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SOLUTE ATTRIBUTES AND MOLECULAR INTERACTIONS CONTRIBUTING TO 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 C18. 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 C18 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.

NMR spectroscopy is demonstrated to be a rapid and useful technique for the determining pK_a values of solutes in HPLC mobile phases. The variation of chemical shift data for protons in the vicinity of a basic nitrogen atom as a function of the medium pH can be related through the Henderson-Hasselbalch equation to estimate analyte pK_a

values. The use of a pH scale based on the measurement following addition of organic component (${}^{s}_{w}$pH) is shown to reflect the true thermodynamic reality of the environment about the analyte as compared to the pH scale based on the purely aqueous component. The present study demonstrates that one can not assume that bases are protonated in high acetonitrile content even if the aqueous pH is adjusted to less than two pK_a units from the literature pK_a value as is commonly practiced. The pK_a values for the basic analytes used in this study were shown to decrease by approximately one pK_a unit in approximately 90 v/v% acetonitrile from their aqueous value. Where ion-exchange mechanisms are present, improved prediction and manipulation of HPLC selectivity results from more accurate knowledge of the analyte degree of dissociation values. NMR spectroscopy provides a non-invasive and direct measure of the equilibria and its dependence on the pH of the media. Knowledge of such equilibria allows one to further explore the fundamental mechanisms of retention in chromatographic processes.

Fluorinated, silica-based stationary phases are becoming increasingly popular alternatives to traditional alkyl phases owing to their differential selectivity and retention for a variety of analyte classes. In this report, the ion-exchange mechanisms characteristic of a fluorinated phase are exploited to rapidly develop separation conditions for ephedrine alkaloids and synephrine using a mobile phase compatible with mass spectrometry. A linear relationship of basic analyte retention with the reciprocal of ammonium acetate concentration is first established. This linear relationship can then be used to optimize retention and selectivity in just two experiments. The relationship of retention with temperature is also explored. Greater retention with increasing temperature is demonstrated on the fluorinated phase at high percentages of organic

modifier, which is in contrast to behavior observed in typical reversed-phase separations. The unexpected observation is explicated based on the reduction in solvent solvating power with increasing temperature. As solvation power of the mobile phase decreases, decreased solvation of both mobile phase and ionized surface groups of the stationary phase leads to stronger interactions between analyte and stationary phase. Both mobile phase ion concentration and temperature are shown to be powerful tools for the manipulation of analyte retention and selectivity.

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Chapter 1

Introduction

1.1 The role of liquid chromatography in the modern analytical laboratory

High-performance liquid chromatography (HPLC) has become the dominant analytical separation tool in the modern analytical laboratory.[1] HPLC is used extensively in pharmaceutical, industrial chemical, agricultural, environmental, food and beverage, cosmetic and other industries. Pharmaceutical companies use HPLC in conjunction with powerful detectors such as mass spectrometers (LC-MS) to identify and quantify active pharmaceutical ingredients (APIs) in raw materials, intermediates and finished products. HPLC is often the tool of choice in determining adsorption, distribution, metabolism, excretion and toxicity (ADMET) of potential therapeutics. The industrial chemical, food and beverage, and cosmetic industries rely on HPLC technology for quality control of assorted intermediate and end products. In environmental and agricultural laboratories, HPLC is the primary analytical technique for the analysis of pesticides, veterinary drug residues and other contaminants in soil, water and foodstuffs. The vast majority of organic analytical laboratories rely on HPLC separations for the bulk of their qualitative and quantitative analyses.

1.1.1 Range of compounds analyzed in drug discovery and development

HPLC and related hyphenated techniques such as LC-MS are primarily used for the analysis of small molecules (< 500 Da) in traditional pharmaceutical and other industrial applications. A growing biopharmaceutical sector has added larger molecular weight analytes such as proteins, peptides and oligonucleotides to the scope of the technology. The plethora of target analytes spans a broad range of chemical space including ionized compounds, polar, non-ionic molecules, and large hydrophobic substances. In addition, HPLC techniques are often called upon to qualitatively or quantitatively evaluate molecular species in a variety of matrices such as soils, plant and animal tissues and wastewater.

1.1.2 Capabilities and limitations of current technology

Successful application of HPLC requires that the target analytes be retained by, or interact, with a given stationary phase. As analyses of pharmaceuticals, their metabolites, and complex mixtures of endogenous metabolites (metabolomics) are growing in importance in drug development, the HPLC practitioner is increasingly faced with the need to retain and separate analytes with disparate physical properties. LC-MS has become the dominant analytical tool in analysis of pharmaceuticals and metabolites.[2] However, LC-MS does not perform well in analysis of many polar, low molecular mass (< 500 Da) analytes, which are often poorly retained on common HPLC stationary phases.[3] The additional mass resolving dimension provided by the mass spectrometer permits less chromatographic resolution, however retention is still necessary to overcome

interference from non-retained sample constituents such as inorganic salts and buffers. Inadequate chromatographic retention can result in suppression of ionization by coeluting species that cause poor quantification in LC-MS analyses.[4] To achieve chromatographic retention of polar ionic solutes, ion-pair reagents are often added to mobile phases. These reagents, however, are usually non-volatile and suppress ionization in LC-MS analyses. Other separation techniques such as capillary electrophoresis (CE) are also employed for retention and separation of ionic analytes, but the predominantly aqueous solvents and non-volatile buffers that are commonly used are less amenable to MS interfacing than many liquid chromatographic systems. It is therefore desirable to design and employ HPLC stationary phases capable of retaining polar analytes using mobile phase constituents compatible with mass spectrometric analysis.

1.1.3 A need for new HPLC stationary phases

The overwhelming majority of HPLC separations are practiced using aqueous-organic mixtures (mobile phases) and chemically modified, silica-based stationary phases. The majority of applications are performed using silica modified with alkyl ligands, such as octadecylsilanes (ODS or C18). Analytes are retained by the C18 bonded phase primarily by dispersive interactions and exclusion from solvation by the mobile phase, in what are commonly referred to as hydrophobic interactions. The C18 support often provides the necessary retention and selectivity to separate moderately polar to non-polar solutes, but the high affinity of polar analytes for polar solvents works against retention of highly polar analytes. Furthermore, for solutes with very similar

solubility properties, C18 stationary phases do not provide the necessary basis for selective retention. There is therefore a need for additional supports in HPLC, particularly for separations of polar analytes. Interactions that contribute to retention in HPLC in addition to dispersive interactions may be classified into two categories: polar and ionic. Stationary phases that provide complementary polar and ionic interactions are therefore actively sought.

1.2 Fluorinated stationary phases for HPLC

Liquid chromatography is most often accomplished on reversed-phase stationary phases based on alkyl-bonded silica particles.[5] Stationary phases manufactured using alternative bonded phases have, however, become increasingly popular owing to the differences in selectivity and retention that they provide. Fluorinated stationary phases, in particular, are gaining acceptance as alternatives to common C18 and C8 phases owing to their unique selectivity.[6] In addition to dispersive interactions available on traditional alkyl phases, the pentafluorophenylpropyl phase also provides enhanced dipole-dipole, pi-pi, charge transfer and ion-exchange interactions.[6,7]

1.2.1 Unique retention characteristics of fluorinated stationary phases

Fluorinated, silica-based stationary phases such as the one depicted in Figure 1-1 have shown unique retention for small, polar analytes.[8-11] In particular, pentafluorophenylpropyl (PFPP) phases exhibit retention behavior for polar analytes that

bears similarity to both reversed-phase and normal-phase retention, which has shown to be dependent on mobile phase composition.[9,12]

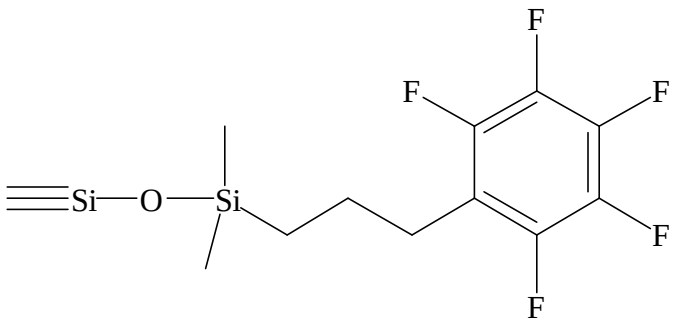

Figure **1-1**: Pentafluorophenylpropyl (PFPP) Stationary Phase

At lower percentages of organic modifier (herein referred to as the reversed-phase region), solute retention resembles that of classical reversed-phase systems, with retention decreasing with increasing concentrations of organic solvents such as acetonitrile or methanol. At higher percentages of organic (herein referred to as the normal-phase region), however, behavior more typical of normal-phase separations is observed, with retention increasing with increasing proportions of organic modifier. This combination of reversed-phase and normal-phase behavior, as shown in Figure **1-2**, 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 compatible with mass spectrometry.[11] Although this "U-Shape" retention has been observed on traditional alkyl columns[13], the magnitude of normal-phase retention is dramatically increased using the PFPP stationary phase, which exhibits

greater than a 10-fold increase in retention for certain solutes at high organic concentrations.[9] Bij, *et. al.*, attributed the irregular retention profiles using the alkyl columns to a dual retention mechanism involving both solvophobic and silanophilic interactions.[14] The solvophobic or hydrophobic interactions are responsible for the commonly observed decrease in retention with increasing organic in reversed-phase chromatography. Silanophilic interactions have been attributed to hydrogen bonding and ionic mechanisms between the analyte and the silica surface. The dual retention mechanism was postulated to be a common phenomenon and thus must be accounted for in HPLC method development practices. 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.

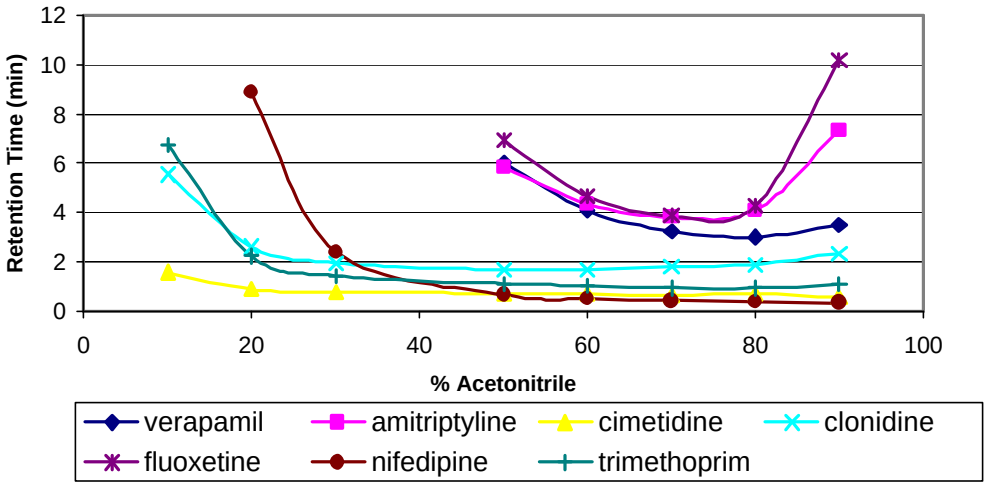

Figure **1-2**: Atypical reversed-phase and normal-phase behavior of some analytes on a pentafluorophenylpropyl stationary phase as a function of percent organic modifier

Column: Discovery HS F5 (PFPP), 50 mm x 4.6 mm, 10 mM ammonium acetate modified with acetonitrile (*Unique Retention and Selectivity of Pentafluorophenylpropyl Phases for High-Throughput LC/MS Analysis*, David S. Bell, Poster, Presented at ASMS Annual Meeting, June 2002, Orlando, FL)

Stationary phases containing perfluorinated functional groups are available from many column manufacturers and are best known for their use in separating taxanes.[15,16] Owing to unique selectivity of the fluorinated phases, they are becoming more widely used as an alternative to traditional alkyl systems.[17] Sadek, *et al.*, used linear solvation energy relationships (LSER) to demonstrate selectivity differences for polar, nonionizable analytes on perfluorinated supports compared to hydrocarbon bonded phases.[18] 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.[19] The "extended polar selectivity" reported by this group was defined as the selectivity, or the relative retention, between two basic components in their test mix normalized to a control C18 stationary phase. The procedure was an attempt to subtract non-specific hydrophobic and silanophilic contributions to retention. The nature of the stationary phase functionality and interpretation of the interactions were not reported. "Phenolic selectivity" interactions were reported as non-hydrophobic contributions to the preferential retention of phenolic analytes using the fluorinated phases as compared to alkyl columns. Again, the fundamental interactions and the stationary phase features responsible for the observations were not reported. The Neue study grouped the

selectivity of fluorinated phases as distinct from C18 and cyanopropyl phases. In a related column classification study, Euerby investigated 135 commercially available stationary phases.[17] The authors observed significant differences in analyte shape selectivity, or the ability of a stationary phase to distinguish between analytes of similar hydrophobic interactions that differ in connectivity, for pentafluorophenyl stationary phases. The unique retention and selectivity of the fluorinated phases in the Euerby study prompted further investigation.[6] 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 provided. Polar stationary phases (relative to C18) are commonly used when the alkyl phases are not suitable for a given separation. Fundamental knowledge of the retention mechanisms that contribute to the alternative selectivity is, however, not well understood.

1.3 Mechanisms of chromatographic retention in HPLC

Reversed-phase (RP) chromatography, where a non-polar stationary phase and a polar mobile phase are employed, represents about 90% of all analytical separations of low molecular weight samples. RP chromatography also plays an important role in higher molecular weight separations such as proteins and peptides. The majority of the discussion will thus be directed toward RP retention mechanisms, however, interactions

based on normal-phase (NP) and ion-exchange interactions (IEX) will also be invoked, as their contribution to an overall description of retention cannot be neglected. Existing chromatographic theories regarding RP chromatography are reasonably accurate in predicting retention and separation of neutral, non-polar compounds, however analytes capable polar and ionic interactions are not well modeled. The following sections describe the current knowledge regarding retention mechanisms in NP, IEX and RP chromatography.

1.3.1 Theoretical basis of retention in normal-phase liquid chromatography

Normal-phase chromatographic processes are defined by the use of a polar stationary phase such as bare silica and a relatively non-polar solvent system. Hydroxyl groups are considered to be the origin of solute interactions on the surface of silica gel. The hydroxyl groups are bound by up to three monolayers of water. Under conditions suitable for liquid chromatography, dry solvents are capable of removing all water except the first monolayer that is tightly bound to the surface via hydrogen bonding. Depending on the polarity of the solvents in a given system, several possibilities exist that describe how the solvents interact with each other and the surface. A non-polar solvent (incapable of hydrogen bonding) will interact with the monolayer of water at the surface of the silica support via dispersion forces. A polar solvent will interact with the water monolayer via hydrogen bonding and dipole-dipole interactions. A strong hydrogen bonding solvent may be able to displace water in the monolayer. A solute may then interact with the bound solvents by a variety of forces. The solvent may simply adsorb onto the

immobilized solvents or it may displace the solvent in the variety of layers.[20] The bare silica surface is generally recognized as being heterogeneous in terms of the available active sites. As depicted in Figure **1-3**, silanols can exist on the surface of the silica support in isolated, geminal or vicinal forms, each of which exhibit different adsorption characteristics or reactivity. Free or isolated silanols are considered the most active.[21] The presence of different silanol forms is dependent upon the preparation of the silica support and the environment to which it is exposed. Heterogeneity of the silica surface due to the presence of the various forms of silanols results in column to column variation as well as irreproducibility based on changing environmental conditions. Trace amounts of a polar solvent such as water have a strong influence on chromatographic performance due to the preferential deactivation, or masking, of the more active sites on the surface. The addition of trace amounts of water or the use of polar bonded phases is a general practice to reduce the effects of heterogeneous silica supports.

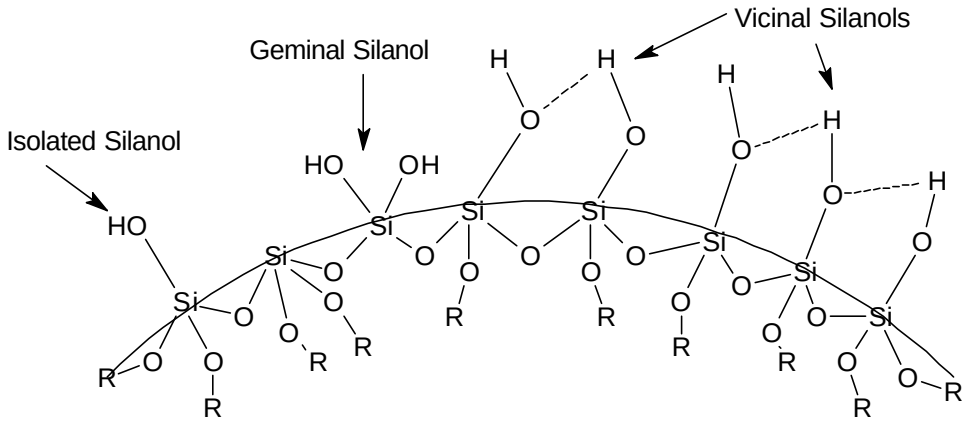

Figure **1-3**: The silanol species present at silica surfaces [21]

1.3.2 Theoretical basis of retention in ion-exchange liquid chromatography

Ion-exchange chromatography is used for the separation of ionic or ionizable analytes. The separation is based on the exchange of the analyte ions with mobile phase ions associated with corresponding counterions on the sorbent surface as shown in Figure **1-4**. The extent of the exchange, and thus the retention, is based on the relative affinities of the analyte and mobile phase ions toward the surface. Retention is primarily controlled through manipulation of the concentration and type of mobile phase ion as well as the charge of the analyte or surface ion through adjustments in mobile phase pH. Most ion-exchange materials are prepared by bonding a suitable ligand such as arylsulfonic acid to a surface such as silica, however, silanol groups on the surface of silica-based bonded phases may also act as ion-exchangers. Although HPLC column manufacturers have made attempts to reduce the effect of silanols in reversed-phase column chemistries, it is currently considered impossible to remove all silanol groups

from interacting with cationic solutes.[1] It is therefore important to understand and account for ion-exchange interactions that may be present even on modern “deactivated” stationary phases.

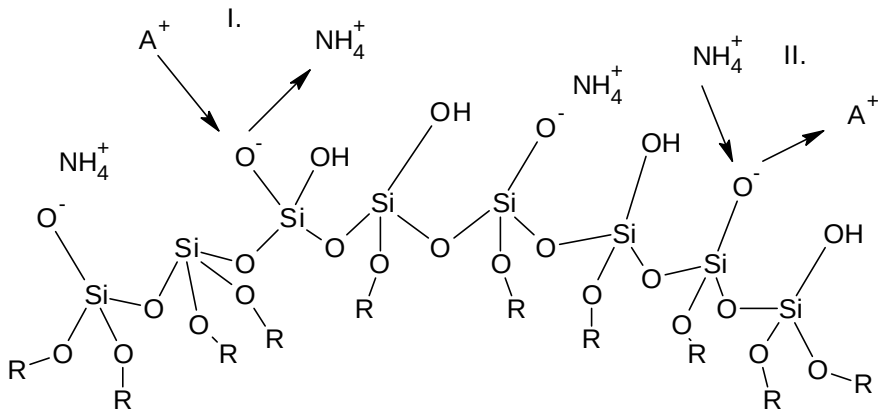

Figure **1-4**: Depiction of an ion-exchange process on bare silica

I. Charged analyte, A+, displaces mobile phase counterion, NH4+. II. Mobile phase ion displaces adsorbed analyte

1.3.3 Theoretical basis of retention in reversed phase liquid chromatography

The oldest accepted theory of RP chromatography is the Hydrophobic or Solvophobic Effect introduced by Horvath *et al.*[22] The retention mechanism can be viewed as the difference in energetics for the formation of cavities in the solvent (mobile phase) in relation to a complex formation between the solute and the stationary phase. Surface tension or cohesive forces of the solvent and the dipole-dipole interactions of the polar functionality of the analyte with the solvent dominate retention. As a result of cavity formation to accommodate an analyte, the surface area of the solvent increases in proportion to the molecular surface area of the solute. The energy associated with cavity

formation should thus be proportional to the surface tension of the solvent and the area of the cavity. Contributions to the energetics of the process due to specific interactions such as dipole-dipole forces between the solute and solvent are also accounted for.

