Scientific paper

Preparation and Chromatographic Evaluation of Columns for Reversed-Phase Liquid Chromatography

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Abstract

Column selection in reversed-phase liquid chromatography is still not a straightforward process. In this study, we have evaluated and compared the performance of several traditional C18 phases with polar-modified phases and found distinct differences in the chromatographic behavior between two groups, as well as a high degree of variability within each group. The polar-endcapped phases exhibit similar hydrophobic selectivity, higher hydrogen bonding capacities and silanol activity with reference to the conventional C18 columns. The polar-embedded phases displayed a greatly reduced hydrophobic nature and a significantly reduced silanol activity compared to the conventional C18 phases. It appears that ionic and/or dipole interactions play a significant role in the overall retention behavior of the polar-modified phases towards basic and acidic analytes.

Keywords: Polar-modified phases, stationary phases, octadecylsilyl, silanols

1. Introduction

Nowadays, a very large number of different brands of reversed-phase liquid chromatography stationary phases are commercially available on the market and new ones are being introduced regularly. Chromatographic performance may alter considerably depending on the properties of the stationary phases. It is apparent that analysts face a difficult problem in selecting the right column for a given application. Moderately or strongly polar pharmaceutical molecules with basic and/or acidic functionalities challenge even the best C18 columns, and make column selection truly confounding. Large differences in retention, selectivity, and peak shape are common obstacles for chemists analyzing drugs. Although many HPLC column manufacturers supply chromatographic information about their products, this information is not always useful because it may be biased towards their own products. The exact data comparison of HPLC columns from different manufacturers is not always available because of differences in testing conditions. Thus, the scientists have to screen several different stationary phases to find out the appropriate one for a separation.

In order to simplify the column selection process, there have been several attempts to create test mixtures and protocols to evaluate and characterize column chemistries.[1–9] These evaluations typically rely on the use of several test probes that have been selected to identify specific column characteristics such as hydrophobicity or silanol activity. There are distinct differences in the testing methodologies that they use to determine those characteristics. For example, Engelhardt proposed using the selectivity factor between ethylbenzene and toluene in a mobile phase of 55:45 (v/v) methanol/water as an indicator of stationary phase hydrophobicity.[1] Kimata *et al* suggested that the same hydrophobicity value be determined from the selectivity value between amylbenzene and butylbenzene in a mobile phase of 80:20 (v/v) methanol/water.[2] Likewise, Neue *et al* suggest that the selectivity value between a base such as amitriptyline, chlorpheniramine, or propranolol and a neutral probe such as acenaphthene be used to gauge silanol activity,[3] while Tanaka test uses the selectivity value between benzylamine and phenol to evaluate the same characteristics.[2] Although the differences in methodologies used for the phase characterization, it was found that there was a relatively high correlation for the evalua-

tion of hydrophobic interactions. However, there was little correlation between the results of the same test methods for the characterization of the silanol activity of different phases.[4]

The goals of this study were to attempt to gain an understanding of how these phases differ from conventional alkyl-bonded phases. We attempted to characterize these phases using a series of assays designed to elucidate fundamental chromatographic behavior and have included several applications that may further illustrate the differences between these phases. We also present examples showing the selectivity difference of the polar-modified columns compared to conventional alkyl-bonded phases due to the nature of the polar group. This evaluation will provide insight to the usefulness and limitations of columns for the HPLC chromatographer.

2. Experimental

2. 1. Reagents and Materials

Organic silane reagents were obtained from Gelest (Tullytown, P. A., USA) or Silar Laboratories (Wilmington, N. C., USA). Hydrofluoric acid and nitric acid were of trace analysis grade from Kanto Chemicals (Tokyo, Japan). The acetonitrile and methanol used in these analyses were HPLC grade from VWR Scientific Products (San Dimas, C. A., USA), and the de-ionized water was prepared using an E-Pure water purification system (Barnstead/Thermolyne, Dubuque, I. A., USA). All reagents were of the highest possible purity and were purchased from Sigma-Aldrich Inc. (Milwaukee, W. I., USA). All of the columns used in the study were based upon the high-purity silica with mean particle size of 5 μm, mean pore diameter of 10 nm and BET surface area of 400 $m^2 g^{-1}$.

2. 2. Determination of Metal Impurities in Silica Gels

Water (5 g) was added to 1 g of silica gel in a PTFE beaker. A 5 mL volume of a 50% aqueous solution of hydrofluoric acid was added to the silica gel to dissolve it. The solution was heated on a hot plate and evaporated to dryness. The residue was dissolved using 0.25 mL of concentrated HNO_3 and the solution was made up to 25 mL with water. The final solution was analyzed using an inductively coupled plasma mass spectrometer (PMS 200, Yokogawa Analytical System, Tokyo, Japan). The total metal content is less than 30 ppm.

2. 3. Preparation of Trimethoxysilylpropylacetamide

A three-neck round-bottomed flask, equipped with a mechanical stirrer, a refluxing condenser and a dropping funnel, was charged with 3-aminopropyltrimethoxysilane (18 g), toluene (50 mL) and triethylamine (13 g). Acetyl chloride (18 mL) was then added dropwise to the flask. The mixture was stirred at room temperature under argon atmosphere for 24 hours. The solvent was removed and the residue was distilled by reduced pressure. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.65 (t, 2H), 1.65 (m, 2H), 1.98 (s, 3H), 3.25 (q, 2H), 3.65 (s, 9H), 5.91 (b, 1H). Calc. C% 43.43, H% 8.59, N% 6.33; Found C% 43.47, H% 7.96, N% 6.25.

