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Solute attributes and molecular interactions contributing to "U-shape" retention on a fluorinated high-performance liquid chromatography stationary phase

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Abstract

The structural attributes and molecular interactions contributing to "U-shape" retention on pentafluorophenylpropyl (PFPP) HPLC stationary phases are systematically investigated. Only basic analytes exhibit retention that increases with the acetonitrile content in mixtures of acetonitrile and aqueous ammonium acetate, with some basic analytes not eluting at all from PFPP columns using 100% acetonitrile. U-shaped retention as a function of mobile phase acetonitrile content was more dramatic on a PFPP column relative to C_{18}. Retention of the quaternary ammonium salt bretylium on these stationary phases and on the same bare silica support showed minimal influence of ion-exchange mechanisms on the C_{18} phase, however, a significant influence of ion-exchange mechanisms was observed for both PFPP and bare silica. The retention of bretylium on PFPP was only slightly less than on bare silica. These findings suggest ion-exchange mechanisms dominate retention of basic analytes in the high acetonitrile realm on PFPP. The PFPP stationary phase exhibits a substantial increase in effects of ionized surface silanol groups compared to the alkyl phase despite similar surface coverage. Retention of some basic analytes on a PFPP phase was enhanced relative to retention on silica alone, and implicates other dispersive interactions that might be exploited for selectivity different from either alkyl phases or silica alone.

Keywords: Liquid chromatography; Mass spectrometry; Pentafluorophenylpropyl; Stationary phase; Fluorinated; Retention mechanisms; Ion-exchange

1. Introduction

Analyses of pharmaceuticals, their metabolites, and complex mixtures of endogenous metabolites (metabolomics) are growing in importance in both drug development and in fundamental studies of cellular responses to genetic or environmental perturbations. The combination of high-performance liquid chromatography (HPLC or LC) and mass spectrometry (MS) has become the dominant analytical tool in analysis of pharmaceuticals and metabolites [1]. LC–MS, however, suffers from serious limitations in analysis of polar, low-molecular-mass (<500) analytes, which are often poorly retained on common HPLC stationary phases [2]. Inadequate chromatographic retention and resolution can result in significant suppression or enhancement of ionizations that can cause poor quantitation in LC–MS analyses [3]. To achieve retention of polar ionic solutes, ion-pair reagents are often added to mobile phases. These reagents, however, are generally non-volatile and suppress ionization in LC–MS experiments. Other separation techniques such as capillary electrophoresis (CE) are also suitably employed for retention and separation of ionic analytes, but the predominantly aqueous solvents and non-volatile buffers employed are less amenable to MS interfacing than many liquid chromatographic systems. It is, therefore, desirable to design HPLC stationary phases capable of retaining polar analytes using mobile phase constituents compatible with mass spectrometric analysis.

Fluorinated, silica-based stationary phases have shown unique retention for small, polar analytes [4–6]. In particular, pentafluorophenylpropyl (PFPP) phases exhibit both reversed- and normal-phase retention for polar analytes,

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which has shown dependence on mobile phase composition. At lower percentages of organic modifier (herein referred to as the reversed-phase region), solute retention resembles that of classical reversed-phase systems. At higher percentages of organic (herein referred to as the normal-phase region), however, behavior more typical of normal-phase separations is observed with increasing proportions of organic modifier. This combination of reversed- and normal-phase behavior forms a "U-shape" relationship between retention and organic modifier percentage. The normal-phase behavior is observed using mobile phase components common to reversed-phase LC that are highly compatible with mass spectrometry [4–7]. Although this "U-shape" retention has been observed on traditional alkyl columns [8], the magnitude of normal-phase retention is dramatically increased using the PFPP stationary phase [5]. Nahum and Horváth attributed the irregular retention profiles using the alkyl columns to a dual retention mechanism involving both silanophobic and silanophilic interactions [9]. To date, the retention mechanisms responsible for the enhanced normal-phase behavior on PFPP phases and the fundamental properties of analytes that exhibit this phenomenon remain unclear.

Stationary phases containing perfluorinated functional groups are available from many column manufacturers and are best known for their use in separating taxanes [10,11]. Due to unique selectivity of the fluorinated phases, they are becoming more widely used as an alternative to traditional alkyl systems [12]. Sadek et al. used linear solvation energy relationships (LSERs) to demonstrate selectivity differences for polar, nonionizable analytes on perfluorinated supports compared to hydrocarbon bonded phases [13]. The small differences they observed in dispersive and polar interactions on fluorinated phases in comparison to alkyl and phenyl stationary phases, however, do not appear to adequately describe the dramatic differences in retention observed for ionizable analytes using these same columns. More recently, perfluorinated stationary phases have shown alternative retention and selectivity in several column classification studies. For example, Neue et al. demonstrated differences in "extended polar selectivity" and "phenolic selectivity" between fluorinated and traditional alkyl phases [14]. The Neue study grouped the selectivity of fluorinated phases as distinct from C_{18} and cyanopropyl phases, however, rigorous studies to elucidate the interactions responsible for this observation were not reported. In a related column classification study, Euerby and Petersson investigated 135 commercially available stationary phases [12]. The authors observed significant differences in analyte shape selectivity for pentafluorophenyl stationary phases. The unique retention and selectivity of the fluorinated phases in the Euerby study prompted further investigation [15]. In this latter study, the authors report orthogonal selectivity of fluorinated phases as compared to phenyl and alkyl phases, especially in the analysis of basic analytes. They also report high retention factors for basic analytes in mobile phases containing in excess of 80% acetonitrile concluding that the retention appears to involve ion-exchange mechanisms. Rationale for the existence of such mechanisms of retention, however, was not given.

To best utilize the retention characteristics of various analytes using the fluorinated phases, recognition of the dominant retention mechanisms and analyte/support properties that govern retention are paramount. This research was designed as a systematic investigation of analyte structure and stationary phase chemistry to elucidate interactions contributing to retention at high organic modifier percentages. Since ion-exchange with surface silanols was initially implicated, further studies were conducted to explore the ion-exchange properties of the PFPP phase and the fundamental reasons for its existence. The availability of surface ionized silanol groups on PFPP, bare silica and C_{18} phases were assessed by measuring the retention of bretylium ion as a function of mobile phase pH. The results demonstrated that the PFPP stationary phase allows analytes to interact with the surface in a similar manner to bare silica, whereas alkyl phases inhibit ionic interactions. The rationale for these observations in terms of differential surface solvation is discussed.