According to the Solvophobic model of retention, the stationary phase plays a passive role in the mechanism by providing only a sorptive site for retention.[23] Experimental observations regarding the selective retention of analytes based on molecular shape for certain stationary phases as well as the dependence of retention on the density of the bonded phase is not well supported by the Solvophobic model as in these cases the stationary phase must play an active role.[23] A more recent model based on the partitioning of an analyte between the aqueous mobile phase and the hydrocarbonaceous bonded phase is built, in part, on the Solvophobic model. In the partitioning model, the solute is described as partitioning fully between mobile phase and the ligands of the stationary phase. As depicted in Figure **1-5,** the partitioning model portrays the bonded phase as an active participant in the interactions pertinent to retention. The density and type of bonded phase ligand will clearly influence analyte retention according to this model. The partitioning theory models retention based on the bulk properties of the mobile phase solvents, but fails to incorporate the effects of preferential solvation of the stationary phase and the resulting impact of interactions with the solvent-modified support.

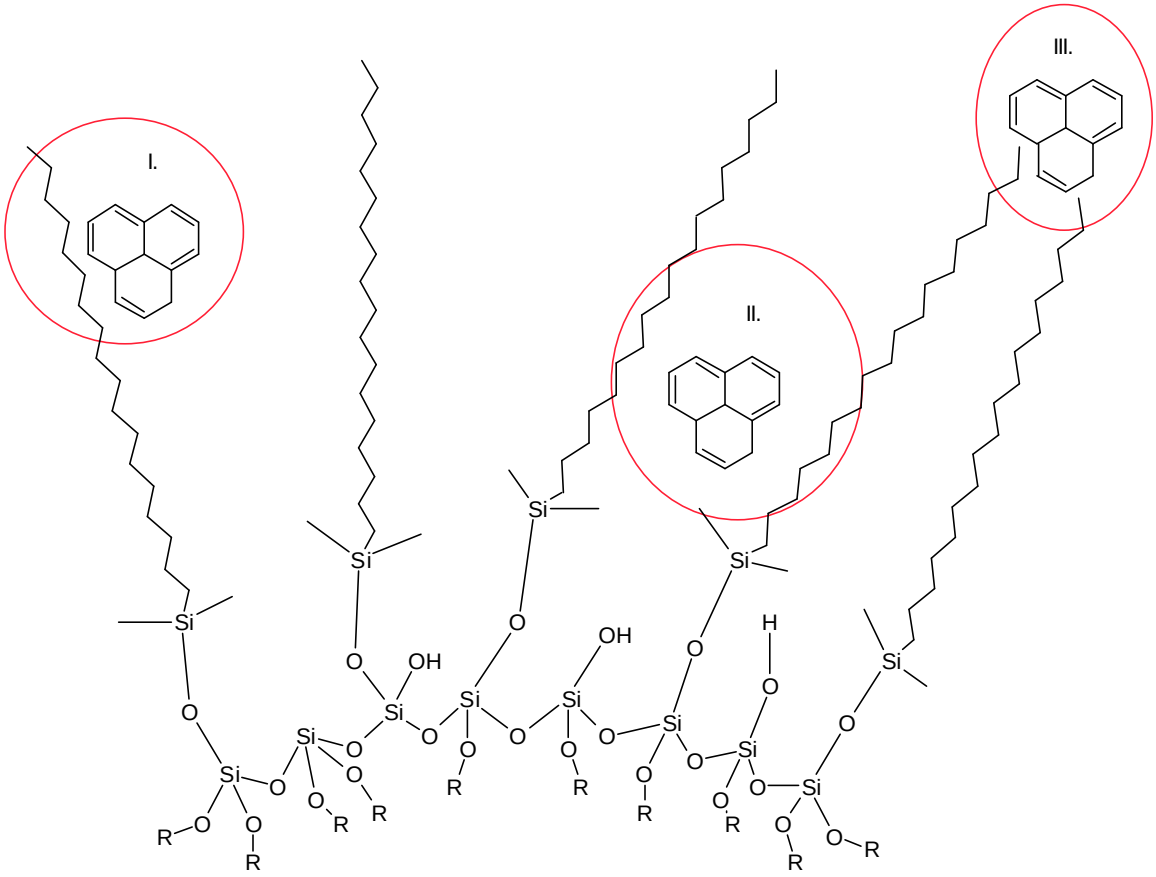

Figure **1-5**: Partitioning model of reversed-phase HPLC retention

I.Analyte interaction with a single stationary phase ligand. II. Analyte interaction with multiple stationary phase ligands. III. Analyte excluded from partitioning

The more advanced theory of Jaroniec treats the mechanism of RP retention as a combination of adsorption or displacement (similar to normal-phase mechanisms) and partition mechanisms.[24] According to this treatment, one should distinguish between two stages of the process of RP chromatography. First, there is a formation of a surface-influenced stationary phase where solvent molecules are incorporated into the stationary phase. A partitioning of the solute between the mobile phase and this modified stationary phase then takes place. In the Jaroniec model, the organic component of the mobile phase is enriched at the (usually) hydrophobic surface as a function of the

thermodynamic equilibrium of the solvent mixture components with the surface. In binary mixtures, the ratio of the solvent components in the solvent shell around a solute may be different than the bulk composition. The tendency is for a solute to be preferentially solvated by the component(s) of the solvent mixture that leads to the more negative Gibbs energy of solvation.[25] In this same manner, the HPLC stationary phase may be preferentially solvated by components of the mobile phase. The extent of surface enrichment (and corresponding bulk mobile phase depletion) of the mobile phase component must therefore be a function of both the mobile phase composition and stationary phase properties. Different solvents are enriched on the surface of a bonded phase to different degrees. For the common organic modifiers used in RP HPLC, methanol is adsorbed less than acetonitrile, which is absorbed less than tetrahydrofuran.

The driving force of RP chromatography is the interaction of the hydrophobic part of the solute molecule with the hydrophobic stationary phase. The interaction of polar groups of the analyte with the mobile phase and residual silanols on a silica-based packing are responsible for much of the selectivity of a given separation. The result is that the solvent that is used as an organic modifier of the mobile phase can influence selectivity of a separation. The amount and activity of surface silanols on the silica-based packing material or their suppression by modification of the bonded phase or components of the mobile phase are significant. In addition, selectivity can be modified by the incorporation of polar functional groups in the stationary phase ligand.

1.3.4 Emerging theories regarding analyte retention in liquid chromatography

Contributions toward retention of ionizable analytes on reversed-phase stationary phases other than those due to dispersive (hydrophobic) interactions have long been known. As stated earlier, protonated bases and other cationic solutes may interact with ionized surface silanol groups via an additional ion-exchange mechanism. The classical treatment of the disparate mechanisms of retention modeled them as separate additive contributions. This approach is equivalent to solutes interacting with hydrophobic and ionic sites in different locations along the column. Simultaneous ionic and dispersive interactions such as those depicted in Figure **1-6** have recently been proposed.

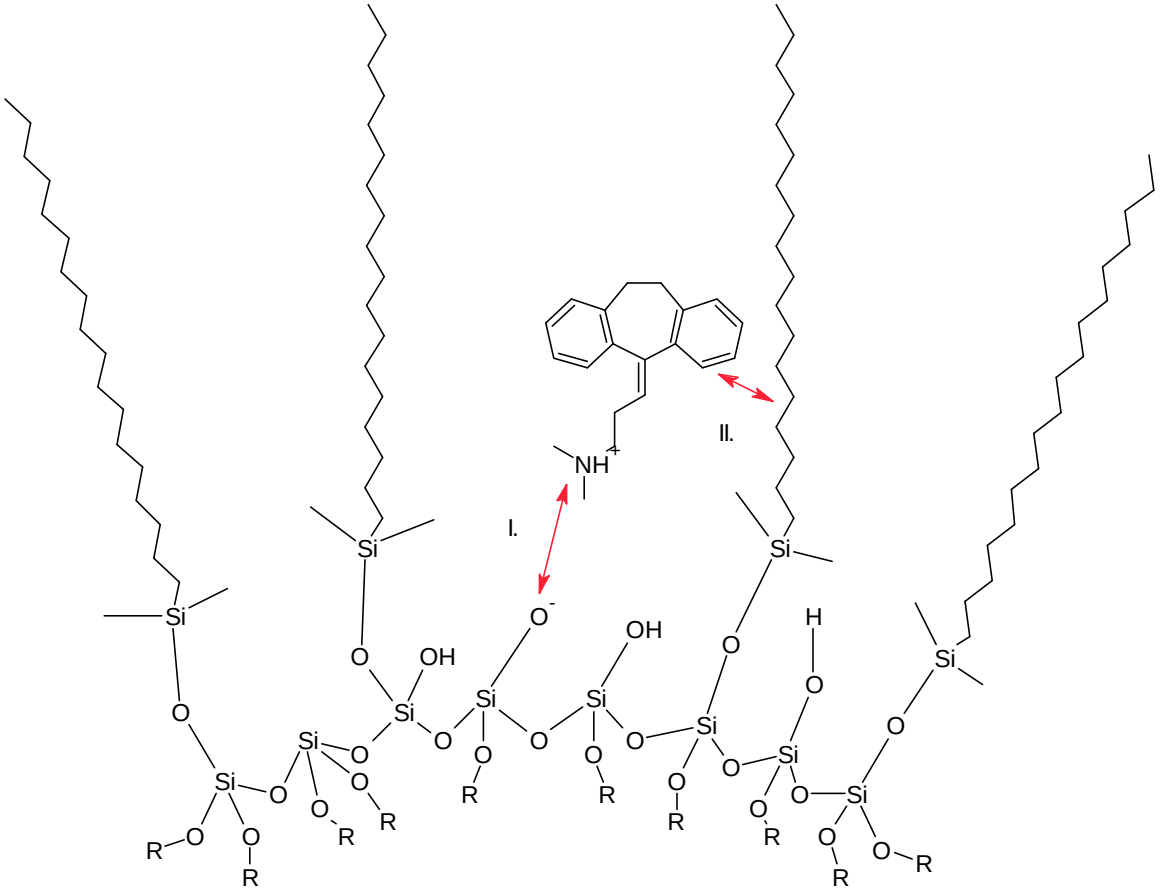

Figure **1-6**: Hydrophobically-assisted ion-exchange mechanism of retention
Interactions may occur as purely ionic (I), purely dispersive (II) or as a simultaneous combination of both I and II

In an attempt to describe the increase in ionic interaction with increased dispersive interactions, a multiplicative model was developed by Neue, *et al*.[26] This model, however, incorrectly predicts that retention will disappear when either the dispersive or ion-exchange contributions are eliminated. Yang, et al, proposed a second dual mode model whereby a hydrophobically-assisted ion-exchange multiplicative mechanism of interaction is combined with a purely hydrophobic mechanism.[27] In this model, as the ion-exchange mechanism is suppressed, the purely hydrophobic mechanism becomes dominant, however, when hydrophobic interactions are eliminated, the model

incorrectly predicts no retention due to ionic interactions. Recently, a third model has been proposed by Neue, et al, that includes a purely hydrophobic term, a purely ionic term and a multiplicative hydrophobically-assisted ion-exchange term.[28]

Traditional chromatographic approaches have attempted to eliminate the contributions of ionic interactions to retention by either suppressing the ionization of surface silanols with pH adjustments or through the use of competitive mobile phase additives. Surface silanol groups exhibit a range of pK_a values with the average pK_a value generally accepted to be about 6 for modern silica materials.[21] By lowering the pH of the mobile phase the degree of surface silanol ionization can be minimized, however, some highly active sites will persist.[1] Mobile phase modifiers such as triethylamine (TEA) are commonly added as their protonated form will interact with ionized surface silanols and effectively prevent them from interacting with a basic solute.[29] These modifiers, however, are often detrimental to desired detection techniques such as mass spectrometry and create even more complex separation mechanisms with outcomes that are difficult to predict.

The modern demands of retaining and separating analytes with widely differing properties has necessitated the exploitation of polar and ionic interactions rather than their suppression. Stationary phases that incorporate polar functionalities provide additional polar and ionic interactions and are becoming desired alternatives to traditional C18 stationary phases. With the addition of polar and ionic character, models such as those proposed by Yang and Neue may be required to better understand and predict retention in the modern analytical laboratory.

1.3.5 Contributions of acid-base equilibria to liquid chromatographic separations

Approximately 80% of active pharmaceutical ingredients are basic compounds, and many are ionized under common chromatographic conditions. Still others exhibit acidic moieties that are also capable of ionization under chromatographic conditions. Improved prediction of analyte retention on various stationary phases requires better understanding of the effects of solute ionization on chromatographic processes as well as the system variables that control the extent of solute and stationary phase ionization.

As mentioned earlier, stationary phases based on silica are most commonly used in modern chromatographic analyses. The role of ionized silanol groups toward the retention of basic analytes has been studied for several decades.[5,30-32] In most cases, it has been determined that the presence of silanol groups in reversed-phase columns is deleterious because ionized surface silanol groups have been related to asymmetrical peak shape for basic analytes. Broadened or tailing chromatographic peaks result in loss of resolution and are difficult to accurately integrate. In addition, the irreproducibility of chromatographic separations is often attributed to the presence of surface silanols and their different activity from column to column.[33] The presence of metal impurities in the silica substrate has been linked to increased silanol activity.[34] The reduction of metal content in silica gel supports and the use of more homogenous silica substrates using various silica pretreatment techniques have been sought by column manufacturers in an attempt to reduce the effects of silanols in modern stationary phases. Efforts to employ the interactions provided by the active silica support, however, have been scarce.

The effect of solute ionization in reversed-phase chromatography has been extensively studied.[35-44] In general, the ionized state of the solute, due to its increased hydrophilicity, is retained less on a reversed-phase stationary phase than the less hydrophilic neutral form. Predictions of the effects of ionization on chromatographic retention, however, often do not account for polar and ionic interactions that may be contribute to analyte retention. As indicated by many column classification studies, stationary phases with polar functionalities provide increased polar and ionic interactions and enhanced retention of polar analytes. It is therefore important to understand the thermodynamics of solute solvation and its influence on acid-base equilibria in a broad range of chromatographic solvent systems.

1.4 High-performance liquid chromatography solvents and additives

Solvents chosen for use in modern reversed-phase HPLC are generally limited to mixtures of water, acetonitrile, methanol and tetrahydrofuran (THF). Additives such as ammonium acetate and other buffers are often included at low millimolar concentrations to control mobile phase pH. Water is commonly employed as the weak solvent, thus the organic modifier must be miscible with water. In addition, organic modifiers are desired that exhibit low ultraviolet wavelength absorption, low viscosity, low toxicity and low reactivity.[45] Selectivity in HPLC separations has been attributed to the dipolar and hydrogen bonding interactions between solutes and the mobile phase components.[46] Retention and selectivity are therefore largely influenced by the dipole moment and hydrogen bond acidity and basicity of the solvents employed. The three organic

modifiers listed above represent relatively dipolar (acetonitrile), hydrogen bond acidic (methanol) and hydrogen bond basic (THF) solvents. Various combinations of these organic solvents with water are likely sufficient to resolve many target analytes. The remainder of the discussion regarding solvent properties will therefore be limited to the aforementioned solvents.

1.4.1 Thermodynamics of solute solvation

Intermolecular forces that govern retention and selectivity in chromatographic processes include transient electronic (dispersive) interactions such as van der Waals and London forces as well as directional interactions such as hydrogen bonding, ion-dipole and electron pair donor-acceptor interactions. Chromatographic retention is the manifestation of the difference in the energetics of solute interactions with the mobile phase components and the solvent modified stationary phase components. Instantaneous dipole-induced dipole interactions or dispersion interactions forces are dependent on the polarizabilities of the interacting molecules and their ionization potentials according to the London formula.[47] The polarizability of a solvent is often correlated to its refractive index. Solvents with large indices of refraction are more polarizable and can interact more strongly via dispersive forces. The refractive indices of water, acetonitrile, methanol and tetrahydrofuran are 1.3330, 1.3441, 1.3284 and 1.4072, respectively at 298.15 K.[25] The ionization energies for water, methanol and acetonitrile are 12.59, 10.85 and 12.22 eV/mol, respectively.[48] The polarizabilities and ionization energies for the common HPLC solvents do not vary considerably. Since retention in RP

chromatography decreases with increased percentages of the organic component of a mobile phase, dispersive interactions alone cannot dominate the operative mechanisms. Polar interactions such as dipole-dipole and hydrogen bonding must therefore play a dominant role in the overall retention mechanism.

Solutes and solvents may interact through various dipole interactions such as ion-dipole, dipole-dipole and dipole-induced dipole forces. The dipole interactions are proportional to the dipole moments of the interacting species and inversely proportional to the molecular distances. Dipole interactions are of generally longer range than dispersive forces (inversely proportional to the square of the molecular distances for ion-dipole interactions, for example). Dipole moments (10^{-30} C m, 298.15 K) for some of the common HPLC solvents are 5.9 for water, 11.8 for acetonitrile, and 5.7 for methanol.[25]

When a covalently bound hydrogen atom in one molecule forms a second bond to another atom in a second molecule, the second bond is referred to as a hydrogen bond.[25] Hydrogen bonding is a special example of a dipole-dipole interaction that involves hydrogen atoms covalently bonded to electronegative herteroatoms and hydrogen atoms. Hydrogen bonding interactions are usually restricted to nitrogen, oxygen and fluorine atoms, however anionic species may also participate. Three molecular orbitals may be formed from the three participating elements where the two lower energy orbitals are occupied by the four participating electrons (two from the existing bond and two from the lone pair of second molecule). Because the highest energy orbital (most antibonding) is vacant, the net effect is a lowering of energy.[47] The most important electron pair donors (hydrogen bond acceptors) are oxygen atoms from carbonyls as well as nitrogen atoms in amines. The strongest hydrogen bonds exist between OH---O, OH--

-N, and NH---O atoms, whereas weaker bonds exist between NH---N atoms. Pi electrons can also act as weak hydrogen bond acceptors. The bond dissociation enthalpy for hydrogen bonds is about 13 to 42 kJ/mol or about 10 times weaker than a covalent bond but about 10 times stronger than dispersive intermolecular interaction forces.[25] When present, hydrogen bonding dominates van der Waals interactions.

As discussed further in sections to follow, it is important to recognize that the solvent-solvent intermolecular interactions play a significant role in determining retention. The energy required to form a cavity to accommodate a solute in a solvent is, according to Horvath, proportional to the surface tension of the liquid. Water exhibits a high surface tension (73 dyn/cm) relative to the common organic cosolvents (29, 22 and 27.6 dyn/cm for acetonitrile, methanol and tetrahydrofuran, respectively[22]) owing to strong intermolecular interactions between the water molecules. As a first approximation, the greater the surface tension of the solvent the greater the retention of a solute as energetics favor exclusion from the mobile phase. Specific interactions between the solvent and solute, however, also play a dominant role.

The solvation or phase transfer of a molecule may be considered as proceeding in several virtual steps:[49]

1. A cavity of appropriate size and shape is created in the solvent to accommodate the solute.
2. The solute particle is inserted into the cavity without interacting with its surroundings.
3. The configuration of the solute molecule relaxes to its equilibrium state in the solution.

4. The solute particle interacts with its environment by dispersion and the various dipole interactions.

5. The molecules of the solvent or solvent mixture adjust themselves to the presence of the solute.

The interactions in steps 4 and 5 are generally of short-range, proceeding over the range of a few molecular diameters only. Dispersive and dipole forces decrease with $1/r^6$ and are thus short-range effects. When ionic solutes are present the solute interactions with the environment and the manner in which the solvent molecules adjust themselves are farther reaching as the resulting ion-ion and ion-dipole forces are on the order of $1/r^1$ and $1/r^2$, respectively. The electrostatic forces are not only longer range, but are also generally stronger than dispersive forces.[49]

In solvent mixtures used for most HPLC mobile phases, microscopic treatment of the solvent-solute interactions suggests that a solute may be preferentially solvated by one of the solvent components more than the other(s). Preferential solvation has been invoked to explain such behavior stems from deviations from the ideal behavior expected from Raoult's law of vapor pressure depression of binary mixtures and spectroscopic studies.[25] Charged molecules are preferentially solvated by the more polar of the solvent molecules in a mixture through both nonspecific solute-solvent association due to dielectric enrichment in the solvent shell about the solvated ion and specific solute-solvent interactions such as hydrogen bonding. Ionic solutes therefore tend to be preferentially solvated with solvents of higher dielectric constants. In addition, solutes that can donate or accept a hydrogen bond preferentially interact with solvent molecules that can accept or donate them, respectively.[49]

Since many of the solutes of interest in HPLC separations are ionizable, it is important to understand the interactions of the solutes with water and the organic modifiers employed as mobile phases. In light of the Jaroniec theory of chromatography and the newly developed theories of Carr and Neue, the nature of the interactions of the solvents with both the analyte and the stationary phase will strongly influence both retention and selectivity of such processes. Binary aqueous-organic mixtures are commonly employed in HPLC separations because the composition of the solvent exerts a strong influence on analyte retention and these effects often show a monotonic dependence on the proportions of the two solvents. Because such influences can differ for solutes with different physical properties, solvent composition can be tailored to optimize selectivity of chromatographic retention for analyte resolution. The properties of the solvents that influence solvation such as dipole moment, dielectric constant, hydrogen bond acidity and basicity change with composition.[50,51] In fact, the structure of the solvent mixture changes significantly upon the increase of one of the components. In water-acetonitrile mixtures, for example, infrared spectroscopic studies have indicated that acetonitrile is solvated by water molecules at low mole fractions of the latter. At mole fractions of acetonitrile greater than 0.3, microheterogeneity is observed where water molecules are preferentially solvated by other water molecules and acetonitrile molecules preferentially interact with other acetonitrile molecules. At mole fractions of acetonitrile greater than 0.7, water exists as isolated molecules, short chains or rings, or associated with acetonitrile molecules via hydrogen bonding.[50,52,53] It is important to gain further understanding of microscopic solvent structure as this may determine the environment about a given solute and thus its potential to interact via the various forces

discussed. The microscopic solvent structure about an ion may have a profound impact on its ability to interact. An ion solvated primarily by water molecules will exhibit relatively low reactivity toward a counterion owing to the shielding effect of this high dielectric solvent. Conversely, an ion solvated by acetonitrile, which possesses a lower dielectric, may interact more strongly with a respective counterion. In addition, the solvation of the stationary phase support material will likewise influence its ability to interact with a given solute. The energetics of solute and stationary phase solvation, and thus chromatographic retention, are fundamentally determined by the physical properties and structure of the solvent mixture.