2. 4. Preparation of N-3-(trimethoxysilyl) Propyl-palmitamide

A three-neck round-bottomed flask, equipped with a mechanical stirrer, a refluxing condenser and a dropping funnel, was charged with 3-aminopropyltrimethoxysilane (184 mL), toluene (1000 mL) and triethylamine (174 mL). Palmitoyl chloride (303 mL) was then added dropwise to the flask. The mixture was stirred at room temperature under argon atmosphere for 24 h. The solvent was removed and the residue was recrystallized from 9:1 *n*-hexane:methylene chloride. m.p. 58–59 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.64 (t, 2H), 0.87 (t, 3H), 1.25 (m, 24H), 1.62 (m, 4H), 2.14 (t, 2H), 3.24 (q, 2H), 3.56 (s, 9H), 5.66 (b, 1H). Calc. C% 63.26, H% 11.34, N% 3.35; Found C% 63.00, H% 11.67, N% 3.37. *N*-3-(dimethylmethoxysilylpropyl)palmitamide can be prepared by a similar method. m.p. 56–57 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.51 (s, 6H), 0.62 (t, 2H), 0.87 (t, 3H), 1.25 (m, 24H), 1.61 (m, 4H), 2.13 (t, 2H), 3.26 (q, 2H), 3.55 (s, 3H), 5.66 (b, 1H). Calc. C% 68.34, H% 12.25, N% 3.62; Found C% 68.39, H% 12.18, N% 3.63.

2. 5. Preparation of ODS Packing Materials

Three types of ODS packing, mono-ODS, di-ODS, and tri-ODS, were prepared by silylating silica gel using octadecyldimethylchlorosilane (ODS-M), octadecylmethyldichlorosilane (ODS-D) and octadecyltrichlorosilane (ODS-T) as the silylating agents, respectively. The following preparation method was employed: a suspension of 10 g of silica gels in 50 mL of concentrated HCl was heated at 100 °C for 16 h, then the suspension was cooled to about 25 °C, and the silica gels were collected, washed with water until free from acid and dried under vacuum at 140 °C for 8 h. A 50% excess of octadecylsilylating agent was added to the stirred suspension of 10 g of the dried silica gels in 50 mL of dry xylene under a nitrogen atmosphere. The calculated amount of dry pyridine equivalent to the amount of octadecylsilylating agent was then added. The suspension was refluxed for 24 h. The silylated silica gels obtained were washed with dry toluene, dichloromethane, THF and methanol. When octadecylmethyldichlorosilane or octadecyltrichlorosilane was used, residual chloro groups on the bonded silane were hydrolyzed in acetonitrile-water (1:1) with refluxing

for 16 h after the silica gels had been washed with acetonitrile. The silica gels in every case were washed with toluene, dichloromethane, THF, methanol and dried under vacuum at 80 °C for 8 h. 3 g of ODS was then placed in a 30 mL glass ampoule and dried under vacuum at 100 °C for 4 h. A silylating agent such as 3 mmol of hexaethylcyclotrisiloxane was added to the ampoule after it had been cooled to about 25 °C under nitrogen atmosphere. The ampoule was cooled to –78 °C and the air in it was replaced with nitrogen. The ampoule was sealed, heated at 250 °C for 24 h, cooled and then opened. The silylated silica gels obtained were washed repeatedly with toluene, dichloromethane, THF, methanol and dried under vacuum at 80 °C for 8 h. For endcapping ODS by the liquid-phase silylation method, the following steps were employed: Hexamethyldisilazane (8 mL) was added to a stirred suspension of 10 g of ODS in 50 mL of dry xylene. The suspension was refluxed for 24 h. The silylated silica gels were washed with toluene, dichloromethane, THF, methanol and dried under vacuum at 80 °C for 8 h. The modification process yielded a modified silica with a ligand surface concentration of 3.38 μmol · m^{-2}. The carbon and hydrogen percentages determined by elemental analysis were 24.82 and 4.68%, respectively. The concentration of the organic groups attached to the silica surface was calculated from the carbon percentages and the BET surface area of the bare silica. At least three batches were made for each material. Microanalyses are in good agreement with the proposed formulation.

2. 6. Preparation of Polar-endcapped Bonded Phases

A suspension of 10 g of silica gels in 50 mL of concentrated HCl was heated at 100 °C for 16 h, The suspension was then cooled to about 25 °C, and the silica gels were collected, washed with water until free from acid and dried under vacuum at 140 °C for 8 h. A 50% molar excess of alkyldimethylchlorosilane such as 20 g of octadecyldimethylchlorosilane and 55 mmol of pyridine and *n*-decane (50 mL) were added. The suspension was refluxed under nitrogen atmosphere for 48 h. The mixture was filtered and washed well with toluene, dichloromethane, THF, methanol and dried under vacuum at 80 °C for 8 h. The silylated silica gels (10 g) were further modified with a stoichiometric excess of 3-cyanopropyltrichlorosilane (18 mmol) or trimethoxysilylpropylacetamide (18 mmol) as described earlier in the primary bonding step. After the secondary bonding step, the silica gels were hydrolyzed with 0.1% trifluoroacetic acid in 5:1 MeOH: H_2O at room temperature for 16 h. The material was filtered and washed with acetone and methanol and dried under vacuum at 80 °C for 8 h. After the bonding steps, the unreacted silanol groups on the surface of the silica gels were blocked by reaction with an endcapping reagent. Briefly, the silanol blocking reaction was performed by refluxing approximately 10 g of the silylated silica gels in 50 mL of *n*-decane with a stoichiometric excess of endcapping reagent such as 8 mL of hexamethyldisilazane (HMDZ). After the mixture was refluxed for 16 h, the silica gels were filtered and washed repeatedly with toluene, dichloromethane, THF, methanol and dried under vacuum at 80 °C for 8 h. The modification process yielded a modified silica with a ligand surface concentration of 3.53 μmol · m^{-2}. The carbon, hydrogen and nitrogen percentages determined by elemental analysis were 25.54, 4.61 and 0.71%, respectively. The concentration of the organic groups attached to the silica surface was calculated from the carbon percentages and the BET surface area of the bare silica. At least three batches were made for each material. Microanalyses are in good agreement with the proposed formulation.