Contributions to retention other than that due to ion-exchange were also addressed. Preferential retention of basic analytes on PFPP compared to bare silica indicates significant contributions from non-ionic retention mechanisms in accordance with a hydrophobically assisted ion-exchange mechanism as proposed by Neue et al. [16] and Carr and co-workers [17]. Selectivity and peak shape issues are also discussed.

2. Experimental

2.1. Reagents and standards

All compounds chosen for the retention studies were obtained from Sigma (St. Louis, MO, USA) with the exception of progesterone (Aldrich, Milwaukee, WI, USA). Separate stock solutions of each analyte were prepared by dissolving a weighed amount of each compound in methanol to obtain concentrations of 1 mg/mL. Stock solutions were stored at 0–4 °C when not in use. Samples for analysis were prepared by diluting stock solutions with the respective buffer for the study to a final concentration of 100 μg/mL. All HPLC reagents were obtained from Aldrich except acetic acid (J.T. Baker, Phillipsburg, NJ, USA) and were of HPLC grade or better and were used without further purification. HPLC-grade water used throughout the study was obtained from a Nanopure Diamond (Barnstead, Boston, MA, USA) source.

2.2. HPLC columns, conditions and apparatus

Discovery HS F5 (pentafluorophenylpropyl bonded), Discovery HS C_{18} and a bare silica column packed with the same proprietary silica used to manufacture the Discovery line of columns were obtained from Supelco (Bellefonte, PA, USA). The columns, packed with 5 μm particles with surface area of 300 m^2/g were 50 mm in length and had 4.6 mm inter-

Table 1
Comparison study column characteristics

Stationary phase	Bonded phase	Particle size (μm)	Surface area (m^2/g)	Pore size (Å)	Coverage (μmol/m^2)	Carbon (%)
Discovery HS F5	Pentafluorophenylpropyl-endcapped	5	300	120	4.0	12
Discovery HS C18	Octadecyl-endcapped	5	300	120	3.8	20
HS silica	None	5	300	120	None	None

nal diameters. The available physical characteristics of the columns are shown in Table 1. Both the PFPP and C_{18} stationary phases are prepared using monofunctional silanes. The same proprietary leaving groups and similar catalysts, solvents and apparatus are used in both manufacturing processes. The columns were chosen for the study to eliminate potential contributions toward retention from differing silica supports.

Retention data were obtained using a Hewlett-Packard (Palo Alto, CA, USA) 1100 series HPLC system equipped with a quaternary pump, autosampler, column temperature controller and a variable-wavelength UV detector. Acquisitions were made using ChemStation software version A.06.01. All pH measurements were carried out using a Corning (Corning, NY, USA) Model 440 pH meter, which was calibrated using standard buffer solutions prior to each use.

Mobile phases employed in the retention profile studies comprised of 10 mM ammonium acetate either adjusted to a pH of 4.0 with acetic acid or unmodified (pH 6.7) and varying percentages of acetonitrile. Mobile phase compositions ranging from 40% to 90% acetonitrile were achieved by mixing in-line using the quaternary pump. Retention data were acquired in triplicate using 25 μl injections, a flow rate of 1 mL/min, a column temperature of 35 °C, and UV detection at 220 nm. System hold-up time (t_0) was estimated by monitoring the first signal disturbance upon injection. Although this is not considered a rigorous measure of hold-up time, the possible retention of traditional t_0 markers such as uracil on the polar phases precluded their use. Hold-up times were consistent within column and mobile phase conditions and varied only slightly with changes in mobile phase composition and column chemistry. The range of hold-up times across all columns and conditions was determined to be 0.49–0.67 min.

For the pH dependence studies, bretylium tosylate was prepared at 1 mg/mL in methanol. Mobile phases ranging in pH were prepared such that the ammonium ion concentration was held constant at 25 mM. For each buffer, 25 mL of a 1 M stock solution of ammonium hydroxide was added to approximately 850 mL of HPLC grade water. The pH of each solution was adjusted to 2, 3, 6, 7, and 8 with concentrated phosphoric acid (J.T. Baker) and 4 and 5 with glacial acetic acid (J.T. Baker), followed by dilution to 1 L with water. The mobile phases consisted of buffer–acetonitrile (80:20) and were proportioned in-line. A flow rate of 1 mL/min, injection volume of 2 μL, and a UV wavelength of 220 nm was used throughout the study. The column temperature was maintained at 35 °C. Retention data at each pH level were obtained at least in duplicate for the three phases investigated.

3. Results and discussion

3.1. Solute attributes contributing to "U-shape" retention

Acid dissociation and octanol–water partition coefficients for the compounds chosen for the study are listed in Table 2, and their structures are presented in Fig. 1. The compounds used are representative of common pharmaceutical acidic, basic and neutral molecules. The retention for each of the analytes was measured in triplicate from 40% to 90% acetonitrile in 10% increments at both pH 4 and 6.7 using PFPP and C_{18} as stationary phases. The capacity factor, k', was calculated using the following equation:

$$k' = \frac{t_R - t_0}{t_0} \quad (1)$$

where t_R and t_0 are the retention time of the analyte and column hold-up time, respectively.

Table 2
Analyte ionization and octanol–water partition coefficients

Class	Compound	pK_a	log P
Bases	Amitriptyline[a]	9.4	4.92
	Nortriptyline[b]	9.7	4.28
	Diphenhydramine[a]	8.98	3.27
	Verapamil[a]	8.92	3.79
	Alprenolol[c]	9.7	3.1
	Lidocaine[a]	8.01	2.44
Neutrals	Hydrocortisone[a]	N/A	1.61
	Hydrocortisone acetate[a]	N/A	2.19
	Progesterone[a]	N/A	3.87
	Corticosterone[a]	N/A	1.94
	Cortisone acetate[a]	N/A	2.1
	Prednisone[a]	N/A	1.46
Acids	Diclofenac[a]	4.15	4.51
	Ibuprofen[a]	4.91	3.97
	Aspirin[a]	3.49	1.19
	Naproxen[a]	4.15	3.18
	Ketoprofen[a]	4.45	3.12
	Piroxicam[a]	6.3	3.06

[a] Denotes data taken from SRC PhysProp Database.
[b] Denotes data taken from Japanese Drug Database (http://chrom.tutms.tut.ac.jp/JINNO/DRUGDATA/55nortriptyline.html).
[c] Denotes data taken from [29].