1.4.2 The effect of solvent composition on acid-base equilibria

Chromatographic retention of ionizable solutes is highly dependent on the form of the species present in the system. The cationic form of a base, for example, may be able to interact via ion-exchange and be preferentially retained as compared to the neutral form. In chromatographic processed dominated by solvophobic mechanisms, the more polar cationic form of a base will be less retained. The degree of ionization for an acid or base is a function of the properties of the surrounding solvent medium. Solvent properties that affect solute ionization include the solvent acidity, basicity, dielectric constant and the ability of the solvent to solvate the various species involved in the equilibrium.[25]

Dissociation of an acid into a proton and anion requires energy to overcome the electrostatic attraction of the ionic products. Some of this energy can be provided by

stabilization of the ions by interactions with solvent molecules. In addition, the solvent has some capacity to shield the ions from the electric field of other solute ions. The Born equation presented in Eq. **1-1** relates the dielectric constant (ε_r) of a medium to the work necessary for dissociation of an acid (HA ? $H^+ + A^-$) in an amphoteric solvent (SH, SH^+),

$$\Delta G^{\circ}_{electrostatic} = \frac{1}{4p \cdot e_0} \cdot \frac{N_A \cdot z_{HA}^2 \cdot e^2}{2 \cdot e_r \cdot r_{HA}} \qquad \text{(Equation 1-1)}$$

The symbols ε_0, z, N_A and r_{HA} correspond to the permittivity of vacuum, number of elementary charges, Avogadro constant and the radius of HA, respectively.

The equation is an approximation because only the effect of dielectric constant on the degree of ionization is considered. The equation assumes a spherical shape for the interacting species and thus fails to account for such effects as electron delocalization for larger anions that will influence solvation. Delocalization results in lower solvation enthalpy than for smaller anions on which the charge in more localized. The equation also fails to take into account potential solvent-solvent molecular interactions or the nonelectrostatic interactions on which the energetics of the overall solvation depend. The Born equation may, however, be used to qualitatively predict how the charge of an acid affects the ionization constant in solvents of differing dielectric constants. Since the Gibbs free energy is related to the equilibrium constant, K_a, through Eq. **1-2,** it may be used to predict how the pK_a of an acid-base reaction may shift as a function of the medium dielectric constant.

$$\Delta G^{\circ} = -RT \cdot \ln K \qquad \text{(Equation 1-2)}$$

Based on this treatment, an acid is predicted to become more acidic and more ionized with an increase in solvent ε_r. Protonation of a basic molecule is also more favorable with increasing solvent dielectric constant, but ionization is less favorable as the solvent dielectric decreases. Such is the case as the proportion of organic modifier is increased. Dielectric constants for the common HPLC solvents span a wide range, and are 78.30, 32.66, 35.94 and 7.58 for water, methanol, acetonitrile and tetrahydrofuran, respectively.[25]The solvation ability of a solvent medium is not fully expressed by its dielectric constant alone because this one constant does not describe other specific interactions such as dipole-dipole, ion-dipole and hydrogen bonding. The specific interactions, however, often play a dominating role in determining ionization equilibria, therefore the bulk solvent properties such as dielectric constants are not sufficient to completely predict ionization nor chromatographic retention.

1.4.3 Acidity scales in aqueous-organic mixtures

Uncertainties persist regarding the extent of ionization of nearly all solutes in water-organic mixtures with greater than 70% (v/v) organic modifier. Additions of organic modifiers such as methanol or acetonitrile are known to alter both mobile phase pH and the pK_a values of solutes including buffers because they provide for differential stabilization of neutral and ionized forms of solutes.[42,54] Since reports of pK_a values for solutes in aqueous-organic mobile phases are scarce [40,55,56], reliable predictions of retention behavior for ionized solutes require more information about the relationship

between composition of solvent mixtures used for HPLC separations and the extent of solute ionization.

The relationships between solvent composition and solute pK_a values depend on the differential energetics of solvation of charged and uncharged forms of solutes.[43] Although acetonitrile has a higher dipole moment than water (11.8 vs. 5.9 10^{-30} Cm), solvation of ionic solutes in acetonitrile is less favorable than in water owing to the smaller dielectric constant in the former (35.1 vs. 76.6 at 298K).[39] The dependence of pK_a values upon the composition of various aqueous-organic mixtures has been established for analytes from several structural classes using both methanol [57] and acetonitrile [40] modifiers. These relationships, however, may not be applicable to different classes of compounds as the dependence of pK_a values on the organic percentage varies significantly for differing structural features. In the case of acetonitrile, knowledge of solute pK_a values is often not known for mixtures with greater than 60 v/v% acetonitrile. Most reports suggest that increasing acetonitrile content shifts acid-base equilibria from charged solutes toward their neutral forms.[35,58-60] Because dielectric constants of the common organic modifiers employed in HPLC separations are lower than the dielectric constant for water, ionization is less favorable as the fraction of organic component increases.

Determination of the extent of solute ionization in acetonitrile/water/ammonium acetate mixtures that are commonly used for LC-MS analyses presents challenges owing to effects of solvent composition on acid-base equilibria and the reference scale used for pH determination. It is common for pH to be adjusted by adding either acetic acid or ammonia to aqueous solutions, followed by addition of acetonitrile. However, the

addition of acetonitrile influences acid dissociation equilibria and can be expected to alter solvent acidity.

Solute pK_a values have been determined in various solvents using potentiometry [55,58,61], conductometry [60-62], ultraviolet spectroscopy [55], liquid chromatography (LC) [63], capillary electrophoresis (CE) [56,60,64-66] and nuclear magnetic resonance (NMR) spectroscopy.[67] Spectroscopic methods such as uv-vis and NMR depend on changes in spectroscopic properties as a function of changing pH. Using LC and CE, the dependence of solute elution as a function of pH is an indirect measure of the extent of solute ionization. Although CE and LC methods allow for simultaneous determination of pK_a values of multiple compounds, interactions of protonated bases with stationary phase or capillary wall surface silanols are common and may give inaccurate measures of solute ionization.[56,63] Potentiometry and conductometry provide measures of changes in electrical properties of a solution that result from solute ionization. The dependence of solute pK_a values on the organic modifier content of the mixture becomes nonlinear at high percentages reportedly due to the effects of ion-pairing and analyte homoconjugation. These effects have largely limited pK_a determinations based on separation techniques and potentiometry to solutions containing about 60 v/v% or less organic modifier, depending on the co-solvent used.[55,56]

Since the interesting retention characteristics of the fluorinated phase occur at high percentages of organic modifier, knowledge of the degree of solute ionization in these media is needed to establish the fundamentals underlying the U-shaped retention behavior described above. It has been well established, however, that both mobile phase pH and the pK_a values of analytes are altered with the addition of organic

modifier.[42,54] Since established pK_a values for analytes in aqueous-organic mobile phases are scarce [40,55,56], there is a need to estimate the pK_a values in such solvent systems.

1.5 Scientific challenges and rewards in studies of chromatography fundamentals

Retention and selectivity in chromatographic separations arise from complex interactions of solutes, mobile phase components and stationary phase supports that are often poorly understood. These interactions influence the energetics of phase transfer, but the properties of solvent, stationary phase, and solute at the interface have not been well characterized. Since the strength of most experimental approaches lies in probing properties of the bulk solution rather than the interface, we build models based on solution properties to explain interfacial phenomena responsible for chromatographic retention. A great deal of research on chromatography fundamentals is based on the assumption that solution and interfacial properties are systematically related, and solution properties can be used as the basis to develop chromatographic separations. It is due to limitations in our knowledge of solution and interfacial properties that over 30 years of HPLC research has failed to develop a complete model for retention and selectivity for chromatographic processes.

To further advance our ability to predict analyte retention and optimize chromatographic separations, the research described in the following chapters has aimed to uncover the molecular attributes of solutes that contribute to retention using a silica-based pentafluorophenylpropyl (PFPP) stationary phase. The richness of potential

interactions with solutes using this phase provides a system complementary to traditional reversed-phase materials for investigating the roles of dispersive, polar, and ionic retention mechanisms. In Chapter 2, an initial survey of solute attributes is undertaken to identify the fundamental interactions that contribute to the unique selectivity observed using the fluorinated phase. The discovery of dominant ionic interactions in certain water-acetonitrile mixtures for some, but not all, basic solutes, necessitated knowledge of solute degree of ionization in a wide variety of solvents used in chromatographic separations. In Chapter 3, proton NMR chemical shifts were determined for several basic solutes as a function of organic modifier and pH. These relationships were employed to determine the degree of solute ionization in water-acetonitrile binary mixtures. Differences between these experimental results and the extent of ionization calculated based upon literature pKa values were used to explain why some basic analytes exhibit U-shaped retention whereas others do not.

Since ion-exchange with stationary phase surface silanols was initially implicated as a dominant interaction on the PFPP phase, further studies were conducted to explore the ion-exchange properties of the phase and the fundamental reasons for its existence. The availability of surface ionized silanol groups on PFPP, bare silica and C18 phases were assessed by measuring the retention of the quaternary amine bretylium ion as a function of mobile phase pH. The results demonstrate 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 and Carr.[27,28] In this model, simultaneous interaction of analytes with the bonded phase ligand (dispersive or polar interactions) occurs in conjunction with ionic interactions with surface silanol groups. This model is fundamentally different than models that treat the ionic and dispersive interactions separately. Finally, the practicality of employing the PFPP phase for the retention and separation of a traditionally difficult set of polar analytes is demonstrated in Chapter 4. The knowledge gained from the fundamental interaction studies was shown to lead to rapid HPLC method development.

This research complements ongoing efforts by many prominent researchers in the field of chormatographic science. Roses, et al, have published extensively in recent years on the subject of solute ionization in solvent mixtures common to HPLC.[33,36-44,57-59,68-70] Their work, however, has centered on the influence of solute ionization in chromatographic systems lacking dominant ion-exchange interactions. In the studies to follow, the influence of ion-exchange mechansms on overall retention are highlighted and the impact of solute ionization in chromatographic solvent systems in light of these ion-exchange mechanisms is investigated. Although much of the current literature related to chromatographic science is application based, several prominent researchers such as Carr and Neue continue to investigate fundamental interactions that contribute to retention and selectivity.[26-28,33,71,72] This effort lends credence to newly proposed retention mechanisms from these groups involving simultaneous nonelectrostatic and ion-exchange

interactions and thus further enhances our knowledge of these complex chromatographic processes.

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Chapter 2

Solute Attributes and Molecular Interactions Contributing to "U-Shape" Retention on a Fluorinated High-Performance Liquid Chromatography Stationary Phase

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2.1 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 Da) 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 ionization 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.[4-7] In particular, pentafluorophenylpropyl (PFPP) phases exhibit both reversed-phase and normal-phase retention for polar analytes, which has shown to be dependent 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-phase 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.[4,6,8,9] Although this "U-Shape" retention has been observed on traditional alkyl columns[10], the magnitude of normal-phase retention is dramatically increased using the PFPP stationary phase.[7] Bij, et. al., attributed the irregular retention profiles using the alkyl columns to a dual retention mechanism involving both solvophobic and silanophilic interactions.[11] 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.[12,13] Due

to unique selectivity of the fluorinated phases, they are becoming more widely used as an alternative to traditional alkyl systems.[14] Sadek, *et al.*, used linear solvation energy relationships (LSER) to demonstrate selectivity differences for polar, nonionizable analytes on perfluorinated supports compared to hydrocarbon bonded phases.[15] The small differences they observed in dispersive and polar interactions on fluorinated phases in comparison to alkyl and phenyl stationary phases, however, does 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.[16] The Neue study grouped the selectivity of fluorinated phases as distinct from C18 and cyanopropyl phases, however, rigorous studies to elucidate the interactions responsible for this observation were not reported. In a related column classification study, Euerby investigated 135 commercially available stationary phases.[14] 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.[17] 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 initial 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 C18 phases were assessed by measuring the retention of quaternary ammonium 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 and Carr.[18-20] Selectivity and peak shape issues are also discussed.

2.2 Experimental

2.2.1 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 (Milwaukee, WI, USA) 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.

2.2.2 HPLC Columns, Conditions and Apparatus:

Discovery HS F5 (silica-based pentafluorophenylpropyl phase), Discovery HS C18 (silica-based octadecyl phase) 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 internal diameters. The available physical characteristics of the columns are shown in Table 2-1. Both the PFPP and C18 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.

Table **2-1**: 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

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 at 25ºC 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 HPLC 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 ultraviolet (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 to 0.67 minutes.

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 liter with water. The mobile phases consisted of 80:20 buffer:acetonitrile 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.

2.3 Results and Discussion:

2.3.1 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 2-2, and their structures are presented in Figure 2-1. 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 C18 as stationary phases. The capacity factor, k', was calculated using Eq. 2-1:

$$k' = (t_R - t_0)/t_0 \qquad \text{(Equation 2-1)}$$

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

Table 2-2: Analyte Ionization and Octanol-Water Partition Coefficients

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

*denotes data taken from SRC PhysProp Database
^denotes data taken from Japanese Drug Database
(http://chrom.tutms.tut.ac.jp/JINNO/DRUGDATA/55nortriptyline.html)
# denotes data taken from [21]

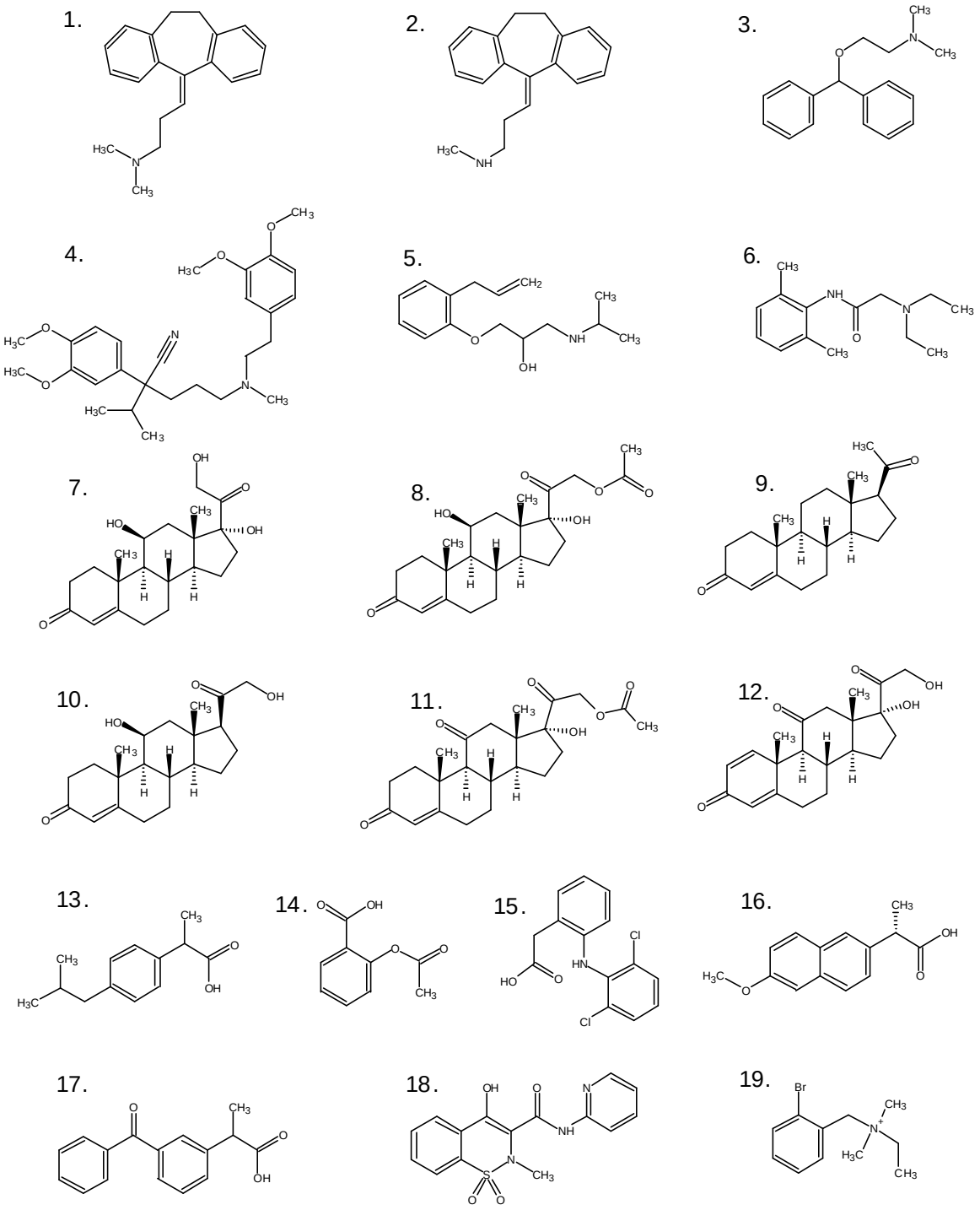

Figure **2-1**: Structures of Analytes Used in the Study:

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. diclofenac 14. ibuprofen 15. aspirin 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 Tables **2-3**, **2-4**, **2-5**, and **2-6.** Comparison of the retention profiles at pH 4 shows that $k'_{PFPP} >> k'_{C18}$ for all basic compounds. The C18 phase exhibits slightly greater retention for both neutral and acidic probes, where the average increase in k' is 1.1 and 0.6 for neutrals and acids, respectively. On both the PFPP and C18 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 15-35 times greater on the PFPP phase compared to C18 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.

Table **2-3**: Capacity Factors as a Function of % Acetonitrile on PFPP Phase, pH 4

Analyte	Capacity Factor (k') at % Acetonitrile					
	40	50	60	70	80	90
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

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

Table **2-4**: Capacity Factors as a Function of % Acetonitrile on C18 Phase, pH 4

Analyte	Capacity Factor (k') at % Acetonitrile					
	40	50	60	70	80	90
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

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

Table 2-5: Capacity Factors as a Function of % Acetonitrile on PFPP Phase, pH 6.7

Analyte	Capacity Factor (k') at % Acetonitrile					
	40	50	60	70	80	90
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	^	^	^

* analytes overly retained

^ analytes exhibited split peaks about the hold-up time

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

Table **2-6**: Capacity Factors as a Function of % Acetonitrile on C18 Phase, pH 6.7

Analyte	Capacity Factor (k') at % Acetonitrile					
	40	50	60	70	80	90
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

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

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, ami triptyline, 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 **2-5**. 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 Figure 2-2 and Figure 2-3. 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 C18 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.