2. 7. Preparation of Polar-embedded Bonded Phase

A suspension of 10 g of silica gels in 50 mL of concentrated HCl was heated at 100 °C for 16 h, the suspension was then cooled to about 25 °C, and the silica gels were collected, washed with water until free from acid and dried under vacuum at 140 °C for 8 h. The silylation reagent *N*-3-(dimethylmethoxysilylpropyl)palmitamide (10 g) and *n*-decane (50 mL) were added. The suspension was refluxed under nitrogen atmosphere for 24 h. The mixture was filtered and washed well with toluene, dichloromethane, THF, methanol and dried under vacuum at 80 °C for 8 h. The silylated silica gels (10 g) were further modified with a stoichiometric excess of trimethoxysilylpropylacetamide (18 mmol) as described earlier in the primary bonding step. After the secondary bonding step, the silica gels were hydrolyzed with 0.1% trifluoroacetic acid in 5:1 MeOH:water at room temperature for 16 h. The material was filtered and washed with acetone and methanol and dried under vacuum at 80 °C for 8 h. After the bonding steps, the unreacted silanol groups on the surface of the silica gels were blocked by reaction with an endcapping reagent. The silanol blocking reaction was performed by refluxing approximately 10 g of the silylated silica gels in 50 mL of *n*-decane with a stoichiometric excess of endcapping reagent such as 8 mL of hexamethyldisilazane (HMDZ). After the mixture was refluxed for 16 h, the silica gels were filtered and washed repeatedly with toluene, dichloromethane, THF, methanol and dried under vacuum at 80 °C for 8 h. The modification process yielded a modified silica with a ligand surface concentration of 3.52 μmol · m^{-2}. The carbon, hydrogen and nitrogen percentages determined by elemental analysis were 25.49, 4.64 and 1.45%, respectively. The concentration of the organic groups attached to the silica surface was calculated from the carbon percentages and the BET surface area of the bare silica. At least three batches were made for each material. Microanalyses are in good agreement with the proposed formulation.

2. 8. Chromatographic Measurements

The resulting bonded phase was packed into two individual stainless steel tubes (150 × 4.6 mm I.D.) by conventional high pressure slurry packing procedures. The columns were used for evaluation of the chromatographic performance. The HPLC system used was a model Agilent 1100 series (Palo Alto, CA, USA) consisting of an Agilent 1100 in-line degasser, an Agilent 1100 autosampler, an Agilent 1100 column thermostat set to 30 °C, an Agilent 1100 quaternary pump, and an Agilent 1100 variable wavelength detector. Agilent Chemstation (version 9.01) was used for data acquisition and analysis. Uracil was used as a void volume marker.

3. Results and Discussuon

Within the field of reversed-phase chromatography, silica-based ODS bonded phases remain among the most widely used of all of the available stationary phases. However, despite the widespread popularity and acceptance of ODS phases, there seems to remain a need, or at least an opportunity, for alternative column chemistries. Two types of newer stationary phases that are gaining popularity are the polar-embedded and the polar-endcapped stationary phases. These phases are modifications of classical C18 chemistry with the addition of a polar functional group, such as an amide or carbamate group, within the alkyl chain itself, or a polar functional group used as an endcapping agent (Fig. 1). Perhaps due to the relatively recent introduction of these types of phases, there have been few studies that have attempted to critically evaluate or characterize their performance. These polar-modified phases are reputed to offer certain advantages such as stability under highly aqueous conditions, improved peak shape for basic compounds, and unique selectivity compared to conventional C18 phases.

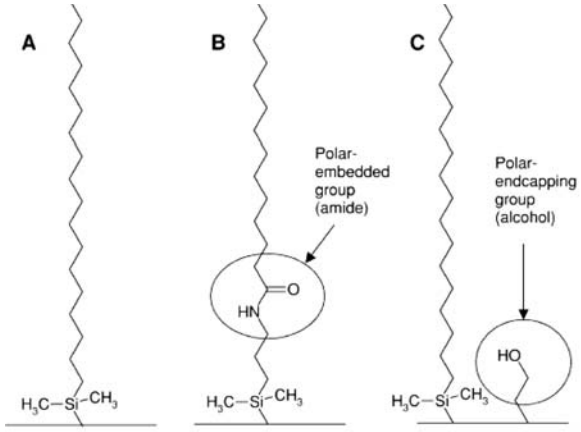

Figure 1. Representative structures for (A) a conventional ODS phase, (B) a polar-embedded phase, and (C) a polar-endcapped phase.

In order to characterize the fundamental chromatographic qualities of these stationary phases, we ran a series of test methods including hydrophobic selectivity, hydrogen bonding capacity, silanol activity at different pHs. These tests involve difficult acidic and basic probe molecules, which clearly indicate the presence or absence of metals and residual silanols. The conditions of the tests cover a broad range of pH and hydrophobicity, indicating the performance of columns for a wide variety of analyses.

3. 1. Hydrophobic Selectivity

The hydrophobic selectivity (methylene selectivity) of columns is defined as the ability of a phase to distinguish between two compounds based upon a single methylene unit substitution, and is determined by injecting a series of *n*-alkylbenzene homologues. For such compounds a linear relationship exists between retention and number of methylene group, $\ln k' = A + B\ \alpha$, where B is the number of methylene groups in alkylbenzene, α gives the slope of the linear relationship, and A gives the intercept value corresponding to the hypothetical retention ($\ln k'$) for benzene. The plots of $\ln k'$ versus B are linear for some limited range of the alkyl chain length. A plot of $\ln k'$ versus the number of carbons in each homologous series for each of the columns is linear. The difference in retention for any given compound between columns is representative of overall hydrophobic interaction associated with that column. The α values next to each pair of column plots are the difference in slope between the two homologous series. Each column gave a linear relationship according to this equation for each of homologous series. The position of each plot against the *y*-axis gives useful comparative information of the overall hydrophobic retention properties of the column and phase. The slope of the regression through the data points of a plot of $\ln k'$ versus number of methylene units was used as the methylene selectivity value (Fig. 2).

