pH on the capacity factors (k′) of solutes on HAAS. Mobile phase: acetonitrile-5 mmol $\cdot$ L^{-1} Na_2HPO_4 buffer solution, (65:35, V/V). Flow rate: 0.7 mL $\cdot$ min^{-1}.

acid. Especially at higher pH (6.5) where the carboxylic acid groups are largely deprotonated, the HILIC interaction or ion exchange interaction effect might be very strong, leading to a great increase in retention.

However, nicotine displayed an unusual behavior compared to other compounds on the HAAS column. Its retention increased initially as the buffer pH increased from 3.5 to 5.5, but leveled off when the buffer pH further increased to 6.5. This unusual behavior of nicotine on the HAAS column could be explained by the pKa value of nicotine (pKa_2 7.9).[25] The pH of the mobile phase is nearly 7.0 via the dilution of acetonitrile though the buffer pH was at 6.5; nicotine was less protonated at buffer pH 6.5 than that at buffer pH 5.5 or others because of its basic property. Thus, the HILIC interaction or ion exchange interaction weakens with the buffer pH increasing to 6.5 inducing the decrease of the capacity factor of nicotine.

Effect of Ionic Strength

The ionic strength of mobile phase also influenced the retentions of four alkaloids and clenbuterol. In this study, the concentration of disodium phosphate in the mobile phase varied from 5 mmol $\cdot$ L^{-1} to 30 mmol $\cdot$ L^{-1}, keeping acetonitrile content constant at 65% (V/V) and buffer pH constant at 4.5. The relationship between salt concentration and capacity factors of the tested solutes are presented in Figure 5. It showed that the capacity factors slightly decreased with increasing ionic strength. As discussed in previous sections, ion exchange interaction between the positively charged tested solutes and the carboxylic acid residues of HAAS played a part in the separation. Higher salt concentrations weakened the ion exchange interaction between the positively charged analytes and partly negatively charged carboxylic acid groups in HAAS column, thus leading to decreasing retention.

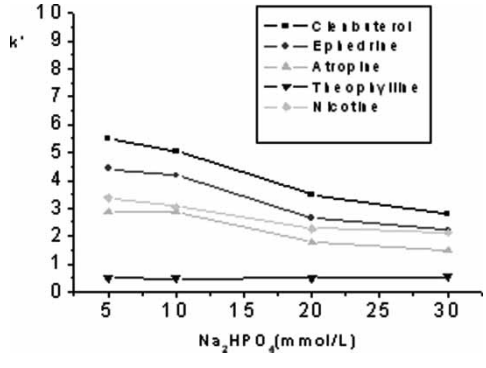

Figure 5. Effect of the Na_2HPO_4 concentration in mobile phase on the capacity factors (k′) of solutes on HAAS. Mobile phase: acetonitrile- Na_2HPO_4 buffer solution (pH 4.5), (65:35, V/V). Flow rate: 0.7 mL $\cdot$ min^{-1}.

Effect of Salt Type in Mobile Phase

Many salts can be used in the mobile phase such as phosphate, acetate, formate, triethylamine, and perchlorate in the separation of liquid chromatography. In this study, disodium phosphate and ammonium acetate were selected as inorganic salt and organic salt to prepare buffers in the mobile phases, respectively, keeping acetonitrile content at 65% (V/V) and salt concentration at 10 mmol $\cdot$ L^{-1} (pH 4.5).

As seen in Figure 6, there was almost no change in retentions of four alkaloids and clenbuterol on the HAAS column using two different salts. However, two salts have respective advantages and disadvantages: first, compared with acetate, phosphate buffer has a wider range of pH in favor of adjusting suitable ambient medium in the mobile phase, and also exhibits low light absorption in the UV region of the light spectrum, which can decrease background absorbency of the mobile phase under low wavelength. In comparison, ammonium acetate is often used as the buffer salt in the mobile phase owing to its relatively high solubility at a high level of organic solvents in HILIC mode. In addition, ammonium acetate is a volatile organic salt when interfacing with the mass spectrometric detector (MS).[5]

Stability of HAAS Column

Many silica based stationary phases show good chromatographic performance in the pH range from 3 to 8. At higher pH, the chemically bonded stationary

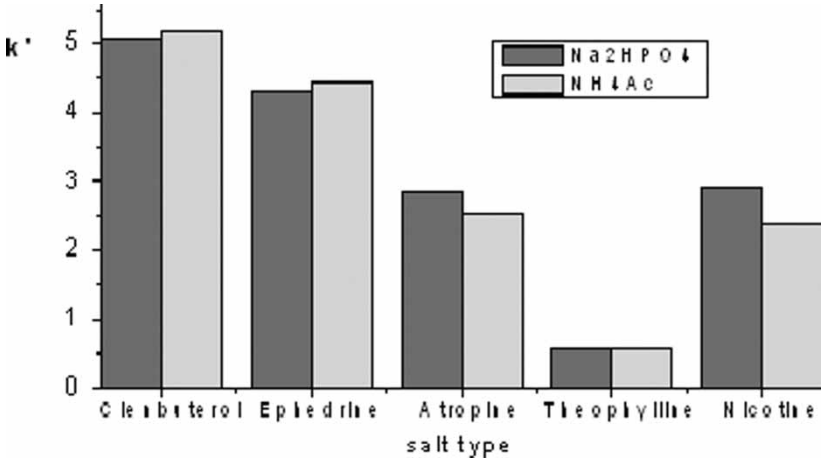

Figure 6. Effect of the salt type of buffer in mobile phase on the capacity factors (k′) of solutes on HAAS. Mobile phase: acetonitrile-10 mmol $\cdot$ L^{-1} buffer solution (pH 4.5), (65:35, V/V). Flow rate: 0.7 mL $\cdot$ min^{-1}.