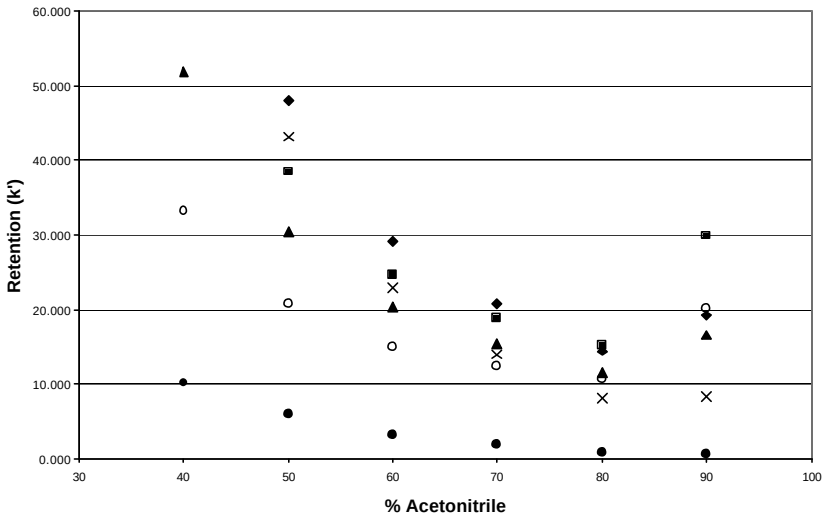

Figure 2-2: Retention Profiles (k') of Basic Probes on PFPP at pH 6.7

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

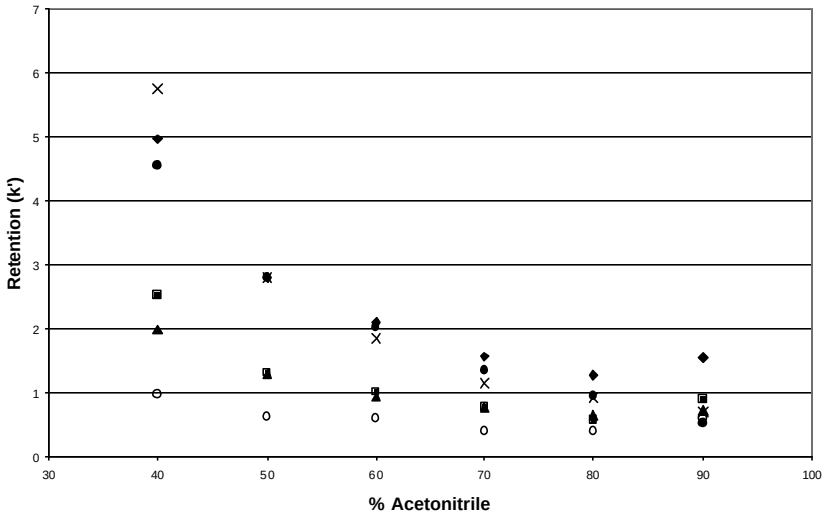

Figure **2-3**: Retention Profiles (k') of Basic Probes on C18 at pH 6.7

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

Table **2-7** provides a comparison of capacity factors obtained for the basic analytes on PFPP at the two pH conditions employed. 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 **2-2**), 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 both a 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.[22,23] 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.

Table 2-7: Comparison of Capacity Factors Obtained on PFPP at Neutral and Acidic pH Values

Compound	**k' at pH 4**	**k' 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 x 4.6mm, 5 µm particles. Other conditions: flow rate: 1 mL/min, temperature: 35°C, detection: 220 nm.

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.[24,25] Studies aimed at the determination of degree of ionization for solutes in highly organic solvents (See Chapter 3) 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 ionization from 99% at the lower pH to just 40% at the higher pH 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 pH 6.7 is more substantial for the more basic secondary amines that are more extensively protonated at both pH values further supports this hypothesis. The effects of organic modifier on silanol pK_a values are discussed in the following sections.

2.3.2 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 C18 and PFPP columns are surprising, as the physical and chemical characteristics for the stationary phases provided in Table 2-1 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 C18 bonded versions of each by acquiring retention data for bretylium ion as a function of mobile phase pH.[18] 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 C18 columns. Mobile phases ranging in pH from 2 to 8 (see experimental section) were prepared such that the ammonium ion concentration was held constant at 25 mM. A plot of retention (log k') for each stationary phase vs. mobile phase pH is presented in Figure 2-4.

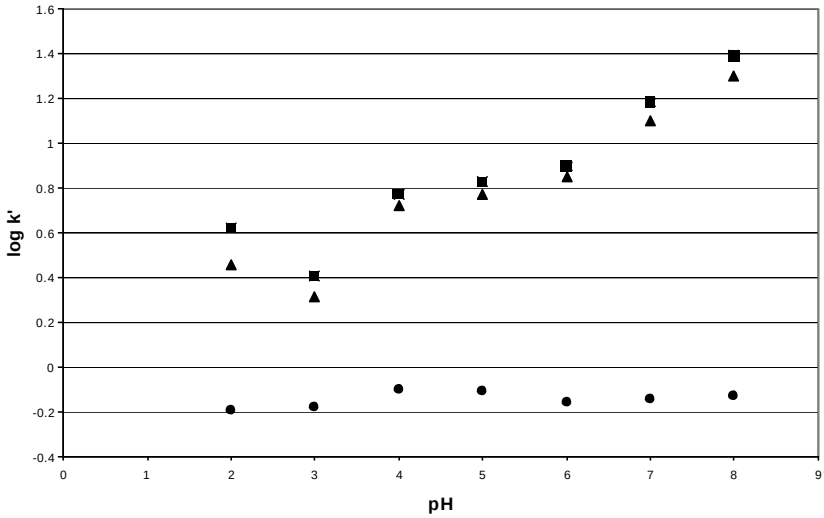

Figure **2-4**: Retention (k') of Bretylium Ion as a Function of pH

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 x 4.6mm, with a 5 µm particle size. Mobile phases consisted of 80:20, buffer:acetonitrile. 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.

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 C18. 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 C18 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 C18-

bonded stationary phase have been observed by others.[23,24] The difference in the silanol pK_a values between the PFPP and C18 bonded phases explains the enhanced retention and "U-Shape" profiles of the basic analytes observed on the PFPP relative to C18. Since the C18 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 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.[26] 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 [27], 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.[28] For example, the pK_a of acetic acid increases from 4.76 in pure water to 6.57 in 60% acetonitrile.[29] 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 C18 stationary phase inhibits the analytes from reaching the surface, rendering the ionized silanols on the C18 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.

2.3.3 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. Retention 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$^{2)}$.[30] The data presented in Table **2-8**, 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.

Table **2-8**: Comparison of Capacity Factors of Basic Analytes at 90% Acetonitrile on PFPP and Bare Silica Stationary Phases

Compound	k' Silica	k' 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 x 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.

Several authors have recently described a hydrophobically-assisted ion-exchange mechanism where the ion-exchange process is enhanced by neighboring hydrophobic sites[18,31]. 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. The simultaneous or separate combination of these interactions, as depicted in Figure **2-5**, provides the enhanced retention observed using the PFPP phase over bare silica and C18-modified columns.

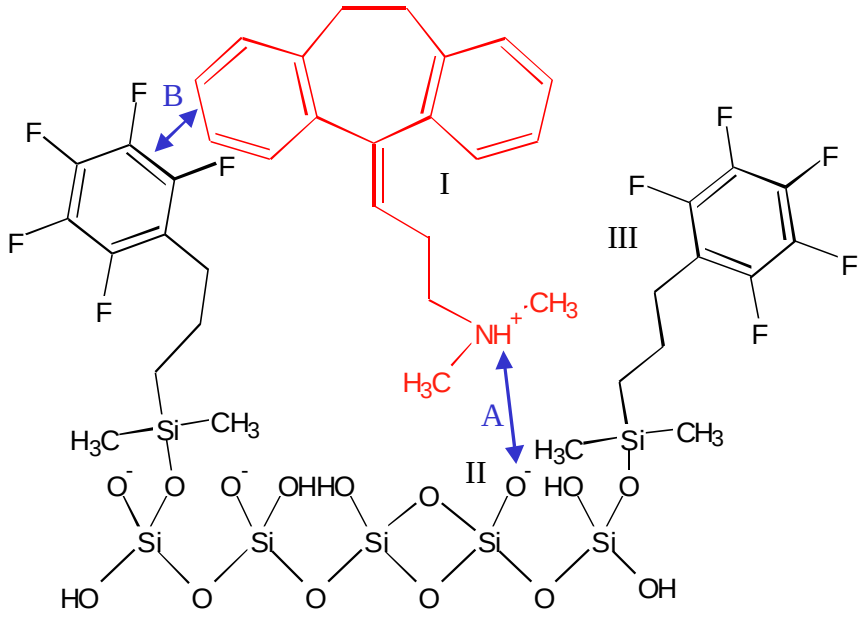

Figure **2-5**: Representation of Potential Interactions of Protonated Basic Analytes with PFPP Stationary Phase

2.3.4 Dependence of "U-Shape" Retention on Analyte pK_a :

Figure **2-6** 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 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.[32] 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 to 7 in 65% methanol mobile phases.[33] 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 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.

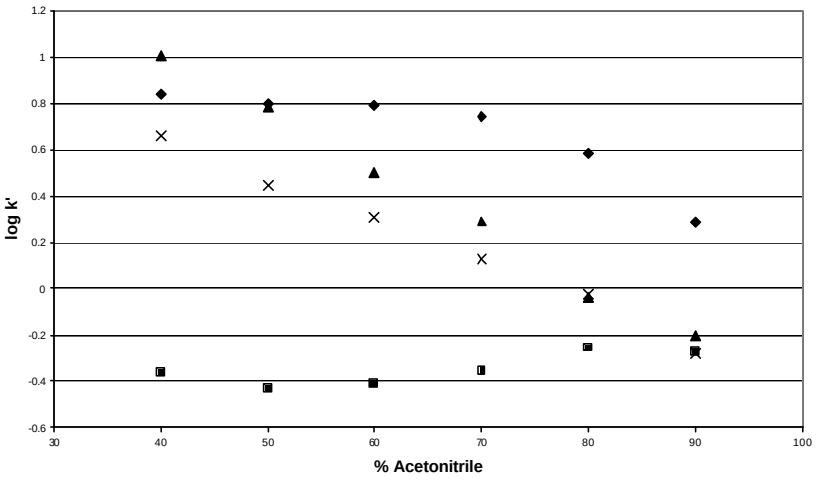

Figure **2-6**: Comparison of Lidocaine Retention Under Various Conditions Studied

Retention of lidocaine on ♦ PFPP at pH 4, ■ C18 at pH 4, ▲ PFPP at pH 6.7 and x C18 at pH 6.7 from 40% to 90% acetonitrile. Columns: PFPP = Discovery HS F5, C18 = 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 x 4.6 mm, 5µm particle size. Other conditions: flow rate 1 mL/min, temperature 35°C, detection UV at 220 nm.

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 (See Chapter 3). This further substantiates the dependence of "U-Shape" retention on ionic interactions.

2.3.5 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.[22] 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.[30] 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 C18 phase. At high organic conditions, however, highly symmetrical peaks shapes were observed for the basic analytes on the PFPP phase. See Figure 2-7. 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.[34] 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 C18 phase is presumably low enough to have minimal impact on peak asymmetry.

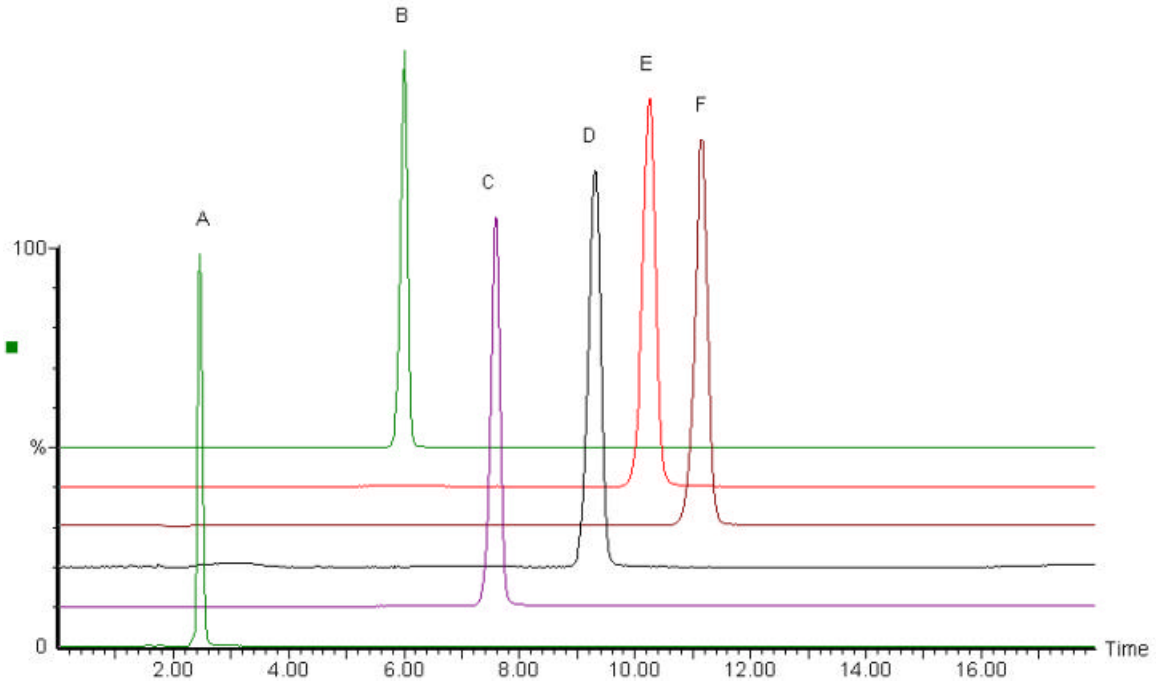

Figure **2-7**: LC-MS Traces of Basic Analytes on PFPP Phase

Retention of basic probes (lidocaine (A), verapamil (B), alprenolol (C), diphenhydramine (D), amitriptyline (E) and nortriptyline (F)) on Discovery HS F5. Column: 15 cm x 4.6 mm, 5 µm particle size. Mobile phase: 14 mM ammonium acetate (pH 6.7) in water:acetonitrile (10:90, v/v). Flow rate: 1 mL/min, temperature: ambient, detection: MS (ESI, positive ion mode).

2.4 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 implicate 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 C18 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 C18 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 C18 phases. The lower hydrophobicity of the PFPP phase compared to C18 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 C18 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 C18 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 [24] and Carr [31] 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 the degree of ionization of the analyte. 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.[35] Excellent selectivity, peak symmetry and LC-MS compatibility make this approach a powerful tool for the analysis of basic analytes.

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Chapter 3

Determination of Acid Dissociation for Protonated Organic Bases in Water-Acetonitrile Mixtures using ^{1}H NMR Spectroscopy: Contributions of Ion-Exchange to Retention in Liquid Chromatographic Separations

Manuscript in preparation for submission to Analytical Chemistry

3.1 Introduction

In Chapter 2 it was reported that ion-exchange makes substantial contributions to retention of organic bases on a silica-based fluorinated stationary phase, particularly when the mobile phase contains a high fraction of acetonitrile. Such effects were more pronounced on the fluorinated phase than on C18, and showed a qualitative relationship with calculations of the degree of ionization of different solutes based on the pH of the aqueous solution before addition of acetonitrile. Additions of organic modifiers such as methanol or acetonitrile are known to alter both mobile phase pH and the pK_a values of solutes including buffers, with ionization less favorable with increasing organic content.[1,2] However, the increased retention of basic solutes at high acetonitrile concentrations conflicted with our expectation that ionization of both solute and silanol groups of the stationary phase material would decrease with increasing acetonitrile concentration. Great uncertainties persist regarding the extent of ionization in water-organic mixtures with greater than 70% (v/v) organic modifier. Since reports of pK_a values for solutes in aqueous-organic mobile phases are scarce [3-5], reliable predictions

of retention behavior for ionized solutes require more information about the relationship between composition of solvent mixtures used for HPLC separations and the extent of solute ionization.

The relationships between solvent composition and solute pK_a values depend on the solvation energetics of the charged and uncharged forms of solutes.[6] Although acetonitrile has a higher dipole moment than water (3.9 vs. 1.85 D), solvation of ionic solutes in acetonitrile is less favorable than in water owing to the smaller dielectric constant in the former (35.1 vs. 76.6 at 298K).[7] The dependence of pK_a values upon the composition of various aqueous-organic mixtures has been established for analytes from several structural classes using both methanol [8] and acetonitrile [5] modifiers. These relationships, however, may not be applicable to different classes of compounds and, in the case of acetonitrile, are often limited to less than 60 v/v% acetonitrile. Most reports suggest that increasing acetonitrile content shifts acid-base equilibria from charged solutes toward their neutral forms.[9-12] As a consequence, positively charged bases such as protonated amines usually become more acidic with increasing organic modifier, whereas compounds with groups such as carboxylic acids that form anions upon proton loss usually become less acidic as organic content increases.

The extent of solute ionization in acetonitrile/water/ammonium acetate mixtures that are commonly used for LC-MS analyses are difficult to predict. The effects of solvent composition on acid-base equilibria vary greatly for solutes possessing different functional groups and structure.. In the practice of HPLC, it is common for pH to be adjusted by adding either acetic acid or ammonia to aqueous mobile phases, followed by addition of acetonitrile. Solute pK_a values have been determined in various solvents

using potentiometry [3,10,13], conductometry [12-14], ultraviolet spectroscopy [3], liquid chromatography (LC) [15], capillary electrophoresis (CE) [4,12,16-18] and nuclear magnetic resonance (NMR) spectroscopy.[19] Spectroscopic methods such as uv-vis and NMR depend on changes in spectroscopic properties as a function of changing pH. Using LC and CE, the dependence of solute elution as a function of pH is an indirect measure of the extent of solute ionization. Although CE and LC methods allow for simultaneous determination of pK_a values of multiple compounds, interactions of protonated bases with stationary phase or capillary wall surface silanols are common and may give inaccurate measures of solute ionization.[4,15] Potentiometry and conductometry provide measures of changes in electrical properties of a solution that result from solute ionization. The dependence of solute pK_a values on the organic modifier content of the mixture becomes nonlinear at high percentages reportedly due to the effects of ion-pairing and analyte homoconjugation. These effects have largely limited pK_a determinations based on separation techniques and potentiometry to solutions containing about 60 v/v% or less organic modifier, depending on the co-solvent used.[3,4]

Solution NMR spectroscopy has the advantage of being independent of and unaffected by interactions of solutes with solids. It is also a static, non-invasive technique that uses chemical shift as a direct probe of the electron density or charge density of functional groups of the target solute. Solution ^1H NMR spectroscopy is applicable to nearly all organic molecules, and the high spectral resolution of NMR spectra makes possible the automated determination of the extent of solute charge for multiple solutes in a single analysis.

In this study we demonstrate a relationship between observed chromatographic retention and the degree of analyte ionization in separations where ion-exchange mechanisms dominate retention. The derived relationship shows that there are two solute-dependent variables relevant to ion-exchange contributions to retention: the solute ion-exchange equilibrium constant and the extent of solute ionization. The latter variable is easily calculated if the pK_a of the solute and pH of the system are known. From the well-known relationship between solute retention and the concentration of the mobile phase counter ion in ion-exchange, a modified ion-exchange equilibrium constant may be determined.

There have been several attempts to model the retention of ionic solutes in terms of reversed-phase mechanisms.[13,15,20] However, there is limited information about the effect of the fraction of solute that is ionized upon retention in terms of ion-exchange mechanisms and the dependence of this value on solvent composition in acetonitrile-water mixtures containing the mass spectrometry-friendly additive ammonium acetate. We report in this study the use of NMR spectroscopy for the determination of the extent of solute ionization in water-acetonitrile mixtures, paying particular attention to mixtures of high acetonitrile concentration. The study investigates the effect of adding acetonitrile to aqueous solvents previously adjusted for pH with ammonia and acetic acid on protonation of some model basic pharmaceuticals. The advantage of using a pH scale based on glass electrode measurement following the addition of the organic modifier is demonstrated.

3.2 Theoretical

3.2.1 Derivation of Ion-Exchange Contributions to Chromatographic Retention

Contributions of ion-exchange to retention are best assessed through the relationships between retention and solvent characteristics that influence ion-exchange retention. The observed capacity factor (k'^*) in liquid chromatography is defined as the ratio of the number of analyte molecules associated with the stationary phase, N^*_s, versus the number of analyte molecules associated with the mobile phase, N^*_m, at any given time as shown in Eq. **3-1**. It provides perhaps the most useful measure of analyte partitioning between stationary and mobile phases.

$$k'^* = \frac{N^*_s}{N^*_m} \qquad \text{(Equation 3-1)}$$

In the present study we used only univalent cationic analytes, therefore N^*_s may be expressed as shown in Eq. **3-2:**

$$N^*_s = N_{A_s} + N_{AH^+_s} = V_s[A]_s + V_s[AH^+]_s \qquad \text{(Equation 3-2)}$$

where V_s is the volume of the stationary phase, $[A]_s$ and $[AH^+]_s$ represent the concentrations of the neutral and protonated forms, respectively of organic base A associated with the stationary phase. It follows in Eq. **3-3** that:

$$N^*_m = N_{A_m} + N_{AH^+_m} = V_m[A]_m + V_m[AH^+]_m \qquad \text{(Equation 3-3)}$$

where V_m is the volume of the mobile phase, $[A]_m$ and $[AH^+]_m$ represent the concentrations of the neutral and protonated species, respectively, residing in the mobile phase.