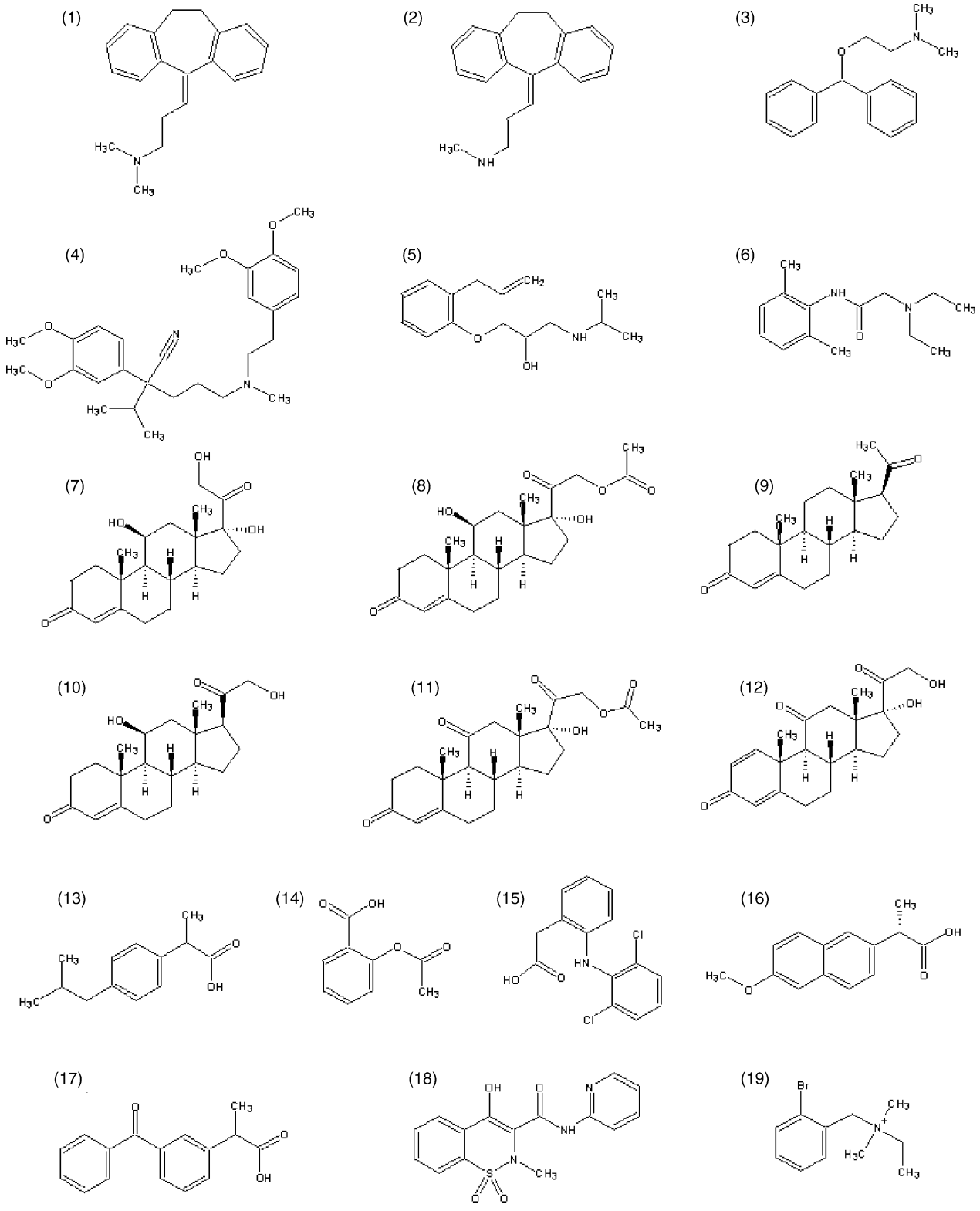

Fig. 1. Structures of analytes used in the study: 1, amitriptyline; 2, nortriptyline; 3, diphenhydramine; 4, verapamil; 5, alprenolol; 6, lidocaine; 7, hydrocortisone; 8, hydrocortisone acetate; 9, progesterone; 10, corticosterone; 11, cortisone acetate; 12, prednisone; 13, ibuprofen; 14, aspirin; 15, diclofenac; 16, naproxen; 17, ketoprofen; 18, piroxicam; 19, bretylium.

The capacity factors obtained for the probes at pH 4 and pH 6.7 for both stationary phases are shown as a function of percent acetonitrile in Table 3. Comparison of the retention profiles at pH 4 shows that $k'_{PFPP} \gg k'_{C18}$ for all basic compounds. The C_{18} phase exhibits slightly greater retention for both neutral and acidic probes, where the average increase in k' is 1.5-fold and 0.8-fold for neutrals and acids, respectively, at both pH conditions. On both the PFPP and C_{18} systems, the acidic and neutral species exhibit classical reversed-phase behavior in that k' monotonically decreases with increasing acetonitrile content. In contrast, basic analytes, with the exception of lidocaine and verapamil, show an increase in retention at both low and high organic on both phases forming a "U-shape" retention profile. With the exception of lidocaine, the k' for basic analytes ranges from about 15 to 35 times greater on the PFPP phase compared to C_{18} at pH 4. The k' for verapamil reaches a minimum and levels off between 70% and 90% acetonitrile, whereas lidocaine retention is observed to continually decrease at high organic mobile phase percentages. The rationale for the observed lidocaine and verapamil retention will be discussed in sections to follow.

Selectivity differences are observed for both phases between the reversed-phase and high organic regions of the profiles. On the PFPP phase at 40% acetonitrile, the elution order for the basic probes is lidocaine, alprenolol, diphenhydramine, nortriptyline, amitriptyline, and then verapamil. At 90% acetonitrile, however, the elution order changes to lidocaine, verapamil, alprenolol, diphenhydramine, amitriptyline, and then nortriptyline. This difference indicates that the relative importance of the various potential interactions has changed between the two regions. The order of retention at 40% acetonitrile monotonically increases with the literature octanol–water partition coefficient ($\log P$) values, showing the expected importance of dispersive interactions under reversed-phase conditions. At high organic percentages the retention monotonically increases with pK_a values. This latter observation implicates the relative importance of ionic interactions at high organic mobile phase compositions. The difference in the two regions is clearly seen when the retention of structurally related amitriptyline and nortriptyline are compared. The more hydrophobic amitriptyline is preferentially retained by 28.5% in k' at 40% acetonitrile, whereas the more polar nortriptyline is retained 12.1% more at 90% acetonitrile. The observation that only bases exhibit normal-phase retention and that the selectivity is at least partially based on the degree of ionization of the basic compounds suggests that ionic interactions are of great importance as the acetonitrile fraction approaches 100%. Further studies aimed at quantifying the relative importance of the mechanisms of interaction using LSER models are underway.