phases tend to degrade, as a result of silica dissolution,[33] while at acidic pH, the siloxane linkage of conventional bonded phase is susceptible to hydrolysis.[34] The stability of the stationary phases are very influencing factors for application. We were often concerned with irreversible adsorption of sample compounds and dissolution of the packing when using these packings containing polar groups for the long applications using polar solvents as eluents. In this study, the separations of five polar compounds were monitored over the period of two months in HILIC. Retentions of five solutes were slightly changed under the same conditions over this period. In order to further investigate the stability of HAAS, 2000 column volumes of mobile phase was continuously passed through the column at different pH values (3.5 and 6.5), while keeping the acetonitrile content at 65% (V/V) and salt concentration at 5 mmol $\cdot$ L^{-1}. The plots of the capacity factors of atropine and ephedrine on HAAS against the volumes of mobile phase are shown in Figure 7. As shown in Figure 7, after the column was purged with 2000 mL of the acetonitrile buffer solution, little variation of the retentions is observed. These indicate that the HAAS is stable in polar solvents. It is reasonable that the humic acid ligands are chemically bonded to the surface of silica rather than being adsorbed to.

Separation of the Tested Solutes on HAAS

The efficient separation of four alkaloids and clenbuterol on the HAAS column can be obtained (Figure 8) with a mixture 65:35 (V/V) of acetonitrile-5 mmol $\cdot$ L^{-1} Na_2HPO_4 buffer solution (pH 4.5) as the mobile phase.

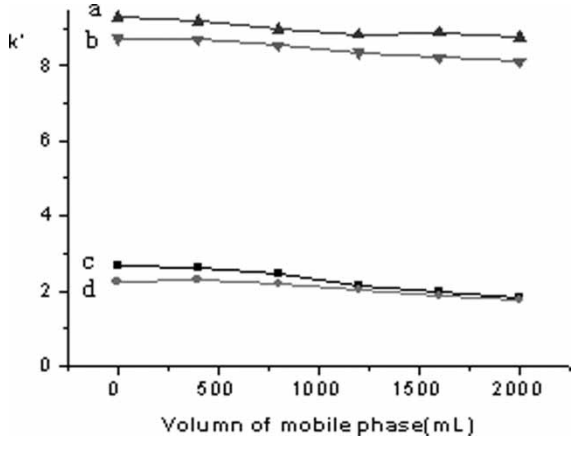

Figure 7. The stability test for HAAS. Mobile phase: acetonitrile-5 mmol $\cdot$ L^{-1} Na_2HPO_4 buffer solution at pH 3.5 (c and d) and pH 6.5 (a and b), (65:35, V/V). Flow rate: 0.7 mL $\cdot$ min^{-1}. Solutes: a, c for ephedrine and b, d for atropine.

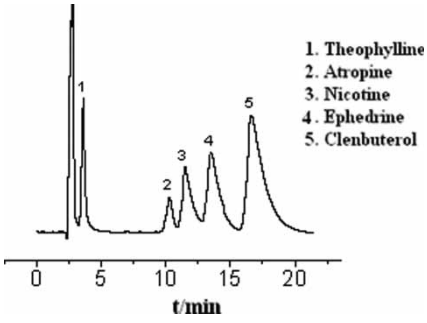

Figure 8. Separation of five solutes on HAAS. Mobile phase: acetonitrile-5 mmol · L^{-1} Na_2HPO_4 buffer solution (pH 4.5), (65:35, V/V). Flow rate: 0.7 mL · min^{-1}.

From Figure 8, it can be seen that the HAAS stationary phase shows good separation selectivity for the five tested solutes. Theophylline is the most hydrophilic compound among the five tested compounds, but its retention was much weaker on HAAS stationary phase. It may be ascribed that HAAS and the tested compounds, except for theophylline, contain phenyl groups. Thus, charge transfer interaction and hydrophilic interaction between the four compounds and HAAS can exhibit the same time and synergy of each other, leading to the stronger retentions than that of theophylline.

CONCLUSION

The influence of mobile phase variables, such as content of acetonitrile, ionic strength and buffer pH, on the retention of five solutes using four alkaloids and clenbuterol as probes on HAAS has been investigated. The chromatographic behavior of the solutes indicates that the hydrophilic interaction can contribute to the retentions of five polar compounds at high acetonitrile content and the stationary phase behaves as a polar material. But, at low acetonitrile content, other interactions may operate besides the hydrophilic interaction. Finally, the separation of five polar solutes was successfully obtained under optimized conditions.

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