For the ionization reaction:

$A + H_3O^+ \rightleftharpoons AH^+ + H_2O$

the acid dissociation constant, K_A, is defined for a protonated base in Eq. **3-4** as:

$$K_A = \frac{[AH^+]}{[A]} \qquad \text{(Equation 3-4)}$$

Rearranging yields Eq. **3-5.**

$$[AH^+] = K_A[A] \qquad \text{(Equation 3-5)}$$

There remains the possibility that the solvation properties of the stationary phase may differ from the bulk solvent, and the acid dissociation constants in the two regions may differ. At present the nature of the difference between the bulk mobile phase and the solvent associated with the stationary phase is not well defined. In the interest of simplification, we assign a single, averaged ionization equilibrium constant to proceed with the derivation. It should be mentioned, however, that the physical properties of the bonded phase may affect the degree of ionization of an analyte in the stationary phase. Enhanced ionization of the analyte in the stationary phase would be expected to result in increased retention via ion-exchange mechanisms. Substituting Eq. **3-5** into Eq. **3-3** yields Eq. **3-6**.

$$N^*_m = V_m[A]_m + V_m K_A[A]_m + V_m K_A[A]_m = V_m[A]_m(1 + K_A) \qquad \text{(Equation 3-6)}$$

Similarly, as shown in Eq. **3-7**, it can be derived that:

$$N^*_s = V_m[A]_m[k'_A + k'_{AH^+}K_A + k''_{AH^+}K_A] \qquad \text{(Equation 3-7)}$$

where *k?*represents the retention from a partitioning mechanism not involving ion-exchange and *k??*represents retention based on an ion-exchange mechanism. Only the positively charged species can interact with the stationary phase via ion-exchange interactions with ionized surface silanols, whereas both forms can be retained through other non-ionic mechanisms. The derivation assumes the contributions to retention are independent of one another.

Substituting Eq. **3-7** and Eq. **3-6** into Eq. **3-1** yields Eq. **3-8**.

$$k'^* = \frac{k'_A + k'_{AH^+}K_A + k''_{AH^+}K_A}{1 + K_A} \qquad \text{(Equation 3-8)}$$

Eq. **3-8** describes that the observed retention is a combination of partitioning of the neutral species, partitioning of the ionic species and ion-exchange based retention of the ionic species. The contribution of ion-exchange to retention in Eq. **3-8** reduces to Eq. **3-9**.

$$k'^* = \frac{k''_{AH^+}K_A}{1 + K_A} \qquad \text{(Equation 3-9)}$$

From the definition of capacity factor Eq. **3-10** can be derived.

$$k''_{AH^+} = \frac{N_{AH^+_s}}{N_{AH^+_m}} = \frac{V_s[AH^+]_s}{V_m[AH^+]_m} \qquad \text{(Equation 3-10)}$$

For the ion-exchange process involving competition between the ionized analyte AH^+ and mobile phase counter ion, C^+ for the ionized surface silanol, $Si\text{-}O^-$, as shown in Eq. **3-11:**

$$AH^+ + [Si-O^- - C^+{}_s] \leftrightarrow [Si-O^- - AH^+{}_s] + C^+{}_m \qquad \text{(Equation 3-11)}$$

the ion-exchange equilibrium constant involving displacement by a mobile phase cation, K_{IEX}, is given by Eq. **3-12**.

$$K_{IEX} = \frac{[Si-O^- - AH^+]_s[C^+]_m}{[AH^+]_m[Si-O^- - C^+]_s} \qquad \text{(Equation 3-12)}$$

Rearranging gives Eq. **3-13**.

$$[AH^+]_s = [Si-O^- - AH^+]_s = \frac{K_{IEX}[AH^+]_m[Si-O^- - C^+]_s}{[C^+]_m} \qquad \text{(Equation 3-13)}$$

Substituting Eq. **3-13** into Eq. **3-10** and rearranging yields Eq. **3-14**.

$$k'' = \frac{V_s K_{IEX}[Si-O^- - C^+]_s}{V_m[C^+]_m} \qquad \text{(Equation 3-14)}$$

Substituting Eq. **3-14** into Eq. **3-9** yields Eq. **3-15**.

$$k'^* = \frac{\dfrac{V_s K_{IEX}[Si-O^- - C^+]_s K_A}{V_m[C^+]_m}}{1+K_A} \qquad \text{(Equation 3-15)}$$

The term:

$$\frac{V_s[Si-O^- - C^+]_s}{V_m[C^+]_m}$$

varies with mobile phase composition but can be treated as a constant (b) for a given mobile phase composition and stationary phase. Substitution of this term into Eq. **3-15** yields Eq. **3-16**.

$$k'^{*} = bK_{IEX}\frac{K_A}{1+K_A} \qquad \text{(Equation 3-16)}$$

Putting **3-16** in terms of pK_a and pH for a basic analyte yields Eq. **3-17**.

$$k'^{*} = bK_{IEX}\frac{10^{(pK_a - pH)}}{1+10^{(pK_a - pH)}} \qquad \text{(Equation 3-17)}$$

If pH and pK_a are known, the degree of ionization of a basic analyte, D, can be calculated using Eq. **3-18**.

$$D = \frac{10^{(pKa - pH)}}{1+10^{(pKa - pH)}} \qquad \text{(Equation 3-18)}$$

Substitution of Eq. **3-18** into Eq. **3-17** yields Eq. **3-19**.

$$k' = bK_{IEX}D \qquad \text{(Equation 3-19)}$$

Eq. **3-19** shows that there are two analyte-dependent variables in an ion-exchange process: the analyte ion-exchange equilibrium constant, K_{IEX} and the analyte degree of ionization, D. The latter variable is easily calculated if the pK_a of the analyte and pH of the system are known.

To assess K_{IEX} we re-expand b in **3-19** as shown in Eq. **3-20**.

$$k' = \frac{V_s[Si-O^-:C^+]_s K_{IEX} D}{V_m[C^+]} \qquad \text{(Equation 3-20)}$$

For a given column and mobile phase composition $\frac{V_s[Si-O^-:C^+]}{V_m}$ = constant (f), which gives Eq. **3-21**.

$$k'^* = \frac{fK_{IEX}D}{[C^+]} \qquad \text{(Equation 3-21)}$$

From Eq. **3-21** it is clear that a plot of k'^* versus $1/[C^+]$ should yield a slope, m, equivalent to $fK_{IEX}D$. If D is known, the modified ion-exchange equilibrium constant, fK_{IEX}, may be determined through Eq. **3-22**.

$$fK_{IEX} = \frac{m}{D} \qquad \text{(Equation 3-22)}$$

Through the variation of counter ion concentration and subsequent measurement of retention, the modified ion-exchange equilibrium constant for a given analyte may be determined. Differences in the modified ion-exchange equilibrium term for various solutes may then be employed to interpret the effects of analyte structure on ion-exchange processes. In addition, the term may aid in elucidating the dependence of ion-exchange processes on bonded phase chemistry.

3.2.2 ^{1}H NMR Determination of Analyte pK$_a$ Values in Aqueous-Organic Solvents

^{1}H NMR spectroscopy can be used to determine the fraction of solute in ionized form based on the dependence of proton chemical shifts upon pH. From chemical shifts gathered at different pH values, analyte pK$_a$ values can be determined in water-organic mixtures as long as pH measurements can be made. Since proton exchange is fast on the NMR timescale, the observed chemical shift (d_{obs}) is a combination of the chemical shift

of the molecules in the neutral state (d_B) and the chemical shift of the molecules in the ionized state (d_{BH+}) weighted by the mole fraction of each state as shown in Eq. **3-23**.

$$d_{obs} = d_B c_B + d_{BH+} c_{BH+} \qquad \text{(Equation 3-23)}$$

Eq. **3-24** can be derived from the Henderson-Hasselbalch Equation.

$$d_{obs} = \frac{d_{BH+} + d_B 10^{(pH-pKa)}}{1 + 10^{(pH-pKa)}} \qquad \text{(Equation 3-24)}$$

If the chemical shifts for the acid and conjugate base forms of an analyte are known, one can obtain a best fit using Eq. **3-24** for chemical shift data at various pH levels to calculate analyte pK_a values. Experiments may be designed to acquire chemical shift values analytes prepared in solvents of specific mole fractions of water and organic (e.g. acetonitrile) over a range of pH levels, enabling the calculation of pK_a values in solvents common to HPLC analyses.

3.3 Experimental Section

All solvents, standards and reagents were obtained from Sigma-Aldrich (St. Louis, MO USA). A 1.0 liter stock solution of D_2O was adjusted to pH (pD) 11 by the addition of 20.0 mL aqueous ND_4OD (25 wt. %) in D_2O. Subsequent solutions at pD values of 10, 9, 8, 7, 6, 5, 4, and 3 were prepared from 100 mL portions of the stock by the addition of acetic acid-d_4 to give the desired pD value. Portions of each of these solutions were individually diluted with acetonitrile-d_3 to prepare 25.0%, 50.0%, 75.0% and 90.0% acetonitrile by volume. The pD of each solution before and after the addition of the organic solvent was measured using a Beckman (Fullerton, CA USA) 390 pH

meter equipped with a Futura ™ glass electrode filled with saturated KCl. The pH meter was calibrated using pH 4, pH 7 and pH 10 NIST standardized aqueous reference buffers (Sigma-Aldrich) prior to each use. Measurements were taken at 25ºC.

Solutions of amitriptyline, nortriptyline, diphenhydramine, verapamil, alprenolol and lidocaine (Sigma-Aldrich) were prepared in acetonitrile-d_3 at 10 mg/mL. Working analyte samples were then prepared by dilution of 100 µL of the stock analyte solutions to 1 mL using each of the prepared D_2O-acetonitrile-d_3 solutions. The resulting final volume percent of acetonitrile-d_3 for the working samples was 32.5, 55.0, 77.5, and 91.0%.

Proton NMR spectra were acquired using a Bruker (Billerica, MA USA) Avance 400 MHz nuclear magnetic resonance spectrometer equipped with a B-ACS 60 autosampler and a 5 mm BBO BB-^{1}H probe (Bruker). The ^{1}H NMR spectra were acquired using 16 scans and a repetition time of 6 s. The probe temperature, as indicated by instrument read back, was maintained at 18ºC. The acquired free induction decay (FID) data were processed using Advanced Chemistry Development Laboratories (Toronto, ON Canada) Spec Manager software (v. 4.60) to obtain chemical shift values. For the analysis of the variation of amitriptyline pK_a values at various acetonitrile concentrations, each of the NMR tubes was fitted with a coaxial insert (Aldrich) containing $CDCl_3$ that served as an external chemical shift reference (7.25 ppm relative to TMS).[21] For the remainder of the studies the acetonitrile pentet (1.94 ppm relative to TMS) was used as the frequency reference to which all chemical shift values were adjusted. Although we demonstrate in this study that the acetonitrile chemical shift is affected by the percentage of acetonitrile in the solvent, the calculated pK_a values using

the acetonitrile pentet differed by less than 0.1 pK_a unit from values obtained utilizing the external reference. The chemical shift values at different pD conditions were fitted using the least-squares method to Eq. **3-24** to calculate analyte pK_a values.

Chromatographic retention time data were acquired using a Waters (Milford, MA USA) 2790 HPLC system equipped with a quaternary pump, autosampler and a Hitachi (San Jose, CA USA) LaChrom Elite column temperature control module. The HPLC system was connected to a Waters/Micromass ZQ single quadrupole mass spectrometer via an electrospray ionization interface operating in positive ion mode. Mobile phases were prepared at nominally 4 mM, 6 mM and 10 mM ammonium acetate in 90% acetonitrile. A bare silica stationary phase (300 m^2/g surface area, 120 Å pore size and 5 µm particle size) was supplied by Supelco (Bellefonte, PA USA). Retention data were acquired at a flow rate of 1 mL/min and a column temperature of 18ºC. The analytes (amitriptyline, nortriptyline, diphenhydramine, verapamil, and alprenolol) were prepared as a mixture in 91% acetonitrile at 5 µg/ml. Retention data were acquired in triplicate.

3.4 Results and Discussion

The most suitable ^1H NMR response to monitor to assess the extent of solute ionization is one that provides a strong response, is sensitive to the ionization state of the analyte and is well resolved from interfering signals. For these reasons the 6 N-methyl protons of amitriptyline were monitored. These protons provide a strong singlet response that is sensitive to the ionization state because of their close proximity to the basic nitrogen atom. Figure **3-1** depicts one ^1H NMR spectrum for amitriptyline dissolved in

91.0 v/v% acetonitrile and $^{w}_{w}$pH 11. In similar fashion, the N-methyl proton responses for nortriptyline, verapamil and diphenhydramine were chosen. For lidocaine and alprenolol, the terminal methyl protons provided the most suitable response based on these criteria (Figure **3-2**). For both alprenolol and lidocaine, the resonances are split by ^{1}H-^{1}H coupling. The downfield chemical shift for the alprenolol doublet and the central peak in the lidocaine triplet were recorded.

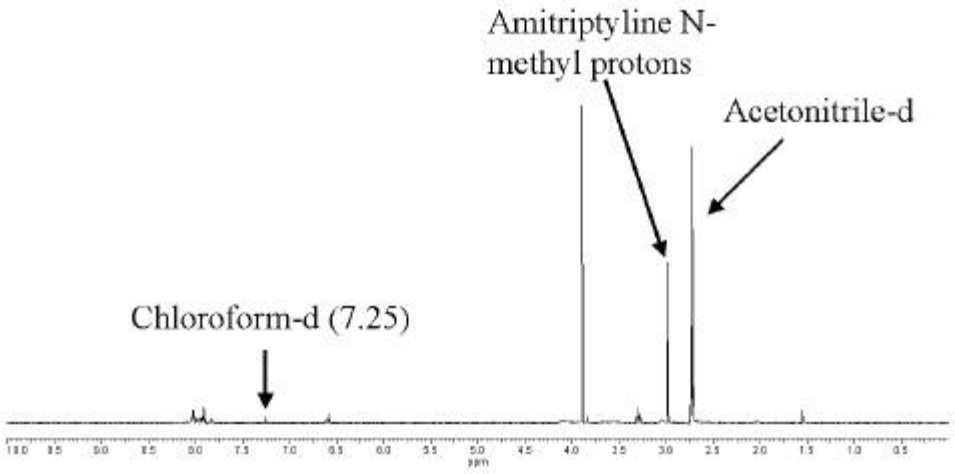
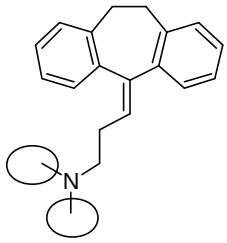
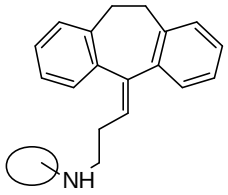


Figure **3-1**: ^{1}H NMR spectrum for amitriptyline, 91.0 v/v% acetonitrile and $^{w}_{w}$pH 11

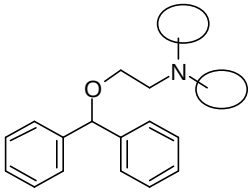
16 scans were acquired with a sweep width of 8278 Hz with a repetition time was 6 s and a probe temperature was 18ºC. The NMR tube was fitted with a coaxial insert containing $CDCl_3$ that served as an external chemical shift reference (7.25 ppm relative to TMS)



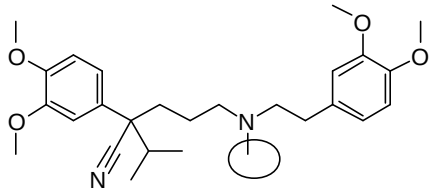
Amitriptyline



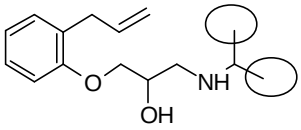
Nortriptyline



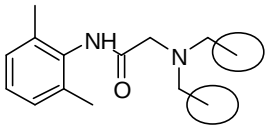
Diphenhydramine



Verapamil



Alprenolol



Lidocaine

Figure **3-2**: Structures of analytes used in the study
Protons designated with a circle were used for chemical shift measurements

The experiments described in this study used concentrations of ammonia and acetic acid to adjust pD in a manner typically used for adjusting mobile phase properties. Table **3-1** details the preparation of the aqueous portion of the solvents utilized in the NMR analyses. The concentrations of ammonia and acetic acid needed for pD adjustment precluded performing all experiments at a standard state with regard to all solute concentrations. However, these effects are predicted to have only minor influence on calculated pK_a values, and the results yield useful information that can be used to

predict ion-exchange contributions to HPLC retention using MS-friendly mobile phases. The use of deuterated solvents is also expected to have a minor effect on the absolute values of pK_a determinations owing to isotope effects on acid-base equilibria. It has been well established that substitution of protons by deuterons can alter pK_a values[22], but the magnitude of such effects is usually small.[22-24] Petersson, *et. al.*, showed an isotope effect of +0.15 pK_a units when the pK_a value for benzylamine was estimated using D_2O versus H_2O.[19] This is in agreement with increased basicity in deuterated solvents reported by Perrin.[25] The utilization of deuterated solvents provides an accurate determination of analyte ionization without the potential interferences associated with spectroscopic interferences from non-deuterated solvents.

Table **3-1**: Preparation of Stock Deuterated Aqueous Solvents

Target pH Level	Measured pD	Volume of CD_3COOD (mL)	Molarity CD_3COOD	Molarity ND_3OD
3	3.33	20.0	3.49	0.132
4	4.04	4.60	0.803	0.132
5	5.03	1.10	0.192	0.132
6	5.98	0.750	0.131	0.132
7	6.93	0.700	0.122	0.132
8	8.11	0.675	0.118	0.132
9	8.88	0.600	0.105	0.132
10	9.98	0.200	0.0349	0.132
11	11.04	0.000	0.000	0.132

3.4.1 Analysis of Amitriptyline Chemical Shifts with pH at Various Acetonitrile Concentrations

Several reports in the literature have discussed the appropriate pH scale in experiments where aqueous-organic mixtures are used. For correlations with chromatographic retention data, for instance, the use of a pH scale based on measurement of pH following the addition of the organic modifier improved predictions of ionizable analyte retention over the pH scale based on aqueous measurements.[9,26-28] For the remainder of this report we will use the recommended IUPAC notation [28] where a left-hand superscript represents the medium in which the pH was measured (s for aqueous-organic mixtures, w for water) and a left-hand subscript denotes the medium in which the electrode was calibrated (herein only aqueous based calibrations were utilized).

The chemical shifts of the methyl protons of amitriptyline (Table **3-2**) were plotted against the ${}^{s}_{w}$pH scale. The data and the fitted line to Eq. **3-24** for each acetonitrile concentration are presented in Figure **3-3**, with each curve giving good fit (R^2 > 0.997) to the experimental data. The calculated pK_a values for all acetonitrile levels are provided in Table **3-3**.