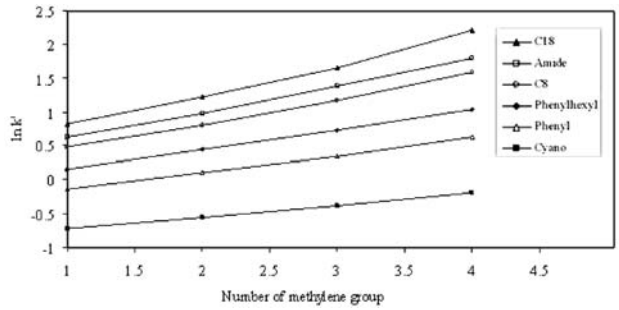

Figure 2. Correlation of $\ln k'$ with number of methylene groups in alkylbenzene probes.

The polar-modified phases exhibited both retention and selectivity differences compared with their alkyl counterparts (Fig. 3). The hydrophobic selectivity values of

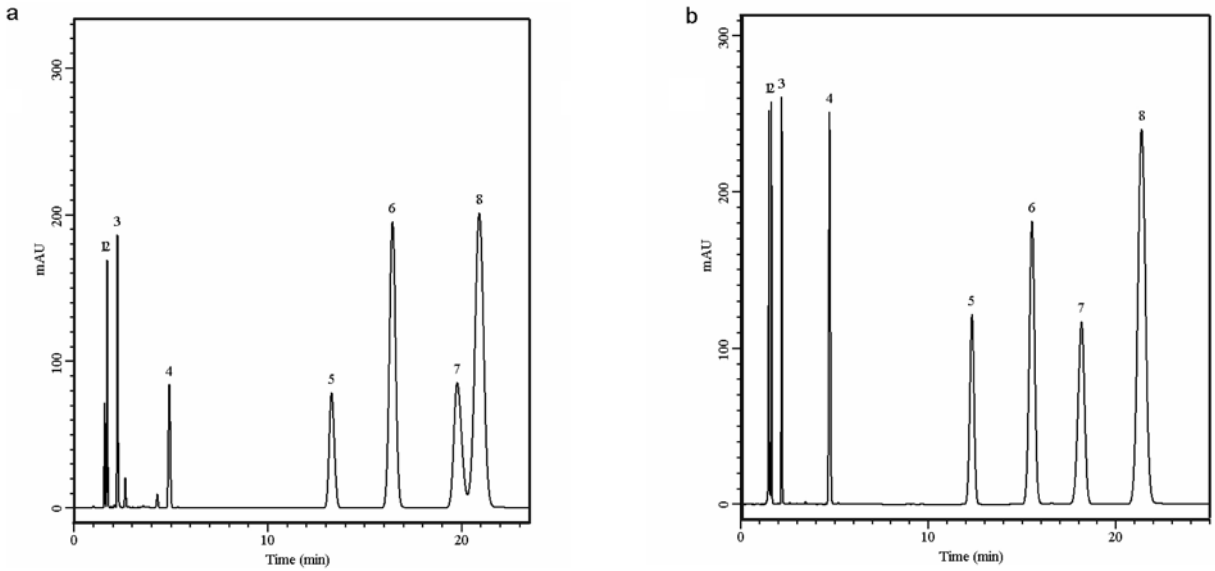

Figure 3. Chromatographic separation of neutral analytes using (a) ODS prepared and (b) polar-modified columns. Chromatographic conditions: mobile phase, MeCN:H_2O, 60:40; flow rate, 1 mL · min^{-1}; detection, UV 254 nm. Peaks: 1 – uracil, 2 – caffeine, 3 – phenol, 4 – toluene, 5 – butylbenzene, 6 – *o*-terphenyl, 7 – amylbenzene, 8 – triphenylene.

butylbenzene and amylbenzene demonstrate the good methylene group selectivity of the bonded phases. The hydrophobic selectivity values of *o*-terphenyl and triphenylene demonstrate a good steric selectivity of phases. The low selectivity values of caffeine and phenol show a negligible amount of the hydrogen bonding interaction (Table 1).

Table 1. Selectivity of ODS and polar-modified columns.

Column	α (hydrogen bonding)	α (hydrophobicity)	α (steric)
C18	0.25	1.56	1.25
polar-modified	0.23	1.51	1.45

3. 2. Base Deactivation Property

Pyridine has often been used to evaluate the column properties because pyridine interacts more strongly with the residual silanol groups than aniline and its derivatives.[1,10] The retention of pyridine is affected by even a small number of residual silanol groups.[11–14] Pyridine was employed in this study to evaluate the effect of the residual silanol groups on the columns prepared. Phenol was used as the reference. The relative retention value ($\alpha_{PhOH/Py}$) was used for the evaluation of the effectiveness of endcapping. The columns prepared