The presence of ionic interactions implies that the pH of the mobile phase may have a significant impact on the retention in the normal-phase region. For this reason, the retention for all of the analytes was monitored using mobile phases prepared using ammonium acetate at near neutral pH (see Table 3). For brevity, the capacity factors obtained for only the basic probes at pH 6.7 for both stationary phases are plotted as a function of percent acetonitrile in Fig. 2. Comparison of the retention profiles at pH 6.7 shows that the neutral and acidic probes (not shown) again exhibit classical reversed-phase retention on both phases. With the exception of lidocaine, the basic analytes are shown to preferentially retain by an average factor of 20 on the PFPP phase when compared to the C_{18} system across the entire range of mobile phase compositions. In addition, the bases exhibit "U-shape" retention profiles on both phases. Verapamil and lidocaine once more are the exceptions.

Table 4 provides a comparison of capacity factors obtained for the basic analytes on PFPP at the two pH conditions employed at 90% acetonitrile. For all analytes that exhibit normal-phase behavior, retention is shown to increase by 10–60% at the higher pH value. This effect is attributed to the greater extent of silanol ionization at the higher pH, and provides further evidence of the importance of ionic interactions. Based on the literature, aqueous-based pK_a values (Table 2), the fraction of analyte molecules protonated at both pH values is greater than 99% for all of the bases except lidocaine. The increase in retention, based solely on ion-exchange, must therefore involve another change in the system. The ion-exchange process necessarily involves positively charged and negatively charged species. In this case, the negatively charged species is presumed to be ionized surface silanols based on the observation of "U-shape" behavior on the alkyl phase. Although the acidity of surface silanols is not homogeneous, an average pK_a value of approximately 7 has been estimated for some modern silicas [18,19]. Using this value, the percentage of silanol groups in an ionized state at pH 4 and 6.7 is estimated to range from about 1% to 33%, respectively. The increased retention at the higher pH is, therefore, attributed to the increase in fraction of silanols in the ionized state.

The contributions of ion-exchange to retention of basic analytes are expected to derive from the extent of ionization of both surface silanols and analyte. For instance, based solely on silanol ionization, each of the protonated bases would be expected to show a corresponding increase in retention at pH 6.7 versus pH 4. However, verapamil shows an 18% decrease in retention at pH 6.7 on the PFPP column at >80% acetonitrile. Such an effect may be attributed to a combination of decreasing analyte protonation and decreasing silanol ionization at high acetonitrile concentrations. Using the aqueous-based pK_a value for verapamil of 8.92, the analyte is greater than 99% protonated at both pH 4 and 6.7. The pK_a values of bases, however, are known to decrease with increasing organic modifier and the apparent pH of aqueous/organic solvents increase with greater proportions of organic [16,20]. Recent studies in our laboratory (manuscript in preparation) demonstrate that the pK_a of verapamil in 90% acetonitrile is 7.97 and the pH measured following the addition of acetonitrile for the aqueous-based pH of 4 and 6.7 is 6.8 and 8.1, respectively. The result is a decrease in the degree of ion-

Table 3
Capacity factors as a function of percent acetonitrile on PFPP and C18 phase

Analyte	Capacity factor (k') at percent acetonitrile					
	40	50	60	70	80	90
I. PFPP, pH 4						
Amitriptyline	41.66	23.37	17.18	13.81	12.67	16.36
Nortriptyline	32.42	18.66	14.04	11.43	11.04	18.34
Diphenhydramine	23.78	15.64	12.67	10.76	10.62	15.11
Verapamil	48.67	23.81	16.18	11.91	10.20	10.10
Alprenolol	15.28	10.56	9.02	8.01	8.39	13.99
Lidocaine	6.94	6.27	6.20	5.54	3.83	1.93
Hydrocortisone	0.66	0.35	0.31	0.23	0.15	0.03
Hydrocortisone acetate	1.90	0.92	0.61	0.39	0.23	0.00
Progesterone	8.12	3.03	1.66	0.95	0.55	0.28
Corticosterone	1.25	0.63	0.48	0.34	0.23	0.03
Cortisone acetate	2.67	1.17	0.73	0.45	0.26	−0.01
Prednisone	0.78	0.41	0.35	0.26	0.17	0.04
Diclofenac	8.16	3.46	2.00	1.26	0.81	0.66
Ibuprofen	6.52	2.53	1.41	0.81	0.49	0.32
Aspirin	0.98	0.61	0.53	0.45	0.32	−0.05
Naproxen	3.82	1.70	1.10	0.73	0.51	0.44
Ketoprofen	2.84	1.28	0.83	0.52	0.34	0.28
Piroxicam	1.30	0.60	0.37	0.16	−0.02	−0.24
II. C18, pH 4[a]						
Amitriptyline	2.52	1.25	0.74	0.53	0.46	0.99
Nortriptyline	2.17	1.08	0.63	0.45	0.37	0.77
Diphenhydramine	1.18	0.70	0.46	0.36	0.30	0.66
Verapamil	2.31	1.10	0.62	0.44	0.36	0.64
Alprenolol	0.92	0.53	0.38	0.29	0.25	0.55
Lidocaine	0.43	0.37	0.39	0.44	0.56	0.53
Hydrocortisone	1.13	0.67	0.48	0.36	0.25	0.19
Hydrocortisone acetate	3.56	1.71	0.96	0.62	0.39	0.24
Progesterone	23.10	8.99	4.12	2.42	1.41	0.85
Corticosterone	2.33	1.25	0.81	0.58	0.41	0.29
Cortisone acetate	4.45	2.00	1.08	0.67	0.40	0.23
Prednisone	1.12	0.66	0.46	0.34	0.23	0.17
Diclofenac	11.81	4.26	1.80	0.93	0.45	0.14
Ibuprofen	17.40	6.39	2.83	1.54	0.83	0.45
Aspirin	0.33	0.29	0.27	0.23	0.07	0.00
Naproxen	4.72	2.15	1.13	0.70	0.40	0.21
Ketoprofen	4.13	1.88	0.99	0.60	0.34	0.13
Piroxicam	2.35	1.26	0.69	0.40	0.21	0.02
III. PFPP, pH 6.7[b]						
Amitriptyline	*	48.07	29.21	20.76	14.43	19.24
Nortriptyline	*	38.46	24.62	18.93	15.18	29.90
Diphenhydramine	51.88	30.42	20.35	15.49	11.55	16.59
Verapamil	*	43.23	22.92	14.05	8.25	8.26
Alprenolol	33.31	20.81	15.06	12.54	10.75	20.21
Lidocaine	10.16	6.05	3.18	1.95	0.92	0.63
Hydrocortisone	0.94	0.55	0.33	0.37	0.17	0.21
Hydrocortisone acetate	2.40	1.17	0.67	0.40	0.16	0.22
Progesterone	9.51	3.73	1.82	1.08	0.44	0.21
Corticosterone	1.64	0.87	0.53	0.36	0.15	0.23
Cortisone acetate	3.24	1.46	0.79	0.48	0.12	0.23
Prednisone	1.11	0.62	0.35	0.37	0.17	0.22
Diclofenac	2.61	1.23	0.76	0.55	0.09	0.15
Ibuprofen	1.58	0.85	0.56	0.37	0.10	0.16
Aspirin	0.42	0.28	0.05	0.04	^	^
Naproxen	1.06	0.63	0.44	0.34	0.08	0.17
Ketoprofen	0.57	0.39	0.32	0.26	0.03	0.09
Piroxicam	0.41	0.28	0.00	^	^	^