Table 3-2: Amitriptyline N-methyl Proton Chemical Shift as a Function of ${}^{w}_{w}$pH and % Acetonitrile

${}^{w}_{w}$pH	Chemical Shift (ppm) in % Acetonitrile			
	32.5	**55.0**	**77.5**	**91.0**
3.33	Not Determined	3.19	3.35	3.44
4.04	2.99	3.16	3.36	3.42
5.03	2.98	3.10	3.37	3.34
5.98	2.97	3.09	3.33	3.27
6.93	2.97	3.08	3.30	3.26
8.11	2.96	3.06	3.28	3.26
8.88	2.86	2.96	3.19	3.23
9.98	2.49	2.63	2.87	3.08
11.04	2.36	2.54	2.76	2.97

Table 3-3: Calculated ${}^{s}_{w}$pK$_a$ Values For Amitriptyline at Various Percent Acetonitrile Levels

% Acetonitrile	**${}^{s}_{w}$pK$_a$**
32.5	9.27
55.0	9.02
77.5	8.79
91.0	8.39

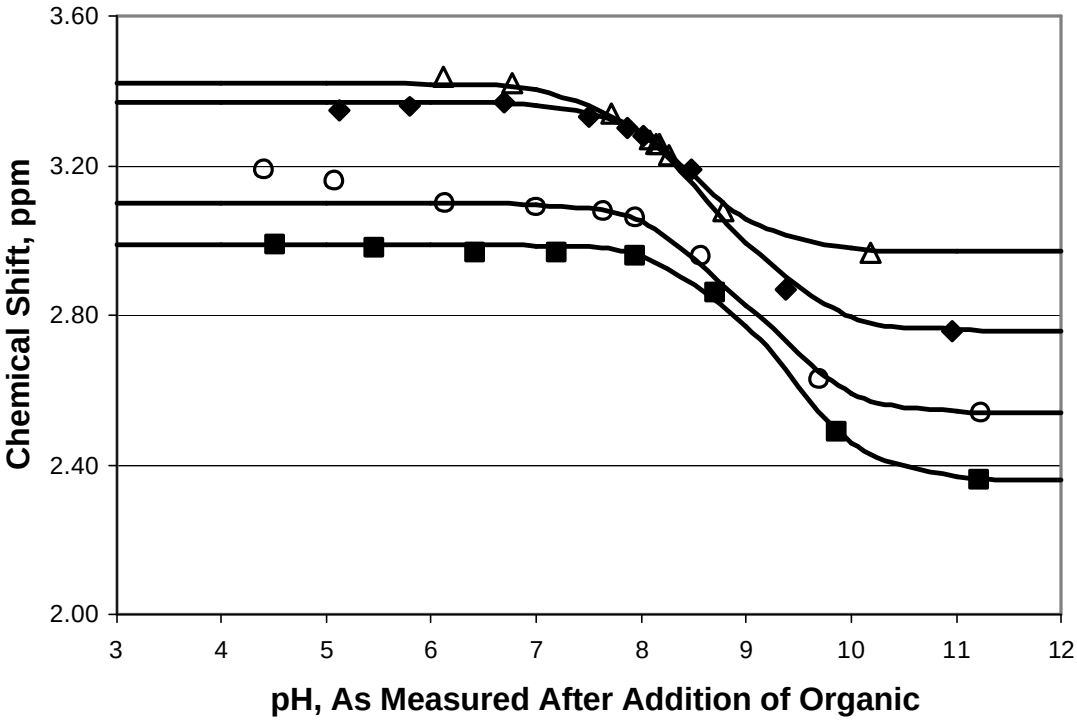


Figure **3-3**: Amitriptyline N-methyl proton chemical shift and fitted lines as a function of ${}^{s}_{w}$pH and v/v% acetonitrile

Open triangle: 91.0%, Diamond: 77.5%, Open circle: 55.0%, Square 32.5%

The calculated ${}^{s}_{w}$pK$_a$ values obtained for amitriptyline in this study are similar to the values reported by Petersson, *et. al*. for a single acetonitrile concentration using NMR spectroscopy (${}^{s}_{w}$pK$_a$ value of 8.89, 50% acetonitrile, 35ºC, phosphate buffer), however, values calculated using the ${}^{w}_{w}$pH scale (data not shown) differ considerably. The value obtained by Petersson (${}^{w}_{w}$pK$_a$ value of 6.92, 50% acetonitrile, 35ºC, phosphate buffer) exhibits a dramatic difference from the value calculated in this study of 9.27 (50% acetonitrile, 18ºC, ammonium acetate buffer).[19] Addition of organic solvent such as

acetonitrile to a buffered aqueous solution causes a shift in pH measured with a glass electrode. The shift depends on the buffering constituents and initial ${}^{w}_{w}$pH because the change in solvent properties may shift acid-base equilibria to a different extent for different buffers.[9,26] The similarity of the values using the ${}^{s}_{w}$pH scale as opposed to the ${}^{w}_{w}$pH scale suggests that the ${}^{s}_{w}$pH scale more accurately reflects the hydrogen ion activity of the solvent and is apparently less dependent on the buffer components used in the preparation.[7,19]

As expected, the ${}^{s}_{w}$pK_a values for protonated amitriptyline decrease with increasing acetonitrile content. At about 90% acetonitrile the ${}^{s}_{w}$pK_a value is approximately one pH unit lower than the literature aqueous pK_a value of 9.4.[29] A decrease in pK_a values with increasing organic is a general behavior of neutral organic bases not only in water-acetonitrile mixtures but also in water-methanol and other aqueous-organic solvents.[9,10,30] Several reports in the literature cite a linear relationship of analyte pK_a value with percent acetonitrile levels up to 60%.[4] At higher acetonitrile levels, however, nonlinear behavior of analyte and buffer pK_a values with increasing organic modifier has been reported based on potentiometric measurements.[10] A plot of pK_a values for amitriptyline against the mole fraction acetonitrile (not shown) was observed to be linear ($R^2 = 0.99$) to $\chi = 0.77$ acetonitrile (91v/v%). The y-intercept of the linear equation corresponding to 100% aqueous is 9.4; equivalent to the literature pK_a value.

An increase in chemical shift is indicative of a decrease in electron density about the target proton. The magnitude of this effect reflects differences in the solvation of an

analyte in the various aqueous-organic solvents. For the water-acetonitrile solvent systems, water is expected to provide better stabilization of the electron deficient cation relative to acetonitrile as it is capable of donating electons.[31] As the percentage of water is decreased, electron density about the solvated analyte decreases, yielding an increase in chemical shift. Examination of the data depicted in Figure **3-3** reveals that both the neutral and ionic forms of amitriptyline (high and low pH, respectively) exhibit methyl group chemical shifts that increase with increasing percent acetonitrile. At $^{s}_{w}$pH 5, for example, the chemical shift increases from 3.0 ppm to 3.4 ppm for the 32.5 v/v% and 91 v/v% acetonitrile solvents, respectively. At $^{s}_{w}$pH 11, where the molecular base is predominant, the chemical shift increases from 2.4 ppm at 32.5 v/v% acetonitrile to 3.0 ppm in the 91 v/v% condition. The magnitude of chemical shift, measured relative to $CDCl_3$ that is not in contact with the analyte, increase is larger for the neutral species than the predominantly ionized form. These data indicate that a loss of electron density about the neutral form exists as well as the expected loss of electron density about the cationic form as the fraction of acetonitrile increased. The neutral species may therefore be increasingly sharing its electrons with the dipolar acetonitrile molecules. A similar effect on the chemical shift of the methyl protons of acetonitrile with decreasing water content was also observed. Figure **3-4** shows that the chemical shift of acetonitrile increases (downfield shift) with increasing percent acetonitrile. The large dependence of chemical shifts upon changes in solvent composition may influence comparisons of NMR spectra to spectrum libraries generated using LC-NMR where acetonitrile or other solvents are utilized as internal frequency reference materials.

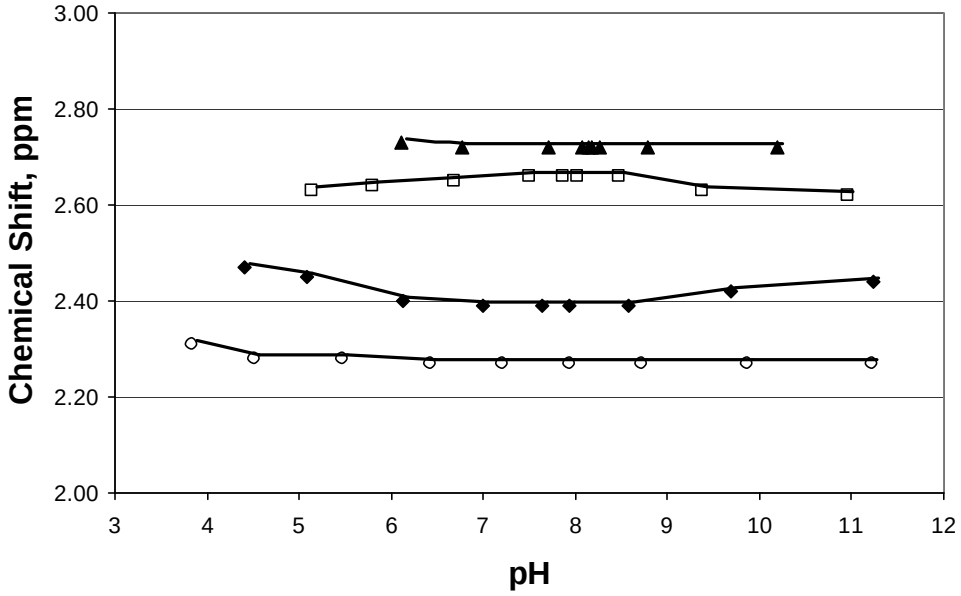


Figure **3-4**: Acetonitrile chemical shift as a function of $^{s}_{w}$pH at different v/v% of acetonitrile

Triangle: 91.0%, Open square: 75.5%, Diamond: 55.0%, Open circle: 32.5%

3.4.2 Acid Dissociation Determination of Other Bases at 91 v/v% Acetonitrile

The pK_a values for acetonitrile were calculated at several levels of organic modifier percentage to better understand how organic content influences the acid-base equilibria. In a similar fashion to the determination of pKa values for amitriptyline at different solvent compositions, ${}^{s}_{w}pK_a$ values were calculated for nortriptyline, diphenhydramine, verapamil and alprenolol for 91 v/v% acetonitrile to ascertain the dependence of pK_a variation with analyte structure in highly organic mixtures. In each case, good fits of experiment to theory ($R^2 > 0.98$) were obtained. The results are listed in Table 3-4.

Table 3-4: Comparison of Literature ${}^{w}_{w}pK_a$ and Experimental ${}^{s}_{w}pK_a$ Values from this study at 91 v/v% Acetonitrile of some basic drugs

Analyte	Literature ${}^{w}_{w}pK_a$	Calculated ${}^{s}_{w}pK_a$
Amitriptyline	9.4	8.39
Nortriptyline	9.7	8.94
Diphenhydramine	9.0	8.31
Verapamil	8.9	7.97
Alprenolol	9.7	8.66

The experimental ${}^{s}_{w}pK_a$ values at 91 v/v% acetonitrile are lower than the literature aqueous pK_a values for each of the basic analytes. The effect of increasing organic on solute pK_a values has been attributed to medium effects based on theintermolecular

interactions of the various species in the different aqueous-organic solvent mixtures. The stabilization of the protonated base due to solvation decreases as the water content of the solvent decreases. The effect of preferential solvation, in this case, is to shift the ionization equilibrium (lower pK_a) such that the ionized species is minimized.[4,6,18,20]

Lidocaine was also included in this study. The chemical shifts for lidocaine at 91% acetonitrile are plotted against the ${}^{s}_{w}$pH scale in Figure **3-5**. The data demonstrate that lidocaine does not approach a fully ionized state even when the solvent was adjusted to pH 3 before addition of acetonitrile. This finding highlights the importance of using the ${}^{s}_{w}$pH scale to predict analyte ionization in acetonitrile-water mixtures because lidocaine, at the ${}^{w}_{w}$pH value of 3.33, should be >99.9% protonated (${}^{w}_{w}pK_a$ = 8.01) before addition of acetonitrile. The pH values of the water-acetonitrile mixtures were measured following the addition of the organic component (${}^{s}_{w}$pH). The relationship between the ${}^{s}_{w}$pH measurements and the initial ${}^{w}_{w}$pH measurements are shown in Figure **3-6**. The data illustrate that the ${}^{s}_{w}$pH measurements are significantly higher at low ${}^{w}_{w}$pH values and that the magnitude of the increase is greater with increasing percent acetonitrile. The two pH scales then coalesce at about pH 8. At high ${}^{w}_{w}$pH values, the ${}^{s}_{w}$pH measurements decrease with increasing percent organic. The data in Figure **3-6** show that the ${}^{s}_{w}$pH measured at the ${}^{w}_{w}$pH of 3.33 is slightly greater than 6. From the data in Figure **3-5** it is apparent that the ${}^{s}_{w}pK_a$ for lidocaine is less than 6.5 in 91% acetonitrile, and this represents a shift of nearly 2 pH units from the pK_a in 100% aqueous solvents. This

finding substantiates the lack of ionic interaction observed for lidocaine chromatographed on a silica-based fluorinated stationary phase ($^{w}_{w}$pH 4, 90 v/v% acetonitrile) in Chapter 2.

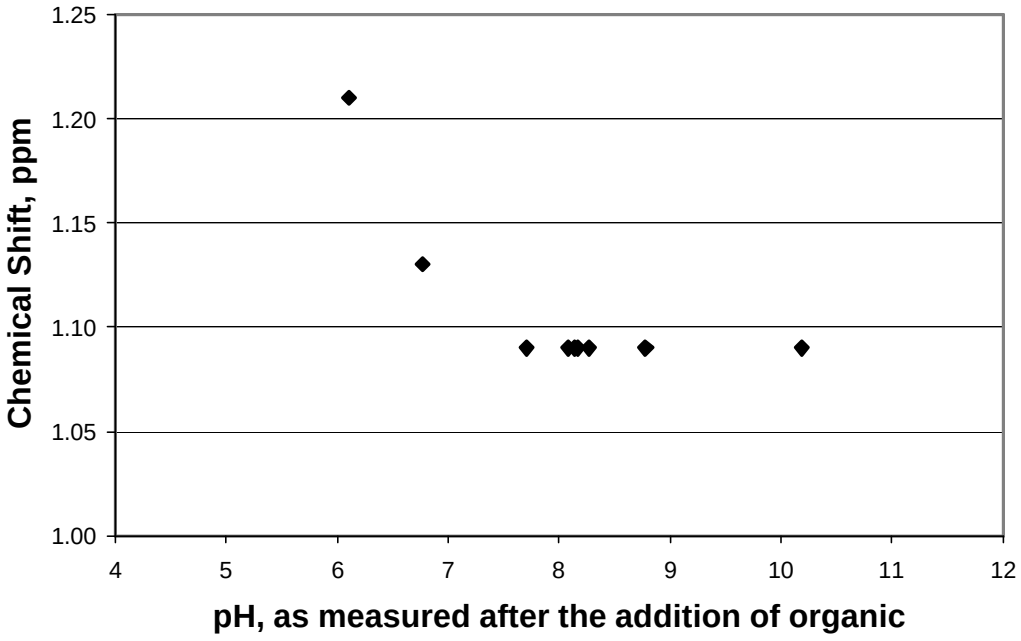


Figure **3-5**: Lidocaine chemical shift as a function of $^{s}_{w}$pH at 91.0 v/v% acetonitrile

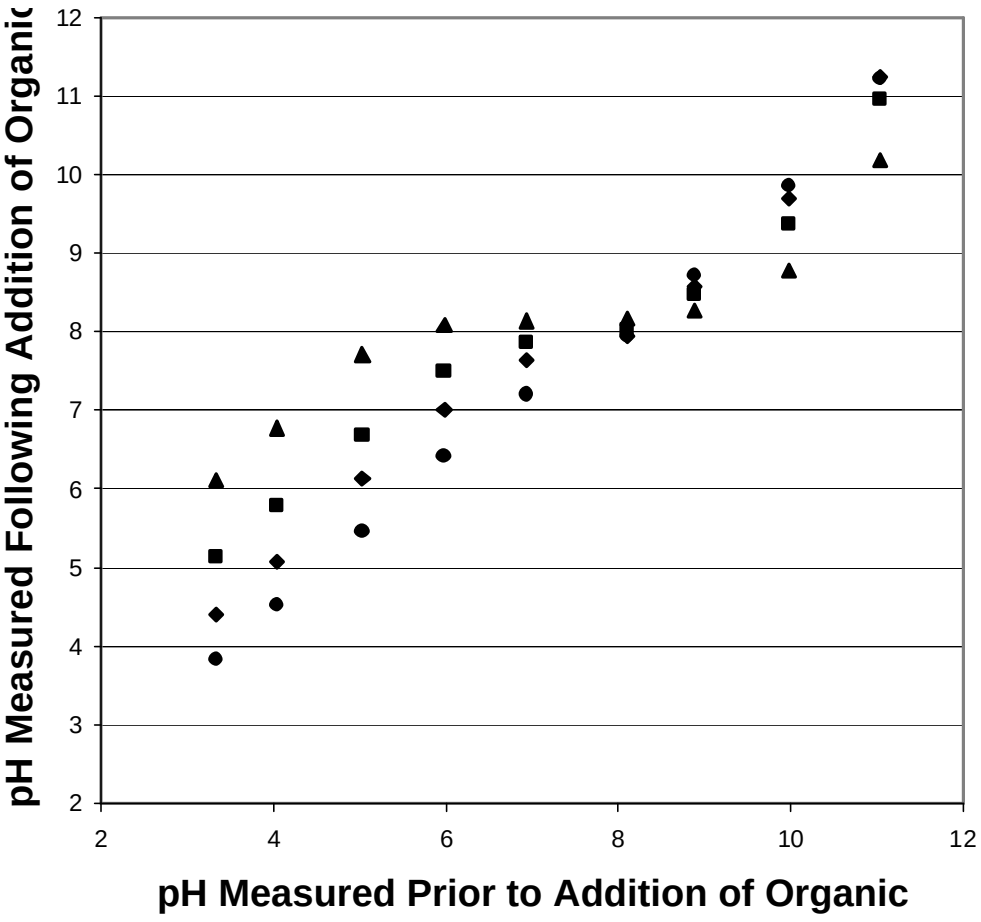


Figure **3-6**: Comparison of ${}^{s}_{w}$pH and ${}^{w}_{w}$pH Measurements

The pH of the aqueous ammonium hydroxide solution was adjusted with acetic acid prior to the addition of acetonitrile (${}^{w}_{w}$pH). Subsequent pH measurement was taken following the addition of acetonitrile (${}^{s}_{w}$pH). Each measurement utilized a glass electrode filled with saturated KCl calibrated using pH 4, pH 7 and pH 10 NIST standardized aqueous reference. Measurements were taken at 25ºC. Triangle: 90.0%, Square: 75%, Diamond: 50%, Circle: 32.5%

3.4.3 Relationship between analyte ionization and chromatographic retention

The accurate measurement of solute pK_a values is essentialfor predicting molecular interactions that depend on the ionization state of the analyte. One such process is the chromatographic retention of basic analytes on silica-based chromatographic media. Stationary phases derived from silica substrates exhibit surface silanol groups that may be present in an anionic form. Ion-exchange interactions between a cationic solute and the anionic surface will therefore be dependent on the extent of ionization of both species. The accurate measure of both analyte pK_a and mobile phase pH are crucial for controlling and manipulating analyte ionization and HPLC retention.[2,32]

Retention of basic analytes at high organic modifier percentages has been shown to exhibit dependence on ion-exchange mechanisms on certain polar stationary phases. In a recent review, Weng pointed out that there is a significant contribution of ion-exchange mechanisms toward the retention of basic analytes on bare silica.[33] From Eq. **3-21** a log-log relationship as shown in Eq. **3-25** between the ion-exchange retention for a monovalent analyte ion and the mobile phase concentration of a singly charged counterion can be derived:

$$\log k'_{IEX} = -\log[C^+] + \log(fK_{IEX}D) \qquad \text{(Equation 3-25)}$$

Eq. **3-25** predicts that the slope of a plot of log k?$_{IEX}$ versus log[C^+] should equal –1 if the retention is based solely on ion-exchange mechanisms. Several singly charged cationic analytes were chromatographed on a bare silica column using 90 v/v% acetonitrile at ammonium concentrations of 4 mM, 6 mM and 10 mM. Slope values for

each of the solutes ranging from –0.913 to –0.947 were obtained from the linear regression analysis of log-log plots according to Eq. **3-25**. The slopes approach the value of –1 indicating that the retention of these bases on bare silica at 90 v/v% acetonitrile is dominated by ion-exchange mechanisms. These results are in agreement with the assumptions underlying Eq. **3-9**, where the contributions to retention from non-ionic mechanisms on bare silica are assumed to be minimal.

Eq. **3-21** predicts that a plot of k?* versus $1/[C^+]$ should yield a slope equivalent to $\phi K_{IEX} D$. The term ϕK_{IEX} represents a modified ion-exchange equilibrium constant for a given analyte that exhibits a particular degree of ionization (D). Retention data obtained on a bare silica stationary phase using 90 v/v% acetonitrile at 4 mM, 6 mM and 10 mM ammonium ion concentrations were obtained. From the linear regression analysis of the retention dependence on mobile phase counterion concentration, the modified K_{IEX} values for each of the analytes were determined through Eq. **3-22**. Using the ${}^{s}_{w}pK_a$ values determined by NMR spectroscopy and the measured ${}^{s}_{w}pH$ of the mobile phase systems (7.95±0.05), the degree of ionization (herein designated ${}^{s}_{w}D$) was calculated through Eq. **3-18**. The results are given in Table **3-5**.

Table **3-5**: Calculated Degree of Ionization and Modified Ion-exchange Equilibrium Constants for Some Basic Drugs.

Retention data obtained on a bare silica stationary phase using 90 v/v% acetonitrile at 4 mM, 6 mM and 10 mM ammonium ion concentrations at pH 7.95 was obtained. The modified K_{IEX} values for each of the analytes were determined through Eq. **3-22**. Using the ${}^{s}_{w}pK_a$ values determined by NMR spectroscopy and the measured ${}^{s}_{w}pH$ of the mobile phase systems (7.95±0.05), the degree of ionization (herein designated ${}^{s}_{w}D$) was calculated through Eq. **3-18**

Compound	${}^{s}_{w}pK_a$	${}^{s}_{w}D$	ϕK_{IEX}
amitriptyline	8.4	0.688	3.34E-02
nortriptyline	8.9	0.906	3.09E-02
diphenhydramine	8.3	0.693	3.22E-02
verapamil	8.0	0.508	1.99E-02
alprenolol	8.7	0.835	3.16E-02

From Table **3-5** it is shown that the modified ion-exchange equilibrium constants vary little between the analytes with the exception of verapamil. As shown in Figure **3-2**, the basic nitrogen in verapamil is located between relatively bulky moieties. As such, the protonated amine may be more restricted (relative to the other 4 bases) from approaching the ionized surface silanol groups. This effect renders the ion-exchange interactions, which are charge- and distance-dependent, weaker. This is an important observation because differences in chemical modification about the amine may be exploited to enhance selectivity in chromatographic systems where ion-exchange makes substantial contributions to retention.