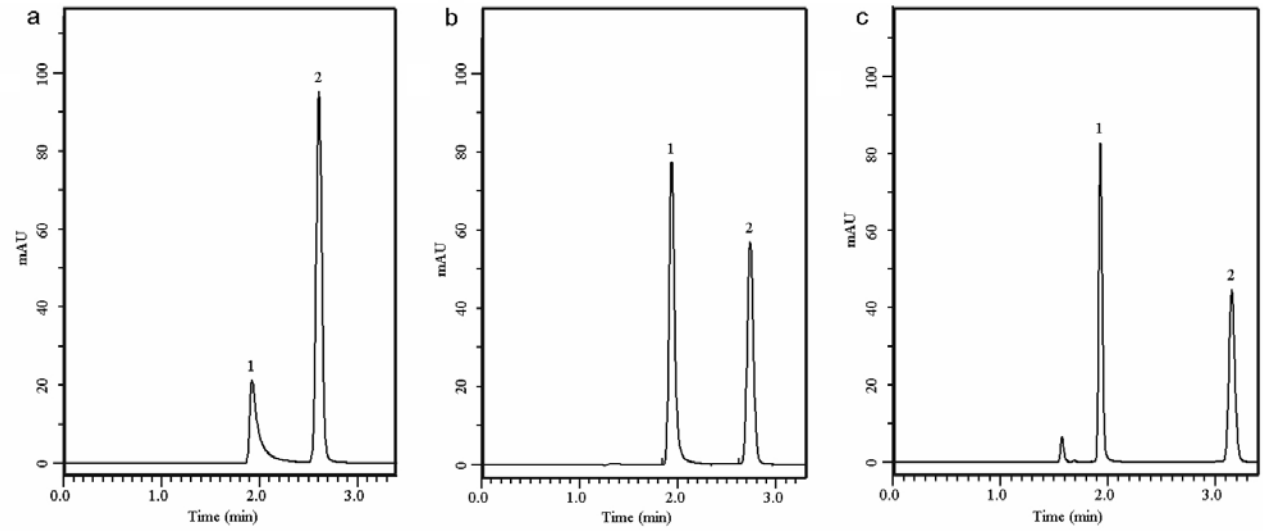

Figure 4. Chromatograms of pyridine and phenol on (a) Symmetry C18 (b) ODS prepared (c) polar-modified columns. Chromatographic conditions: mobile phase, acetonitrile:water, 1:1; flow rate, 1 mL · min^{-1}; detection, UV 254 nm. Peaks: **1** – pyridine, **2** – phenol.

were tested by separating a mixture of pyridine and phenol using acetonitrile-water (1:1, v/v) as the mobile phase. Since acidic mobile phases prevent peak tailing of basic solutes, a mobile phase without a buffer solution may reveal the silanol effect. Therefore, the bonded phases prepared here were evaluated from $\alpha_{PhOH/Py}$ values measured with the mobile phase in the absence of a buffer solution. Figure 4 shows chromatograms of the mixture of pyridine and phenol on the Symmetry C18 and bonded phases prepared. The columns we prepared have a higher separation factor of pyridine and phenol and a lower tailing factor of pyridine than Symmetry C18 column.

3. 3. Hydrogen Bonding and Metal Chelation Activity at pH 2.0

Silanol activity, apart from purely ionic interactions, comprises a number of stationary phase-solute interactions, usually indicated as Van der Waals forces. These interactions may include ion-ion (ion-exchange), ion-dipole, dipole-dipole (*e.g.* hydrogen bonding), dipole-induced dipole, and induced dipole-induced dipole forces. The latter two interaction types particularly depend on the polarisability of the involved solutes and stationary phase. In the past, the effects of active silanols have been eliminated by addition of mobile phase modifiers, endcapping, and utilization of polymers as stationary phase support. On the contrary, sufficiently clean silicas with minimal residual silanol content and no significant amounts of metal impurities contribute to superior chromatographic performance, especially for certain polar analytes such as amine bases. A low pH test with strong chelating analytes such as salicylic acid amply demonstrate the absence of silanophilic activity under different mobile phase conditions. Figure 5 measures hydrogen bonding and metal chelation activity for a selection of highly sensitive compounds at pH 2.0. Hydrogen bonding capacity is a measurement of the amount of accessible hydrogen bonding sites of silanol groups on the silica surface after bonding and is determined either from the selectivity value between caffeine and phenol in neutral pH conditions or from the asymmetry value of sorbic acid in low pH conditions. The peak asymmetry factor of sorbic acid, a probe for the silanol hydrogen bonding activity, is excellent, indicating a negligible amount of silanol activity on these columns. Metal impurities may influence the properties of reversed-phase liquid chromatography phases drastically. Metal contamination may enhance the acidity of silanols, polarity and chelate formation especially for biomolecules. The metal chelating interaction can be determined from the asymmetry value of salicylic acid in low pH conditions. The specifications for retention and peak shape indicate the lack of metal contamination in the silica and provide further assurance of reproducible chromatography. The peak asymmetry factor of salicylic acid, a probe for the metal chelation activity, is very good indicating negligible amount of metal chelation activity on these columns.

3. 4. Basic Drugs at Neutral pH

Since their discovery in the late 1950s, the tricyclic antidepressants (TCAs) have been considered the most effective agents for treating moderate to severe depression.[15–17] The group name is derived from these molecules' dibenzoazepin or dibenzocycloheptane tricyclic ring structures, but all of these compounds also are secondary or tertiary amines, whose basic character is reflected by pK_a values ranging from 8.2 to