Table 3 (*Continued*)

Analyte	Capacity factor (k') at percent acetonitrile					
	40	50	60	70	80	90
IV. C18, pH 6.7[c]						
Amitriptyline	4.97	2.80	2.09	1.57	1.27	1.55
Nortriptyline	2.52	1.31	1.02	0.77	0.58	0.89
Diphenhydramine	1.99	1.30	0.94	0.77	0.64	0.73
Verapamil	5.75	2.80	1.86	1.16	0.92	0.69
Alprenolol	0.98	0.62	0.60	0.40	0.39	0.60
Lidocaine	4.57	2.79	2.03	1.35	0.95	0.52
Hydrocortisone	0.69	0.45	0.40	0.31	0.23	0.15
Hydrocortisone acetate	2.56	1.30	0.95	0.63	0.35	0.21
Progesterone	17.57	7.16	4.14	2.42	1.56	0.99
Corticosterone	1.64	0.94	0.79	0.57	0.37	0.24
Cortisone acetate	3.22	1.54	1.07	0.68	0.37	0.21
Prednisone	0.69	0.44	0.38	0.30	0.22	0.14
Diclofenac	0.60	0.22	0.25	0.19	0.13	0.02
Ibuprofen	1.22	0.47	0.56	0.34	0.28	0.03
Aspirin	0.13	0.16	0.23	0.04	0.03	0.01
Naproxen	0.12	0.18	0.27	0.22	0.09	0.02
Ketoprofen	0.09	0.19	0.27	0.22	0.07	0.02
Piroxicam	0.09	0.18	0.31	0.23	0.08	0.01

[a] Data acquired on Discovery HS F5 and Discovery HS C18 using 10 mM ammonium acetate, pH adjusted to 4.0 with acetic acid (pH 4): acetonitrile. Columns were 5 cm × 4.6 mm, 5 μm particles. Other conditions: flow rate, 1 mL/min; temperature, 35 °C; detection, 220 nm.

[b] (*) Analytes overly retained; (^) analytes exhibited split peaks about the hold-up time.

[c] Data acquired on Discovery HS F5 and Discovery HS C18 using 10 mM ammonium acetate, pH unadjusted (pH 6.7): acetonitrile. Columns were 5 cm × 4.6 mm, 5 μm particles. Other conditions: flow rate, 1 mL/min; temperature, 35 °C; detection, 220 nm.

ization from 99% at the lower pH to just 40% at the higher level. The decrease in pK_a coupled with an increase in apparent pH substantially neutralizes the basic analyte, thereby decreasing the overall ion-exchange interactions. The observation that the increase in retention on going from pH 4 to 6.7 is more substantial for the more basic secondary amines that are more extensively protonated at both pH values further supports this hypothesis. More quantitative studies on variation of pK_a values and pH measurements with organic composition are a subject of current work. The effects of organic modifier on silanol pK_a values are discussed in the following sections.

3.2. Dependence of apparent silanol pK_a values on stationary phase chemistry

The observations above provide evidence that ion-exchange interactions between ionized silanols and positively charged analytes have an important role in their retention, particularly on the PFPP phase. The differences between C_{18} and PFPP columns are surprising, as the physical and chemical characteristics for the stationary phases provided in Table 1 indicate that a similar number of unmodified silanols should exist on both bonded phases. The difference then, must be a function of either the degree of silanol ionization on the two phases or the availability of the ionized silanols to interact with the basic analytes.

Neue et al. approximated the pK_a values of surface silanols for several silica supports, an organic-silica hybrid particle and the C_{18} bonded versions of each by acquiring retention data for bretylium ion as a function of mobile phase pH [16]. The quaternary bretylium ion exhibits a permanent positive charge and thus will not change in retention due to altered ionization as a function of pH. A change in retention with pH is thus a measure of the degree of ionization of the stationary phase. In a similar fashion to the Neue experiments, the retention of bretylium ion was monitored as a function of pH using the PFPP, bare silica and C_{18} columns. Mobile phases ranging in pH from 2 to 8 (see Section 2) were prepared such that the ammonium ion concentration was held constant at 25 mM. A plot of retention ($\log k'$) for each stationary phase versus mobile phase pH is presented in Fig. 3.