Based on Eq. **3-19**, if the modified ion-exchange equilibrium constants for a given set of analytes are similar, the degree of dissociation would account for the order of retention in a chromatographic system based on ion-exchange interactions. Excluding verapamil due to the different ϕK_{IEX} value, the data in Table **3-5** predicts a retention order of amitriptyline=diphenhydramine<alprenolol<nortriptyline. The retention of the basic analytes on bare silica using 4 mM ammonium acetate in 90% acetonitrile is shown in Figure **3-7**. Amitriptyline and diphenhydramine are shown to coelute and are followed by alprenolol then nortriptyline as predicted.

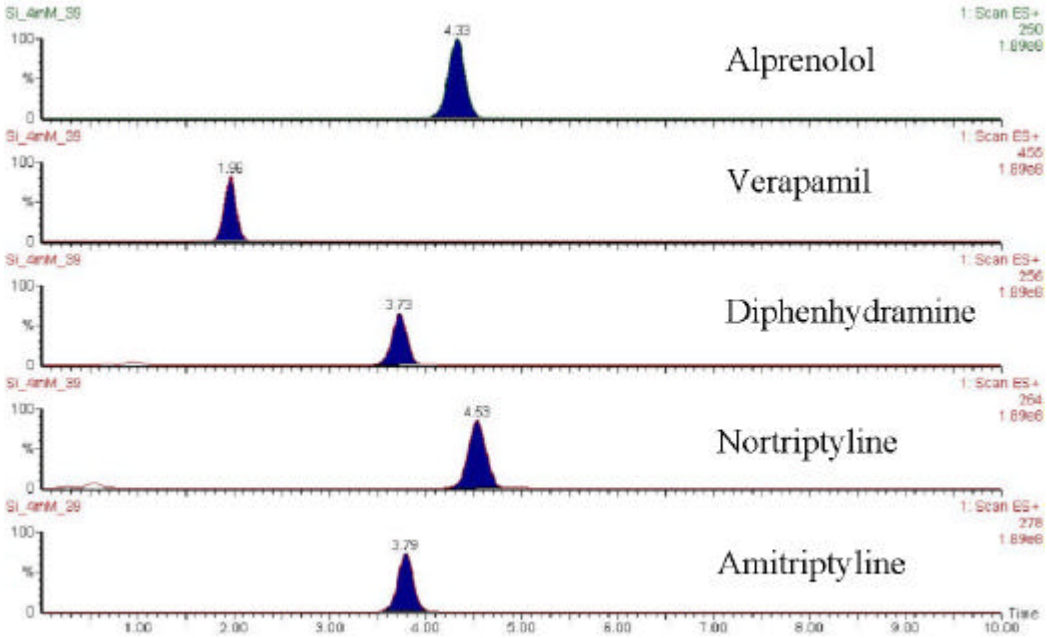


Figure **3-7**: Reconstructed ion chromatograms of basic analytes run on a bare silica HPLC column

Electrospray ionization interface operating in positive ion mode. Mobile phase: 4 mM ammonium acetate in 90% acetonitrile ($^{S}_{w}$ pH = 7.95), Column: bare silica 50 cm x 4.6 mm, 5 µm particle size, flow rate 1 mL/min, temperature: 18ºC. The analytes were prepared as a mixture in 91% acetonitrile at 5 µg/ml

3.5 Conclusions

NMR spectroscopy is a rapid and useful technique for determining the degree of ionization and pK_a values for solutes in HPLC mobile phases. Such investigations can be performed in a relatively high-throughput mode using NMR autosamplers, and are accessible to many laboratories. The variation of chemical shifts for protons in the vicinity of a basic nitrogen atom as a function of the medium pH can be related through the Henderson-Hasselbalch equation to calculate analyte pK_a values. The use of a pH scale based on the measurement following addition of organic component ($^{s}_{w}$pH) was shown to better reflect the thermodynamic reality of the environment about the analyte as compared to pH measurements made in aqueous solvents before addition of organic.

Our findings in the case of lidocaine highlight the notion that one should not assume that bases are protonated in high acetonitrile content solvents even if the aqueous pH is adjusted to two pH units lower than the literature (aqueous) pK_a value, as is commonly practiced. For control of interactions that depend on analyte degree of ionization, it is critical to measure the pH following the addition of organic and to recognize that pK_a values change significantly with the addition of organic modifier. The pK_a values for the basic analytes used in this study were shown to decrease by approximately one pK_a unit in approximately 90 v/v% acetonitrile from their aqueous value. Where ion-exchange mechanisms are present, improved prediction and manipulation of HPLC selectivity results from more accurate knowledge of the analyte degree of dissociation values.

In summary, NMR spectroscopy provides a non-invasive and direct measure of solute acid-base equilibria and their dependence on the pH of the media. Knowledge of such equilibria enhances our ability to further explore the fundamental mechanisms of retention in chromatographic processes. Specifically, we used the results to explain the lack of ion-exchange behavior of lidocaine on a fluorinated stationary phase at $^{w}_{w}$pH 4, 90 v/v% acetonitrile reported in Chapter 2.

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Chapter 4

Rational Method Development Strategies on a Fluorinated Liquid Chromatography Stationary Phase: Mobile Phase Ion Concentration and Temperature Effects on the Separation of Ephedrine Alkaloids

This chapter has been submitted for publication in Journal of Chromatography A

4.1 Introduction

Liquid chromatography is most often accomplished on reversed-phase stationary phases based on alkyl-bonded silica particles.[1] Stationary phases manufactured using alternative bonded phases have, however, become increasingly popular due to the differences in selectivity and retention that they often provide. Fluorinated stationary phases, in particular, are gaining acceptance as alternatives to common C18 and C8 phases owing to their unique selectivity.[2] In addition to dispersive solute-stationary phase interactions available on traditional alkyl phases, the pentafluorophenylpropyl phase also allows for dipole-dipole, pi-pi, charge transfer and ion-exchange interactions.[2,3]

Phases with perfluorinated functional groups have shown unique selectivity in several column classification studies. Neue grouped fluorinated phases separate from C18 and cyanopropyl phases, claiming differences in “extended polar selectivity” and “phenolic selectivity.”[4] In an investigation of 135 commercially available stationary

phases, Euerby noted significant selectivity differences for a set of fluorinated phases.[5] These findings prompted further study in which the authors reported orthogonal selectivity of the fluorinated phases compared to both phenyl- and alkyl- based columns.[2] Orthogonal selectivity was especially evident for the retention of basic analytes, which are notorious for being difficult to retain on reversed phase columns. Needham reported the exceptional retentivity of a series of tricyclic antidepressants and calcium channel blockers at high percentages of organic modifier on a fluorinated phase, noting the substantial increase in LC-MS response under such conditions.[6] In a recent report (Chapter 2), we investigated the molecular interactions contributing to retention on a pentafluorophenylpropyl (PFPP) stationary phase at high organic modifier content.[7] A major conclusion from this study was that retention of protonated bases at high percentages of organic modifier is characterized by strong ion-exchange interactions with ionized surface silanol groups plus additional nonionic interactions.[7]

The objective of the present study was to rapidly develop a method for the separation of several related alkaloids using the knowledge that ionic interactions dominate mechanisms of retention on the PFPP phase. First, a linear relationship of basic analyte retention with the reciprocal of mobile phase ammonium ion concentration is established. The linear dependence is consistent with ion-exchange contributions to retention, and allows for optimization of mobile phase ion concentration in just two experiments. For demonstration purposes, we chose a set of ephedrine alkaloids as a representative set of polar, basic analytes that are difficult to retain and separate on traditional alkyl stationary phases. Synephrine was included as it is a constituent of currently available herbal dietary supplements indicated for weight loss with structural

features similar to the banned ephedrine-based supplements.[8] Several literature methods have been reported for separation of ephedrine alkaloids, however these often call for time-consuming derivatization procedures[9,10], ion-pairing modifiers[11] or strong cation-exchange stationary phases.[12] The latter methods are inappropriate for LC-MS analyses owing to the nonvolatility of the mobile phase additives. Gay and White recently reported a system suitable for LC-MS analysis of ephedrine alkaloids, however, the high aqueous content of the mobile phase employed is likely to limit the sensitivity of the method.[13] Although our intent here was not to rigorously develop a method for the analysis of all ephedrine-related compounds, suitable separation for the analytes under the high organic conditions on the PFPP phase provides a starting point for further development of a sensitive LC-MS method.

In addition to mobile phase ion concentration, the dependence of retention on temperature at high organic modifier on the PFPP phase was also investigated. The dependence of retention on temperature in classical reversed-phase chromatography has been well studied[14-17]. In most reports, retention is shown to decrease as a function of elevated temperature, however the anomalous observation of increasing retention with increasing temperature has been reported.[17-19] In each case the anomalous behavior involved the retention of a basic solute, suggesting that there may be some connection to underlying ion-exchange phenomena. Temperature effects for systems dominated by ion-exchange retention mechanisms are not well understood. The unexpected relationship of analyte retention with temperature observed in this study is discussed in terms of the effects of mobile phase solvating power and its dependence on temperature.

4.2 Experimental

4.2.1 Reagents and Standards

All compounds chosen for the retention studies were obtained from Sigma (St. Louis, MO, 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 mobile phase for the study to a final concentration of 100 µg/mL or 10 µg/mL. All HPLC reagents were obtained from Aldrich (Milwaukee, WI, 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 Barnstead Nanopure Diamond ™ (Boston, MA, USA) source.

4.2.2 HPLC Columns, Conditions and Apparatus

Pentafluorophenylpropyl-bonded liquid chromatography columns (Discovery HS F5) were obtained from Supelco (Bellefonte, PA, USA). The columns, packed with 5 µm particles with surface area of 300 m^2/g were either 50 mm or 150 mm in length and had 4.6 mm internal diameters. Mobile phases employed in the study were prepared by dissolving ammonium acetate in either 85% or 90% aqueous acetonitrile mixtures to obtain the desired molar concentration of ammonium counter ion. All mobile phases were premixed. The pH values of the mobile phases were unadjusted (pH 6.7 prior to the addition of organic modifier). HPLC-UV analyses were conducted using a Hitachi (San

Jose, CA USA) LaChrom Elite HPLC system equipped with a quaternary pump, autosampler, in-line degassing unit, temperature control unit and photo-diode array UV detector. Acquisitions were made using EZChrom Elite version 3.1.3 from Scientific Software Inc. (Pleasanton, CA USA). Retention data were acquired in triplicate using 10 µl injections, a flow rate of 1 mL/min and UV detection at either 220 nm or 215 nm. Temperature was varied within the specifications of the instrument (10ºC to 65ºC). System hold-up time (t_0) was estimated by injecting pure methanol. The possibility of retention for traditional t_0 markers such as uracil on the PFPP phase precluded their use. Hold-up times measured in this manner were consistent throughout the studies.

LC-MS data were acquired using a Waters (Milford, MA USA) 2790 HPLC system equipped with a quaternary pump, autosampler and a Hitachi LaChrom Elite column temperature control module. The HPLC system was connected to a Waters/Micromass ZQ single quadrupole mass spectrometer via an electrospray ionization interface operating in positive ion mode. Retention data were acquired at a flow rate of 1 mL/min and a temperature of 45ºC. The analytes (ephedrine alkaloids and synephrine) were prepared as a mixture in 90% acetonitrile at 10 µg/ml.

4.3 Results and Discussion

4.3.1 Dependence of Retention on Mobile Phase Ion Concentration

At high organic mobile phase compositions, the dominant mechanism contributing to retention for cationic analytes on the PFPP phase is ion-exchange.[7] For

an ion-exchange process involving singly charged analytes, the dependence of retention on mobile phase counter ion concentration (ammonium in this case) may be expressed as given in Equation **4-1**:

$$\log k' = -\log[C^+]_m + \log\beta_{IEX} \qquad \text{Equation } \mathbf{4\text{-}1}$$

where $[C^+]_m$ represents the concentration of counter ion in the mobile phase and β_{IEX} is a constant for a given system which incorporates the phase ratio, ϕ, ion-exchange capacity of the stationary phase, $[A^-]_s$, and the ion-exchange equilibrium constant, K_{IEX} as shown in Equation **4-2.**[20]

$$\beta = \phi K_{IEX}[A^-]_s \qquad \text{Equation } \mathbf{4\text{-}2}$$

The relationship of retention dependence on mobile phase counter ion concentration was investigated by monitoring the retention of amitriptyline [See Figure **4-1**] on the PFPP phase from 2 mM to 20 mM ammonium acetate in 85% acetonitrile . As shown in Figure **4-2**, a linear dependence of capacity factor (k') with the reciprocal of mobile phase ammonium ion is demonstrated by the coefficient of variation of 0.9990. The linear relationship has also been verified using a number of basic analytes under similar conditions on the PFPP phase in our laboratories [data not shown]. Note that the slope of the regression line in **4-2** is only 0.67, and not 1 as predicted by Equation **4-1**. A slope of 1 is obtained where ion-exchange is the only mechanism contributing to analyte retention. The PFPP phase is thus shown to interact with the solutes via additional mechanisms of retention as reported previously.[7]

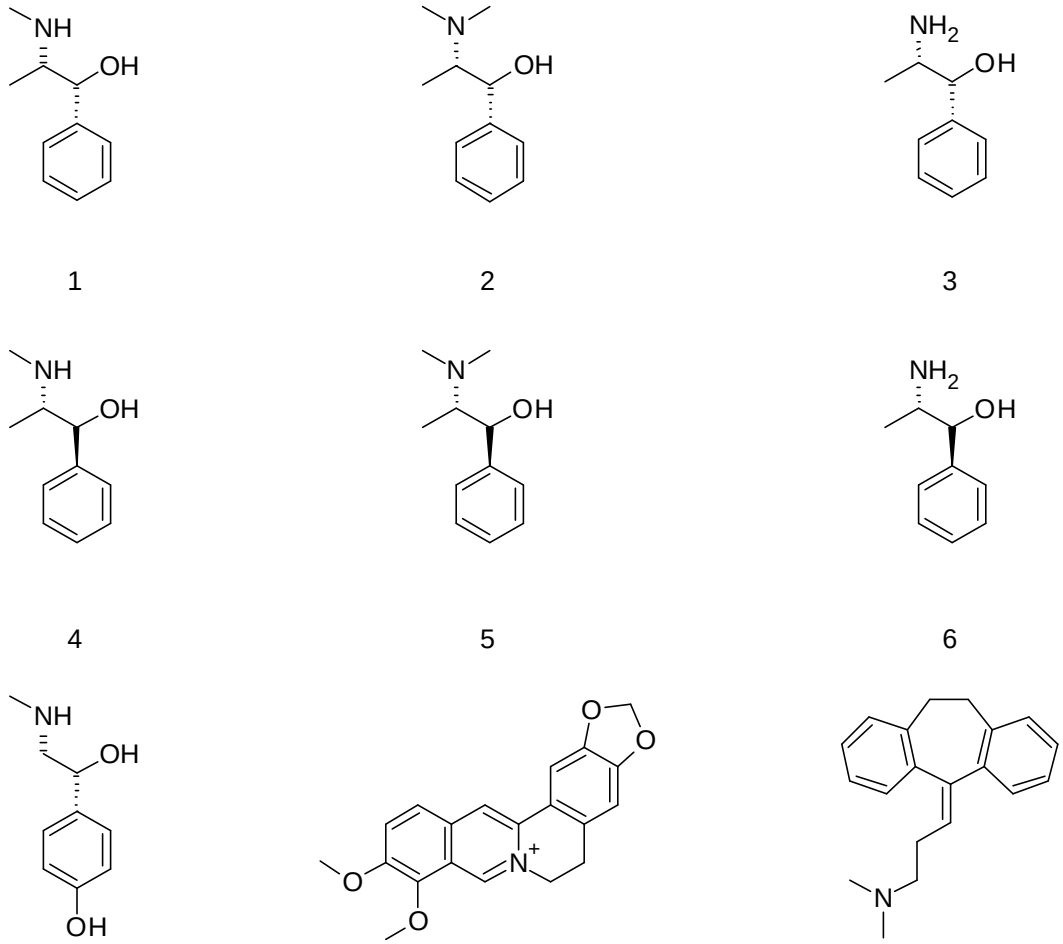

Figure **4-1**: Structures of ephedrine alkaloids

synephrine, berberine and
amitriptyline. 1. ephedrine, 2. methylephedrine, 3. norephedrine, 4. pseudoephedrine, 5. methylpseudoephedrine, 6. norpseudoephedrine, 7. synephrine, 8. berberine, 9. amitriptyline

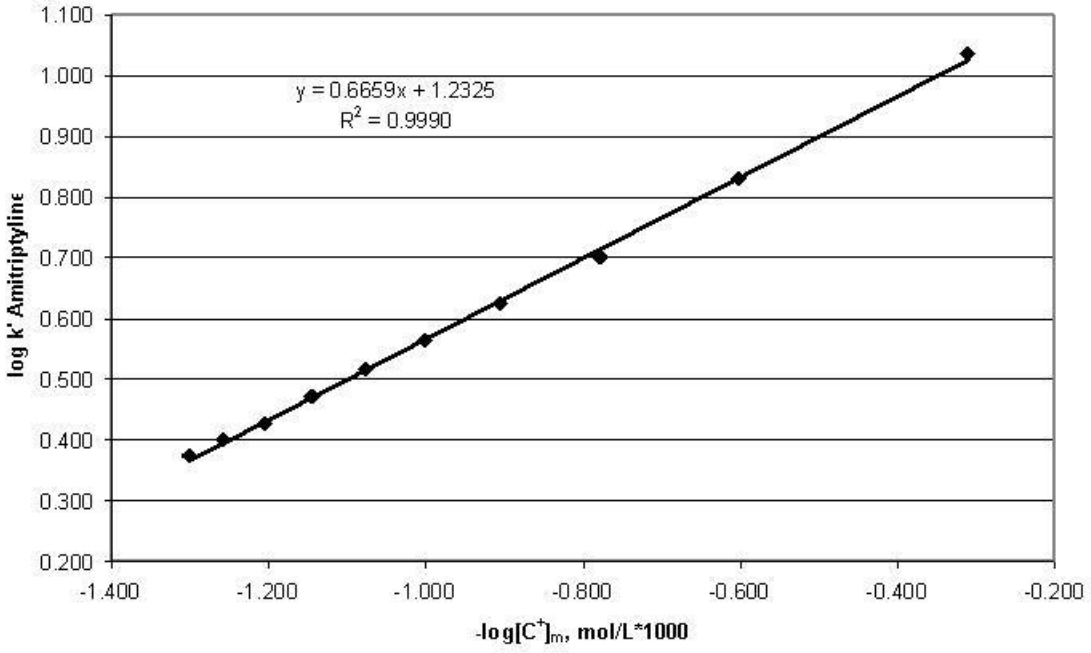

Figure **4-2**: Dependence of amitriptyline retention on mobile phase ion concentration at 85% acetonitrile using the PFPP stationary phase

Conditions: column: Discovery HS F5 (50 mm x 4.6 mm, 5µm particle size), mobile phase: ammonium acetate (pH 6.7, unadjusted) varying in concentration from 2 mM to 20 mM in 85% acetonitrile, temperature: 35ºC, flow rate: 1 mL/min, detection: UV at 220 nm

The linear relationship of retention with mobile phase counter ion concentration allows for facile optimization of this parameter with only two experiments. Retention data for norephedrine, synephrine, methylephedrine, ephedrine, methylpseudoephedrine and pseudoephedrine were acquired at 2 mM and 10 mM ammonium acetate (pH 6.7, unadjusted) in 90% acetonitrile on the PFPP phase. The structures of the ephedrine alkaloids along with synephrine are presented in Figure **4-1**. The compounds are all basic and expected to form protonated cationic forms at the mobile phase pH, and thus readily

interact via ion-exchange mechanisms with the ionized silanol groups on the PFPP phase using mobile phases at high organic percentages. The analytes are also difficult to retain on typical reversed-phase stationary phases because they are ionized at pH values suitable for HPLC analysis. The analytes represent a class of compounds well suited for analysis using fluorinated stationary phases.