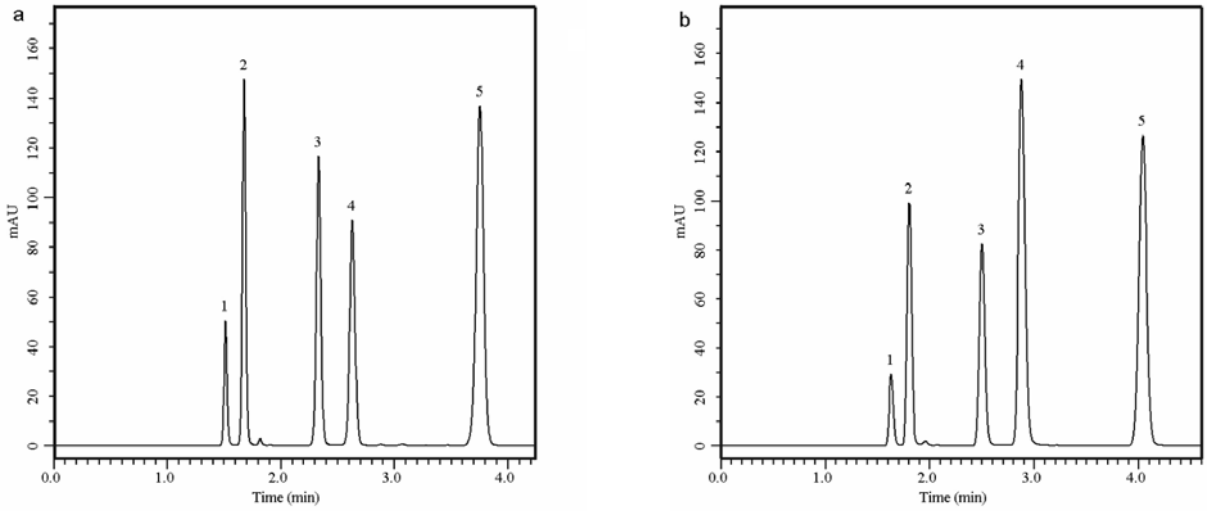

Figure 5. Silanophilic acidic probes on (a) ODS prepared and (b) polar-modified columns. Chromatographic conditions: mobile phase, MeCN:H_2O, 1:1, plus 0.1% formic acid; flow rate, 1 mL · min^{-1}; detection, UV 254 nm. Peaks: **1** – uracil, **2** – caffeine, **3** – sorbic acid, **4** – salicylic acid, **5** – propylparaben.

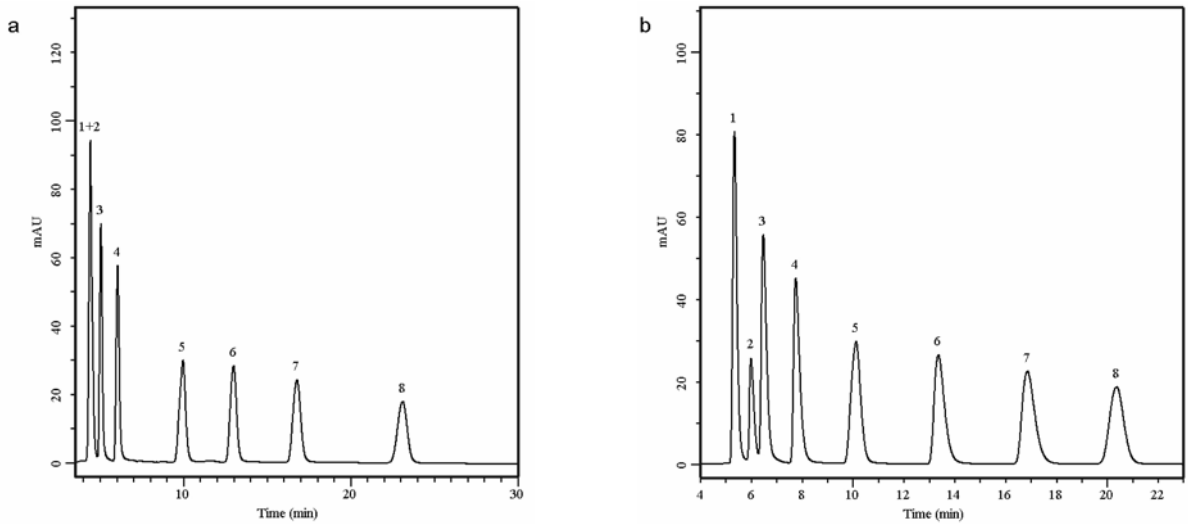

Figure 6. Separation of tricyclic antidepressants at pH 7.0 on (a) ODS prepared and (b) polar-modified columns. Chromatographic conditions: mobile phase, MeCN:MeOH:10 mM phosphate buffer, pH 7.0, 1:1:1; flow rate, 1 mL min^{-1}; detection, UV 254 nm. Peaks: **1** – desmethyldoxepin, **2** – protriptyline, **3** – desipramine, **4** – nortriptyline, **5** – doxepin, **6** – imipramine, **7** – amitriptyline, **8** – trimipramine.

9.4. We have investigated the applicability of C18 and polar-modified reversed phase columns for analyses of TCAs without mobile phase additives under different pH conditions. The chromatogram shows excellent peak symmetries and efficiencies for TCAs from a C18 column under acidic conditions. At acidic pH, the amine groups of the TCA structure are highly protonated, and the analytes will be eluted more quickly than the weakly protonated moieties present at higher pH. However, at neutral pH, TCAs undergo significant ion exchange interactions with residual silanols on the silica backbones of reversed phase packings, and are problem drugs for HPLC analysis. Reversed-phase HPLC on C18, C8, cyano, and phenyl phases is most widely used for analyses of TCAs. In most cases, mobile phases with basic additives have been needed to reduce peak tailing. An eight-component mix on ODS and polar-modified columns at pH 7.0 was studied without additives (Fig. 6). The polar-modified column provides a quantifiable separation of all components. Of particular interest is the separation of protriptyline from desipramine, compounds which differ by only one double bond in the central ring.

Polar-modified phases show dual selectivity benefits. The separation of a complex mixture of tricyclic antidepressants, having both hydrophobic and polar characteristics, demonstrates their unique selectivity, and the power of stationary phase manipulation in HPLC method development. The polar-modified phases seem to impart a unique selectivity with good separation not observed in ODS phases. The peak symmetries and efficiency values obtained for these drugs, which are considered to be difficult HPLC candidates, furnish a good measure of performance of these columns for similar problem drugs compared to ODS columns.