Both the PFPP and silica stationary phases show an increase in bretylium ion retention of approximately 80% at pH 8 versus pH 4 while no significant increase is observed over the pH range studied on C_{18}. The concomitant increase in retention on the PFPP and bare silica phases with pH suggests that the extent of available ionized silanol groups are similar. Bretylium retention does not increase on the C_{18} phase up to a pH value of 8 suggesting that the extent of effective silanol ionization does not change in this pH range. Similar differences in silanol pK_a values between bare silica and the C_{18}-bonded stationary phase have been observed by others [16,19]. The difference in the silanol pK_a values between the PFPP and C_{18} bonded phases explains the enhanced retention and "U-shape" profiles of the basic analytes observed on the PFPP relative to C_{18}. Since the C_{18} exhibits few effective ionized silanols at pH values less than 8, the degree of effective silanol dissociation is minimal under pH conditions typically employed using silica-based stationary phases (pH

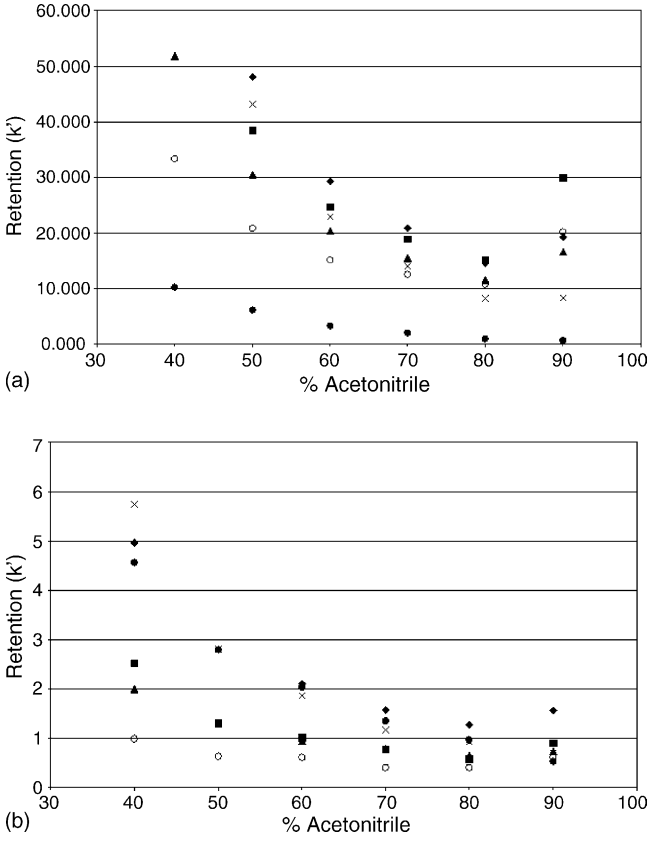

Fig. 2. Retention profiles (k') of basic probes on PFPP at pH 6.7. Retention (k') of basic probes: ♦, amitriptyline; ■, nortriptyline; ▲, diphenhydramine; ×, verapamil; ◯, alprenolol; and ●, lidocaine using (a) PFPP and (b) C18 from 40% to 90% acetonitrile under pH 6.7 conditions. Aqueous component: 10 mM ammonium acetate, pH unadjusted. Column: Discovery HS F5, 5 cm × 4.6 mm, 5 μm particle size. Other conditions: flow rate, 1 mL/min; temperature, 35 °C; detection UV at 220 nm.

2–8). Under these same conditions, the PFPP phase is likely to exhibit a significant degree of accessible ionized silanols and is therefore more likely to retain basic analytes via ion-exchange processes.

The PFPP phase is less hydrophobic than the alkyl phase [21]. The difference in the overall hydrophobicity of the surface likely influences the solvation of the silanol groups. The more hydrophobic alkyl phase may induce a greater concentration of the organic phase near the surface, whereas the more polar PFPP ligands likely promotes a more polar composition owing to more favorable interactions with polar solvent molecules. This is consistent with the HILIC mode of retention as proposed by Alpert [22], however, the dominant interaction mechanism appears to be related to ion-exchange rather than partitioning between the organic-rich mobile phase and a layer of semi-immobilized aqueous-rich solvent at the surface. The pK_a values of acids are known to increase with greater proportions of organic content [23]. For example, the pK_a of acetic acid increases from 4.76 in pure water to 6.57 in 60% acetonitrile [24]. Silanol groups exhibit an average pK_a of approximately 7 in modern silicas. If the trend of increasing pK_a with increasing organic proportion holds for silanol groups, the pK_a values may approach 9 or 10 in the presence of the alkyl ligand.

A second rationale for the observed behavior might be that the C_{18} stationary phase inhibits the analytes from reaching the surface, rendering the ionized silanols on the C_{18} phase inaccessible to the bretylium ion. Studies in our laboratory (data not shown) have shown that the retention of basic analytes decreases with increasing ammonium acetate concentration. It stands to reason that if an ion such as ammonium has free access to the surface, a slightly larger analyte such as bretylium would also experience free access.

Table 4
Comparison of capacity factors obtained on PFPP at neutral and acidic pH values at 90% acetonitrile

Compound	k'	
	At pH 4	At pH 6.7
Amitriptyline	16.4	19.2
Nortriptyline	18.3	29.9
Diphenhydramine	15.1	16.6
Verapamil	10.1	8.3
Alprenolol	14.0	20.2
Lidocaine	1.9	0.6

Data acquired on Discovery HS F5 and Discovery HS C18 using 10 mM ammonium acetate, pH adjusted to 4.0 with acetic acid (pH 4) and unadjusted (pH 6.7): acetonitrile (10:90, v/v). Columns were 5 cm × 4.6 mm, 5 μm particles. Other conditions: flow rate, 1 mL/min; temperature, 35 °C; detection, 220 nm.

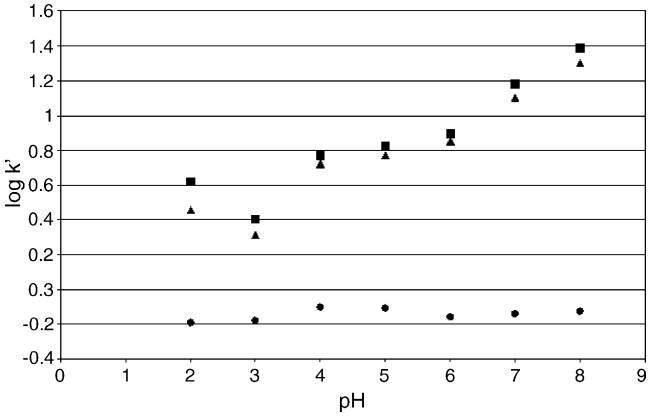

Fig. 3. Retention (k') of Bretylium ion as a function of pH retention of bretylium ion from pH 2 to 8 on ■ bare silica, ▲ PFPP and ● C18. PFPP, Discovery HS F5; C18, Discovery HS C18; and bare silica, proprietary unbonded silica support for Discovery HS column line. Each column was 5 cm × 4.6 mm, with a 5 μm particle size. Mobile phases consisted of 80:20, buffer:acetonitrile. Buffer ammonium ion concentration held constant at 25 mM. pH adjusted as described in the experimental section. Other conditions: flow rate, 1 mL/min; temperature, 35 °C; detection UV at 220 nm.