3. 5. Selectivity Differences

Tricyclic antidepressants are used to treat depression. They are also used to treat some other conditions such as migraine, panic disorder, obsessive compulsive disorder, recurrent headaches, and some forms of pain. Structurally related benzodiazepines are widely used in neurological and psychiatric disorders as antiepileptic, hypnotic, or sleep-inducing drugs. Benzodiazepines, antidepressants, and their combinations are primary medications used today for panic disorder. The separation in Figure 7 demonstrates a challenging pharmaceutical mixture run on ODS prepared and commercially available C18 columns. Superior base-line resolution and outstanding peak symmetry of these polar compounds is observed on ODS prepared whereas the commercially available columns exhibit coelution, incomplete resolution, and peak tailing.

Catecholamines are polar compounds occurring naturally in the body serving the function of neurotransmitters and hormones. These polar compounds are extremely difficult to retain and separate using reversed-phase chromatography, which is the most common means of separation in analytical HPLC. Traditional options for retaining polar analytes include ion pairing, manipulation of mobile phase pH to neutralize charged compounds (utilize pH extremes) or RP phases specifically designed for enhanced polar retention. While offering a potential cure for difficult separations, ion pairing agents are often MS-incompatible and pH extremes can affect some compounds. Polar-modified phases offer an alternative for polar compounds and work well with a wide variety of unmodified mobile phases, both hydrophobic and polar. These phases provide a novel separation mechanism unavailable with hydrophobic phases allowing the chromatographer to avoid troublesome additi-

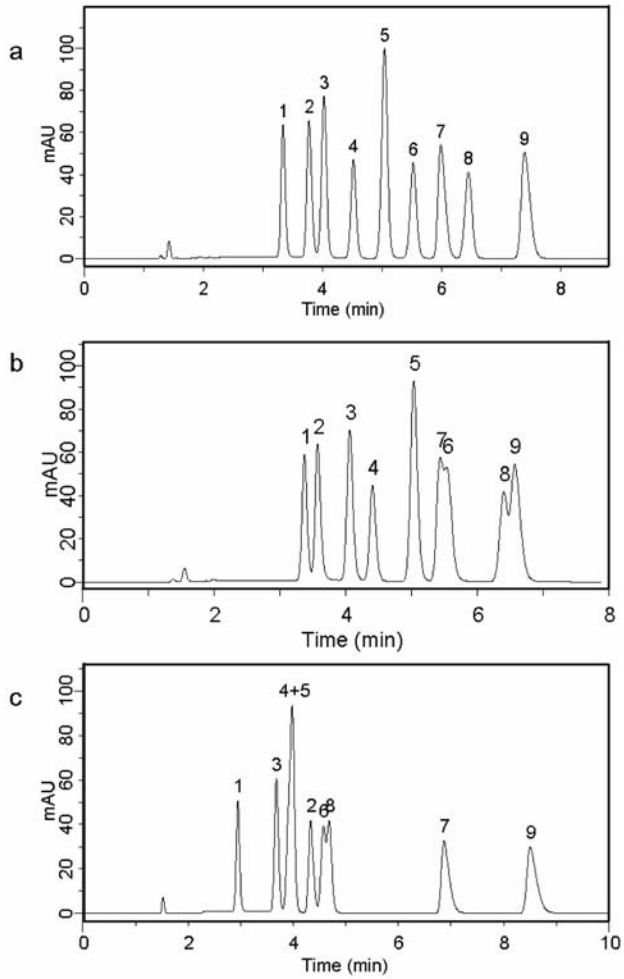

Figure 7. Chromatographic separation of tricyclic antidepressants and benzodiazepines on (a) ODS prepared (b) Luna C18 (2) and (c) XBridge C18 columns. Chromatographic conditions: mobile phase, 0.1% TFA in MeCN:0.1% TFA in H_2O, 40:60; flow rate, 1 mL · min^{-1}; detection, UV 254 nm. Peaks: **1** – nitrozepam, **2** – nordoxepin, **3** – alprazolam, **4** – diazepam, **5** – oxazepam, **6** – triazolam, **7** – nortriptyline, **8** – clonazepam, **9** – trimipramine.