3.3. Comparison of basic probe retention using PFPP and bare silica

To further explore the contribution of silanol interactions, the retention data for each of the six basic probes were recorded using both the PFPP and bare silica phases. Re-

Table 5
Comparison of capacity factors of basic analytes at 90% acetonitrile on PFPP and bare silica stationary phases

Compound	k'	
	Silica	PFPP
Amitriptyline	4.63	12.71
Nortriptyline	6.92	18.98
Diphenhydramine	4.69	10.98
Verapamil	1.76	5.40
Alprenolol	6.09	12.59
Lidocaine	0.09	0.46

Data acquired on 5 cm × 4.6 mm, 5 μm Discovery HS PFPP and a custom bare silica phase based on HS silica using 2 mM ammonium acetate, pH 6.7 in 90% acetonitrile. Other conditions: flow rate, 1 mL/min; temperature, 35 °C; detection, 220 nm.

tention data were acquired using a mobile phase consisting of 2 mM ammonium acetate in 90% acetonitrile. If the retention of the basic analytes were explicitly based on silanol interactions, the magnitude of retention should be greater on bare silica owing to the greater number of available silanol groups (about twice that available on the PFPP phase using the accepted silanol density of 8 μmol/m^2 [25]). The data presented in Table 5, however, show that the retention for the basic analytes significantly increase (by a factor of 2–3) on the PFPP column compared to the bare silica phase. This points to a significant contribution of non-ionic mechanisms to the retention of bases on PFPP stationary phases as minimal contribution from reversed-phase mechanisms is expected on the bare silica support under these conditions. In other words, a significant contribution to retention from the PFPP bonded phase is demonstrated.

Several authors have recently described a hydrophobically assisted ion-exchange mechanism where the ion-exchange process is enhanced by neighboring hydrophobic sites [16,17]. The increased retention observed on the PFPP phase may be due to simultaneous electrostatic interactions of the analyte with ionized silanols and hydrophobic or polar interactions with the pentafluorophenylpropyl moiety, which would be consistent with the hydrophobically assisted ion-exchange mechanism. Alternatively, a two-site model may be invoked where a combination of effects from separate ion-exchange and non-electrostatic interactions result in enhanced retention on the PFPP phase. Current research is aimed at further elucidation of these potential mechanisms. The simultaneous or separate combination of these interactions, as depicted in Fig. 4, provides the enhanced retention observed using the PFPP phase over bare silica and C_{18}-modified columns.

3.4. Dependence of "U-shape" retention on analyte pK_a

Fig. 5 shows the dependence of lidocaine retention on mobile phase percent acetonitrile content obtained under each of the conditions studied. At high pH the retention of lidocaine exhibits a linear dependence on percent acetonitrile on both stationary phase systems. At pH 4, however, non-linear

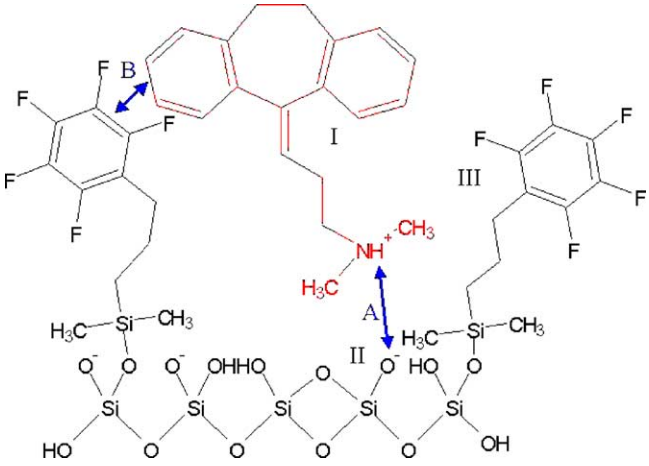

Fig. 4. Representation of potential interactions of protonated basic analytes with PFPP stationary phase. The protonated amitriptyline (I) molecule is shown to interact via ion-exchange (A) with an ionized surface silanol (II) while simultaneously interacting via dispersive and polar mechanisms (B) with the PFPP bonded phase (III).

dependence is observed. In addition, the slightly basic analyte does not exhibit the "U-shape" profile under any of the conditions investigated. The major difference between lidocaine and the other basic probes lies in its pK_a value. The aqueous pK_a for lidocaine is about 1 pH unit less than that for the next lowest test probe. In general, the pK_a of the protonated form of basic analytes decrease with increasing percentages of organic modifier [26]. For instance Neue et al. showed that amitriptyline, with an aqueous pK_a value of 9.4, exhibits an apparent pK_a value of 6.5–7 in 65% methanol mobile phases [27]. It is proposed that the pH of the higher organic-containing mobile phases is approaching the actual pK_a of lidocaine in this solvent system. This leads to a decrease in the degree of ionization, which reduces the potential of the analyte to interact electrostatically with ionized

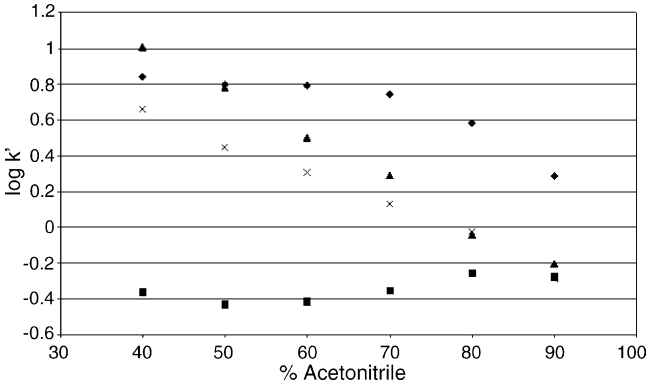

Fig. 5. Comparison of lidocaine retention under various conditions studied. Retention of lidocaine on ♦ PFPP at pH 4, ■ C18 at pH 4, ▲ PFPP at pH 6.7 and × C18 at pH 6.7 from 40% to 90% acetonitrile. Columns: PFPP, Discovery HS F5; Cl8, Discovery HS C18. pH 4, 10 mM ammonium acetate; pH to 4 with acetic acid. pH 6.7, ammonium acetate, unadjusted. Both columns were 5 cm × 4.6 mm, 5 μm particle size. Other conditions: flow rate, 1 mL/min; temperature, 35 °C; detection UV at 220 nm.

silanols. At pH 6.7, lidocaine retention is shown to exhibit a near linear correlation with percent acetonitrile. At the higher pH, lidocaine is expected to be significantly neutralized and therefore should act like a more "ideal" solute as appears to be the case.