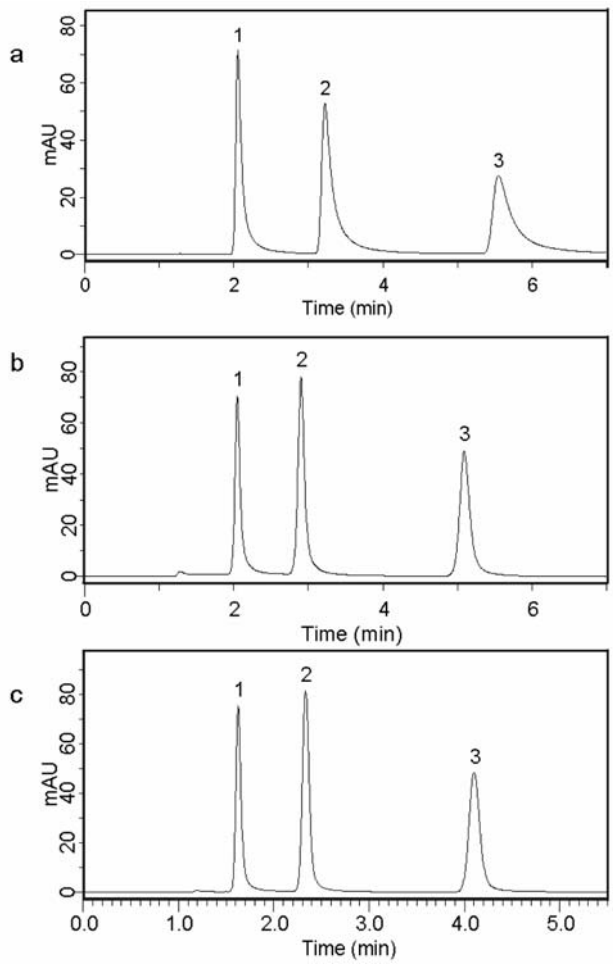

Figure 8. Chromatographic separation of catecholamines in 100% aqueous mobile phase conditions on (a) Symmetry C18 (b) ODS prepared and (c) polar-modified columns. Chromatographic conditions: mobile phase, 20 mM phosphate buffer, pH 7.0; flow rate, 1 mL · min^{-1}; detection, UV 270 nm. Peaks: **1** – norepinephrine, **2** – epinephrine, **3** – dopamine.

ves and pH extremes. Figure 8 shows chromatograms of the three catecholamines on the Symmetry C18 and bonded phases prepared. The polar-modified column provides a noticeable reduction in retention time and improvement in peak shape.

The polar-modified phases demonstrate differences in selectivity and better separation characteristics for phenol derivatives compared to traditional alkyl phases. A mixture of nine compounds was chromatographed on ODS and polar-modified columns using 0.1% formic acid and acetonitrile mixture as the mobile phase (Fig. 9). A dramatic differences in peak order and run time are observed on ODS and polar-modified columns. The unique selectivity of polar-modified columns provides a superior separation of phenol derivatives compared to standard reversed phase chemistries. Data demonstrates that there is not only a significant selectivity difference, but also a reversal in the elution order of 2-nitrophenol and 4-nitrophenol between ODS and polar-modified phases.

4. Conclusions

We attempted to characterize and compare the behavior of commercially available C18, ODS and polar-modified phases prepared in this work using a series of diagnostic probes and several generic applications. Relative to C18 phases, polar-endcapped phases were typified by equivalent hydrophobic interaction capacity, enhanced hydrogen bonding and silanol activity. The polar-embedded phases were typified by significantly reduced hydrophobicity, reduced hydrogen bonding capacity and silanol activity, and a unique selectivity due to ion exchange or dipole interaction with polar embedded groups.

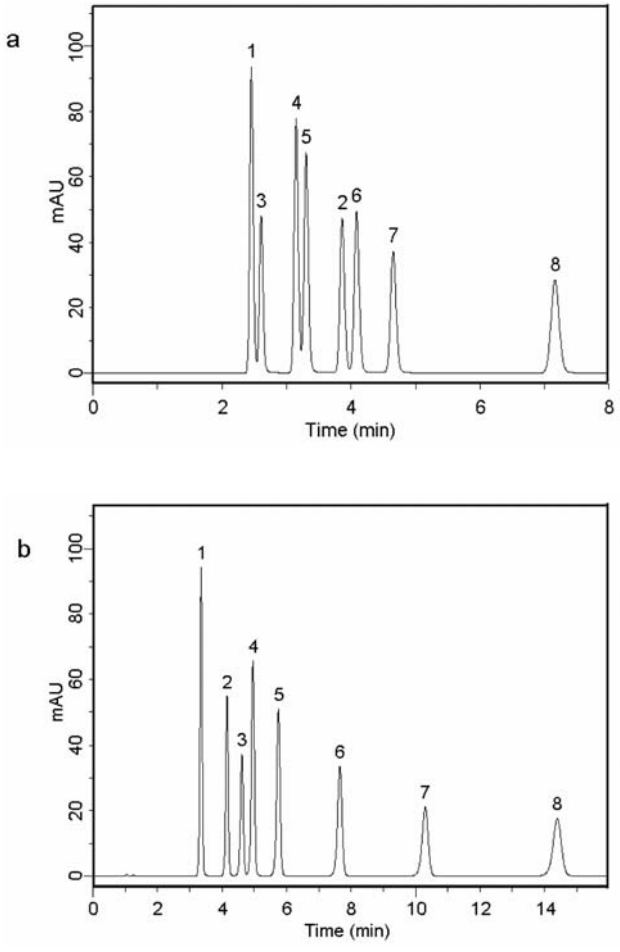

Figure 9. Separation of phenol derivatives on (a) ODS prepared and (b) polar-modified columns. Chromatographic conditions: mobile phase, 0.1% HCOOH in acetonitrile:0.1% HCOOH in H_2O, 55:45; flow rate, 1 mL · min^{-1}; detection, UV 280 nm. Peaks: **1** – phenol, **2** – 2-nitrophenol, **3** – 4-nitrophenol, **4** – 2-chlorophenol, **5** – 4-chlorophenol, **6** – 4-chloro-3-methylphenol, **7** – 2, 4-dichlorophenol, **8** – 2, 4, 6-trichlorophenol.

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6. References

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Povzetek

Izbira kolon za reverzno fazno tekočinsko kromatografijo ni enostavna. V predstavljeni raziskavi smo primerjali in ovrednotili lastnosti običajnih C18 faz s polarno modificiranimi fazami in našli izrazite razlike v kromatografskih karakteristikah med obema skupinama, kakor tudi visoko stopnjo spremenljivosti v vsaki skupini. Dodatno zaščitene polarne faze imajo podobno hidrofobno ločljivost, višjo kapaciteto vezave vodika in silanolno kapaciteto v primerjavi z navadno C18 kolono. Vtisnjene polarne faze kažejo zelo zmanjšane hidrofobne lastnosti in pomembno zmanjšano silanolno aktivnost v primerjavi z navadno C18 kolono. Ionske in/ali dipolne interakcije imajo pomembno vlogo pri polarno modificiranih fazah, ki se kaže pri retenciji alkalnih in kislih analitov.