Recent determinations of pK_a values of basic analytes in highly organic solvents using NMR spectroscopy have shown that verapamil and lidocaine exhibit significantly lower pK_a values than the remainder of the basic analytes (manuscript in preparation). This further substantiates the dependence of "U-shape" retention on ionic interactions.

3.5. Ion-exchange and peak shape

The interaction of basic analytes with ionized surface silanols is well known and has been implicated as the primary cause of excessive peak tailing and selectivity differences between different manufacturer's reversed-phase columns [18]. Older, type A reversed-phase columns are notorious for exhibiting peak asymmetry for basic analytes. Although advances in bonding procedures and in the manufacture of pure (type B) silica has succeeded in reducing these effects, the silanol surface cannot be completely deactivated [25]. In these studies, it was noted that the basic analytes exhibited greater asymmetry on the PFPP phase in the reversed-phase regions as compared to the C_{18} phase. At high organic conditions, however, highly symmetrical peaks shapes were observed for the basic analytes on the PFPP phase (see Fig. 6). This observation indicates that at high organic percentages there is an increase in available ionized surface silanol concentration. As such, the entire molecular population of the probes has equal access to surface silanol groups on the PFPP phase. Water can interact strongly with surface silanols, thereby modulating the ion-exchange interactions and effectively reducing the available concentration of ionized silanols [28]. In the reversed-phase region, where the water concentration is relatively high, peak tailing results from an overload of the limited concentration of available ionized silanols on the PFPP phase. In contrast, the ionized surface silanol concentration on the C_{18} phase is presumably low enough to have minimal impact on peak asymmetry.

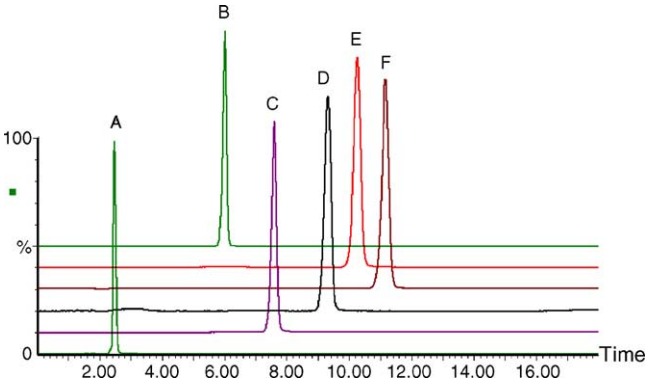

Fig. 6. LC–MS traces of basic analytes on PFPP phase. Retention of basic probes (lidocaine (A), verapamil (B), alprenolol (C), diphenhydramine (D), amitriptyline (E) and nortriptyline (F)) on discovery HS F5. Column: 15 cm × 4.6 mm, 5 μm particle size. Mobile phase: 14 mM ammonium acetate in water:acetonitrile (10:90, v/v). Flow rate: 1 mL/min, temperature: ambient, detection: MS (ESI, positive ion mode).

4. Conclusions

In this study we have shown that only basic analytes exhibit "U-shape" retention, that both the fluorinated phase and the alkyl phase exhibit this phenomenon and that retention depends on mobile phase pH. Each of these observations imply that ionic interactions with surface silanols are present. The increased retention observed in the high organic region on the PFPP phase as compared to the same region on the C_{18} column was found to be due to greater degree of dissociation of surface silanol groups on the PFPP phase. It was shown that the PFPP and bare silica systems exhibit similar silanol activity over the pH range studied, whereas the C_{18} system showed minimal effects that could be attributed to ionized silanols over this region. The degree of dissociation of surface silanols is explained by the different solvation states of the silica surface between the PFPP and C_{18} phases. The lower hydrophobicity of the PFPP phase compared to C_{18} column induces a more aqueous solvent composition whereas the hydrophobic alkyl phase promotes a richer organic composition, which results in a change in the ionization state of the surface silanols. The difference in silanol pK_a values between PFPP and C_{18} suggest that bonding chemistry plays an important role in both reversed-phase and ionic interactions. With this knowledge, further developments in bonded phase chemistry are expected to yield stationary phases with enhanced reversed-phase and ion-exchange properties.

Retention of basic analytes was found to be substantially greater using PFPP relative to either C_{18} or bare silica. Excess retention of basic probes on PFPP as compared to bare silica supports the presence of a hydrophobically assisted ion-exchange mechanism as proposed by Neue et al. [16] and Carr and co-workers [17] or additional independent interactions due to the presence of the PFPP ligands.

It was noted that not all bases exhibited retention in the normal-phase region and that there was significant selectivity between basic analytes of identical charge state. This observation signifies the importance of combined ion-exchange and non-ionic mechanisms to retention of basic compounds. Lidocaine and verapamil exhibited limited ionic interactions due to their relatively low degree of ionization under the conditions studied.

The substantial ion-exchange property of the PFPP phase presents new opportunities for manipulating retention and selectivity. Mobile phase pH has been shown to be a valuable tool for the manipulation of basic analyte retention. In addition to controlling the analyte ionization state, the ionization state of the silanol surface is governed by mobile phase pH.

The potential to retain basic analytes at high percentages of organic modifier and LC–MS compatible buffers offers an opportunity to increase the sensitivity and selectivity of LC–MS experiments. Since the interactions responsible for the retention are predominantly ionic, neutral and acidic endogenous species are not likely to retain and interfere with the analysis. In addition, the mobile phase volatility under these conditions results in facile solvent evaporation, which increases MS sensitivity [7]. Excellent selectivity, peak symmetry and LC–MS compatibility make this approach a powerful tool for the analysis of basic analytes.

Acknowledgements

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