The capacity factors for the ephedrine alkaloids and synephrine are plotted in Figure **4-3** and the respective slopes, intercepts and known octanol-water partition coefficients (Log P) are presented in Table **4-1**. Slope values approaching 1 demonstrate that the primary mechanism contributing to retention is ion-exchange. Deviation from the value of 1, however, shows that other mechanisms contribute to retention in addition to ion-exchange. The intercept value serves as a measure of the retention of the analytes due to interactions other than ion-exchange. The data in Table **4-1** show that the more polar (negative Log P values) norephedrine and synephrine analytes exhibit the lowest y-intercept values for this set, as would be expected based on purely hydrophobic mechanisms. . The ephedrine/pseudoephedrine and methylephedrine/methylpseudoephedrine pairs show similar y-intercept values as would also be expected from their similar Log P values. The magnitude of the y-intercept for these two pairs of analytes however, does not correlate with their expected retention based solely on their octanol-water partition coefficients. This is a significant observation as it suggests that the non-ionic interactions other than purely hydrophobic mechanisms such as hydrogen bonding and dipole interactions are present. The different slope and y-intercept values obtained for these similar analytes demonstrates the power of

mobile phase ion concentration for manipulation of selectivity and retention on the fluorinated phase.

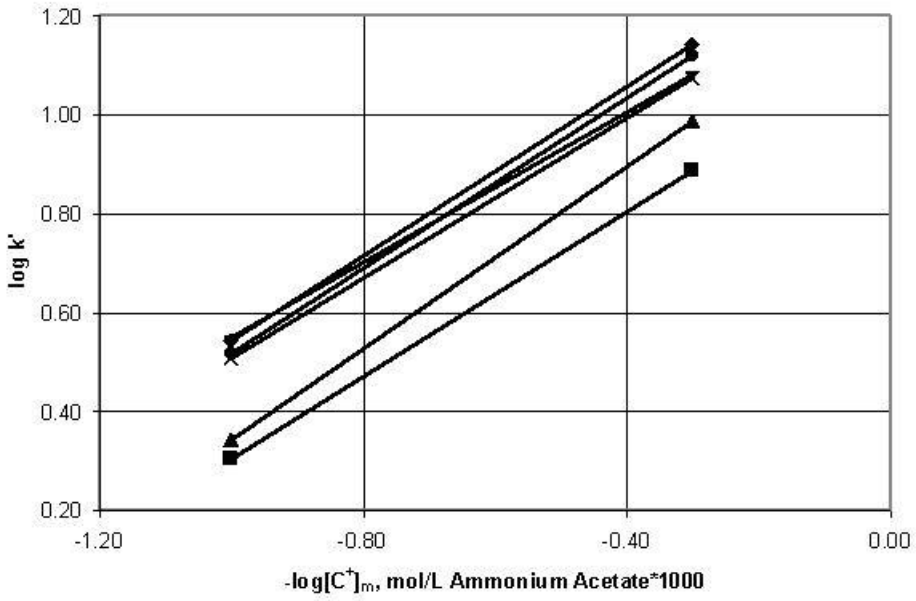

Figure **4-3**: Retention of norephedrine (¦), synephrine (?), methylephedrine (X), ephedrine (?), pseudoephedrine (?) and methylpsuedoephedrine (–) as a function of mobile phase ammonium ion concentration on a pentafluorophenylpropyl stationary phase

Mobile phase: 2 or 10 mM ammonium acetate (pH 6.7, unadjusted) in 90:10 v/v acetonitrile:water. Flow rate: 1 mL/min. Temperature: 35ºC. Detection: UV at 215 nm

Table **4-1**: Slope and intercept values from log-log Plots of capacity factors vs. reciprocal of mobile phase ammonium ion concentration for ephedrine alkaloids and synephrine

Conditions: column: pentafluorophenylpropyl stationary phase, mobile phase: 2 or 10 mM ammonium acetate (pH 6.7, unadjusted) in 90:10 v/v acetonitrile:water, flow rate: 1 mL/min, temperature: 35ºC, detection: UV at 215 nm

Analyte	Slope	Intercept	Log P*
Norephedrine	0.84	1.14	-1.24
Synephrine	0.92	1.27	-0.45
Methylephedrine	0.81	1.32	1.70
Ephedrine	0.86	1.38	1.13
Pseudoephedrine	0.86	1.40	0.89
Methylpseudoephedrine	0.76	1.31	unknown

*Data taken from SRC PhysProp Database, http://esc.syrres.com

As shown in Figure **4-3**, the selectivity for all analyte pairs except for ephedrine and pseudoephedrine changes as a function of the mobile phase ammonium concentration. The differences in slopes (for k' vs. 1/[NH4+]) suggest that selectivity can be manipulated by changing the ammonium acetate concentration in the mobile phase. Methylephedrine and methylpsuedoephedrine coelute at 2 mM ammonium acetate, however this pair is well resolved at a 10 mM concentration. At 10 mM ammonium acetate the methylephedrine and ephedrine as well as methylpseudoephedrine and psuedoephedrine pairs are unresolved. The obtained slopes that describe the effects of ammonium acetate concentration on retention can be used to predict optimum ammonium concentrations for resolving these analytes. Resolution of the analytes can be achieved between 3 mM and 4 mM ammonium acetate. A concentration of 4 mM was chosen as a

compromise between resolution and the speed of analysisas shorter retention is observed with increasing mobile phase ion concentration. In addition to selectivity changes, it is readily observed from the retention data that elution time can also be rapidly optimized using this approach.

4.3.2 Dependence of Retention on Temperature

Temperature can be used as a variable to optimize retention selectivity and run time in chromatographic analyses. The retention dependence on temperature is related to the van't Hoff equation for chromatography as given in Equation **4-3:**[21]

$$\ln k' = \ln \beta - \frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R} \qquad \text{Equation 4-3}$$

where retention is linearly related to 1/T(K) assuming that the adsorption enthalpy (ΔH^o) does not vary with temperatureover a limited range. In initial retention studies for the ephedrine alkaloids performed at 35ºC and 60ºC (data not shown) elution order differences were observed for the analytes, demonstrating the potential for controlling selectivity employing temperature. To further investigate the relationship of retention with temperature, retention data for norephedrine, synephrine and methylephedrine were acquired from 10ºC to 45ºC in increments of 5ºC. In addition to these three analytes, a quaternary ammonium compound, berberine, was also included in the study. Berberine [See Figure **4-1**], due to its permanently charged state, will not change in retention as a function of changes in its degree of ionization and therefore is suitable for probing the ionization state of the surface silanols as a function of temperature.[22]

Figure 4-4 shows the van't Hoff plots for the four analytes in the study. Berberine is shown to increase in retention in a linear fashion (R^2 = 0.9621) from 10ºC to 45ºC. In reversed-phase separations a decrease in retention with increased temperature typically occurs. Since the dispersive (hydrophobic) mechanism is exothermic and the enthalpy term dominates the Gibbs free energy of the interaction, retention decreases with increasing temperature[18], however, several reports have shown behavior for basic compounds where retention increases with increasing temperature.[17] McCalley reported negative slopes in the van't Hoff plots for nortriptyline and quinine on a base-deactivated C18 stationary phase.[19] The increase in retention with temperature was explicated by a decrease in the degree of ionization of the analytes combined with an increase in effective pH as a function of increasing temperature. The decrease in degree of ionization was said to lead to increased hydrophobic retention and thus a negative slope in the van't Hoff plot. Since the degree of ionization of berberine is unaffected by pH or temperature, the explanation cannot account for the observations of this study. Temperature adjustments may induce changes in pH and pK_a values for the surface silanols, however evidence in the literature suggests that acid dissociation constants for benzoic [23] and phosphoric acids in 50% methanol [18,19] are relatively independent of temperature (15ºC to 50ºC) . The observed independence of acid pK_a values on temperature is likely a result of a concomitant increase in pH and the acid pK_a value. If the pK_a values of surface silanol groups increase at a greater rate than the rise in pH, the increase in berberine retention could be explained by the enhanced degree of surface silanol ionization. It is assumed here, however, that the acidic silanol groups act in accordance with the acids noted in the literature.

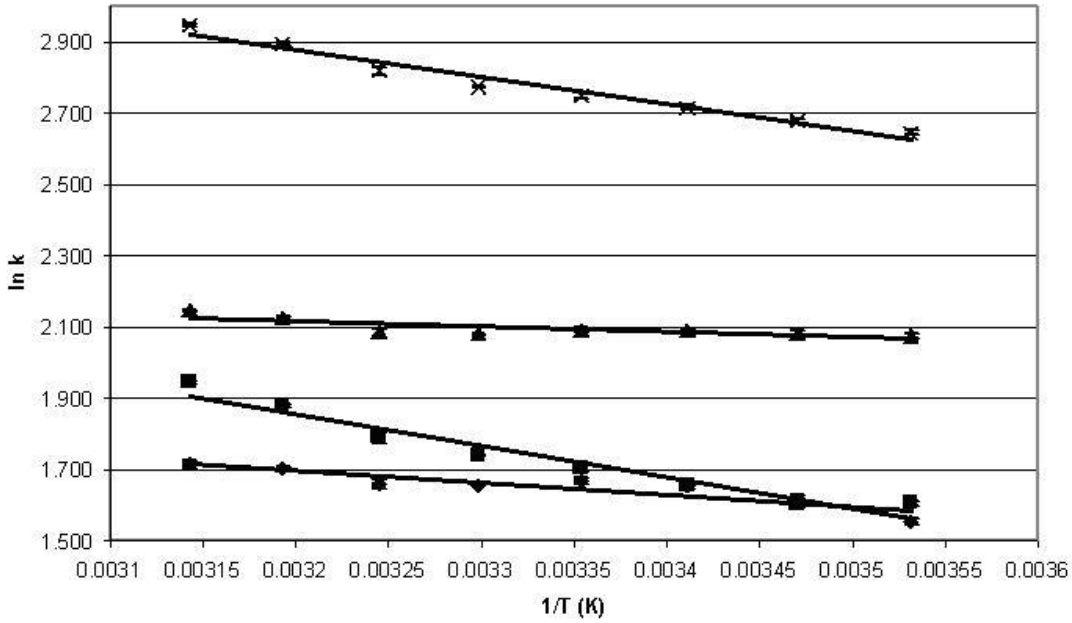

Figure **4-4**: Retention of berberine (X), synephrine (¦), methylephedrine (?) and norephedrine (?) as a function of temperature on a pentafluorophenylpropyl stationary phase

Mobile phase: 4 mM ammonium acetate in 90:10 v/v acetonitrile:water. Flow rate: 1 mL/min. Detection: UV at 220 nm

The increase in retention of berberine is best explained by a decrease in solvation energy with increasing temperature. It is well established that solvent solvating power decreases with increasing temperature. This effect has been demonstrated through the thermosolvatochromism of many spectroscopic probes in various solvent systems.[24-28] Solvating power is based on an empirical scale intended to describe the solute/solute and solute/solvent interactions in addition to contributions to solvation from quantifiable solvent physical parameters such as dielectric constant, dipole moment and polarizability.

Because the quantitative parameters represent bulk properties of the solvent, they do not account for specific solvent/solute or solute/solute interactions at the molecular level.[24]

A change in solvating power may alter the acid-base equilibrium constants for the solutes as well as the surface silanol groups because of differential effects on solvation of the neutral and ionized forms. As noted by McCalley, if the degree of ionization changes, the dispersive interactions of the solute with both the solvent and the stationary phase change based on reversed-phase chromatographic theory.[29] In addition, and apparently of great importance under the chromatographic conditions of this study, decreased solvation power results in an increase in ionic interactions between cationic solutes and the anionic support. Stronger solvent-solute interactions at low temperatures are more effective at shielding the ions from interacting. As the temperature increases, weaker solvent-solute interactions render the ions more interactive, resulting in an increase in ion-exchange interactions.

The slope value for synephrine close to 1 (0.92) shown in Table **4-1** indicates that synephrine retention is primarily due to ion-exchange mechanisms. Norephedrine and methylephedrine show a relative increase in non-ionic retention (slope values of 0.84 and 0.81, respectively) as their slope values greater deviation from 1. Synephrine retention increased by 2 k' units as temperature was increased from 10ºC to 45ºC. The retention for norephedine and methylephedrine increased by only 1.1 and 0.6 k' units over the same temperature range. The magnitude of analyte retention response to temperature thus appears to be dependent on the relative importance of ionic and non-ionic contributions. The differential response in analyte retention to temperature for these similar solutes demonstrates that temperature is a powerful tool to manipulate selectivity in this mode of

chromatography. The ammonium acetate concentration and temperature for the separation of the ephedrine alkaloids and synephrine was empirically established at 4 mM and 45ºC, respectively and is shown in Figure **4-5**. Our aim in this study was to investigate the dependence of retention and selectivity on ion concentration and temperature in systems dominated by ion-exchange mechanisms, not necessarily to develop a fully optimized set of conditions. The linear dependence of retention on both ion concentration and temperature, however, should provide for facile method optimization.

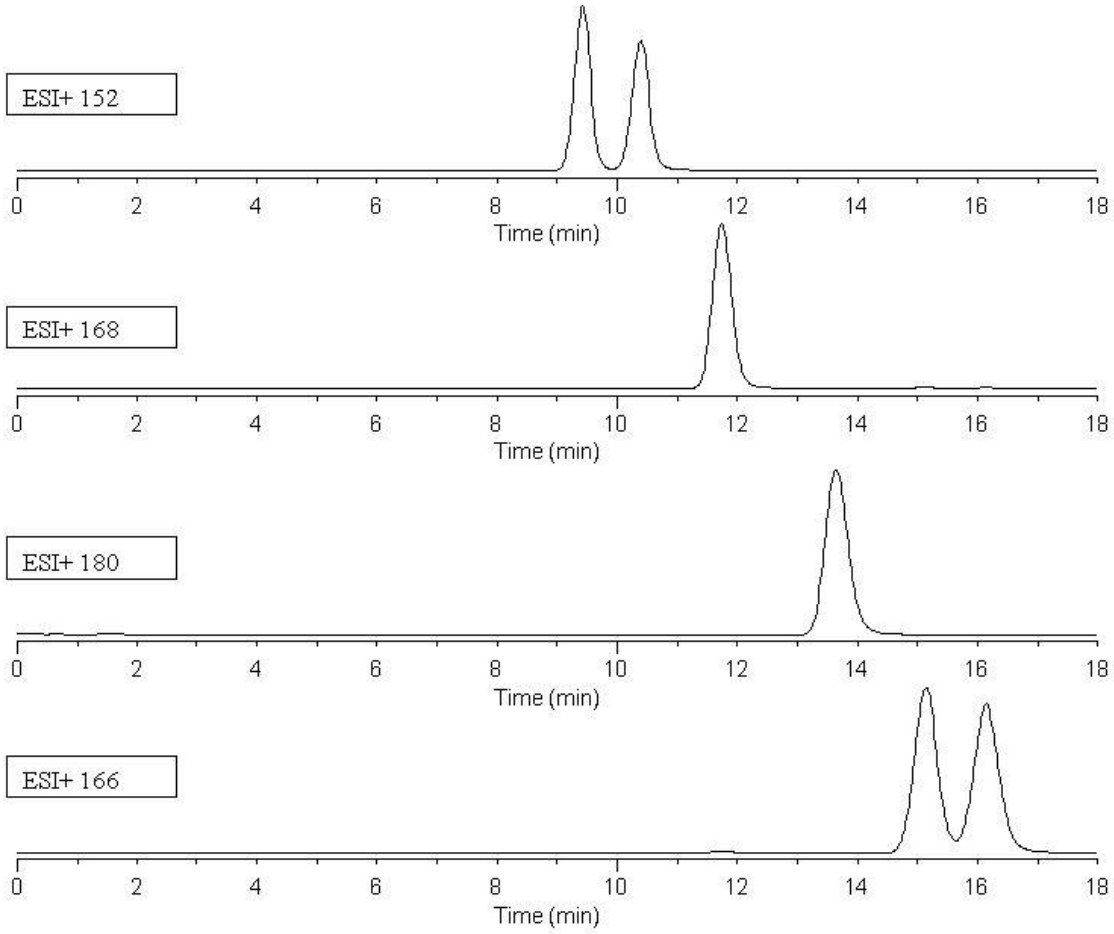

Figure **4-5**: Reconstructed ion chromatograms at m/z 152, norephedrine and norpseudoephedrine, m/z 168, synephrine, m/z 180, methylephedrine, and m/z 166, ephedrine and pseudoephedrine in order of elution on a pentafluorophenylpropyl stationary phase

150 mm x 4.6 mm, 5 µm. Mobile phase: 4 mM ammonium acetate (pH 6.7, unadjusted) in 90:10 v/v acetonitrile:water, flow rate: 1 mL/min, temperature: 45ºC, detection: ESI-MS operating in positive ion mode

4.4 Conclusions

Strategies for rapid method development can be established through knowledge of the dominant molecular interactions that contribute to retention and selectivity and the

parameters that control such interactions. At high percentages of organic modifier, a pentafluorophenylpropyl stationary phase has been shown to retain basic analytes via dominant ion-exchange mechanisms. In this study we have demonstrated that retention and selectivity of basic analytes on a fluorinated stationary phase are strongly dependent on mobile phase ammonium ion concentration. Only two experiments are required for optimization of this parameter due to the linear dependence of log k' on mobile phase ionic concentration. The result is facile method development in terms of both selectivity and run time. Temperature was shown to be an effective parameter for the manipulation of retention and selectivity. Retention at high organic modifier percentages on the fluorinated phase increases with increasing temperature in contrast to chromatographic processes dominated by dispersive interactions (RPLC). This observation was explained by the lower solvation strength of the mobile phase at higher temperatures that consequently results in more loosely solvated ions at higher temperatures. The relatively poor solvation renders the ions more active toward ion-exchange interactions.

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Chapter 5

Conclusion

5.1 Solute Attributes and Molecular Interactions Contributing to Unique Retention on Fluorinated HPLC Stationary Phases

Chromatographic separation of compounds with disparate properties requires novel stationary phases that impart diverse retention mechanisms that can be manipulated through choice of mobile phase composition and temperature. The primary accomplishment of these studies has been in revealing analyte properties and molecular interactions that contribute to the unique retention observed on fluorinated stationary phases, particularly the phenomenon of "U-Shape" retention. Enhanced understanding of these chromatographic processes should lead to an improved basis for rational design of chromatographic stationary phases and separation protocols.

The systematic comparison of the effects of mobile phase composition on solute retention on pentafluorophenylpropyl (PFPP) and C18 stationary phases revealed that ion-exchange interactions play a substantial, and perhaps dominant, role in retention of cationic solutes on the PFPP phase. Both the fluorinated phase and an alkyl (C18) phase bonded to the same silica substrate exhibited evidence of cation-exchange retention in water-acetonitrile mobile phases with high acetonitrile content. However, the contribution of ion-exchange to retention of organic cations was more pronounced on the fluorinated phase than on the C18 column. The cumulative observations of these studies implicate ion-exchange retention of cationic solutes by deprotonated surface silanols that

is amplified on the PFPP phase. Elemental analyses for both the PFPP and C18 stationary phases indicated comparable bonded phase coverage, so the surface density of silanols is similar on both phases.

The increased ion-exchange character of the PFPP phase relative to C18 must be attributed to of the effects of the bonded phase ligand on the environment at the particle-solution interface. The bonded phase can influence the composition of solvent at the particle surface [1], may provide inductive effects or specific intermolecular interactions that modify surface silanol acidity, and may effectively shield solutes from ionic interaction with the surface by influencing interfacial physical properties such as the dielectric constant of the interfacial layer.

A primary focus of these studies has involved the effects of the bonded ligand and the mobile phase composition on the retention of cationic solutes. Increasing acetonitrile content in the mobile phase is expected to decrease ionization of the acidic silanol groups as has been demonstrated for a number of acidic solutes.[4] Furthermore, protonation of organic bases is also less favorable as acetonitrile content increases. However, the increase in cationic solute retention with increasing acetonitrile content would appear to be in conflict with these expectations. Retention of the permanently-charged bretylium served as a probe of the contributions of silanols to retention based on ion-exchange. Bretylium retention increased with mobile phase pH on the PFPP phase, but retention on C18 was largely insensitive to pH. Since the density of silanol groups on PFPP and C18 phases are similar, differences in pH-dependent retention are likely due to differences in silanol acidity or differences in the interface environment that shield cationic solutes from the influence of the anionic silanol groups. Bonded phase functional groups may

influence the composition and properties of the interface that can either shield or enhance surface silanol activity. It was anticipated that the electron-withdrawing properties of the PFPP ligand might increase the acidity of neighboring surface silanol groups either through induction or via hydrogen bonding with the silica surface. However, initial semi-empirical quantum mechanical calculations based on the AM1 Hamiltonian has failed to provide support for such claims.

It is clear from the above discussion that the interfacial properties such as electric field strengths and interfacial dielectric constants that govern the thermodynamics of ion-exchange interactions are not well characterized. Limitations in our knowledge are probably the result of experimental difficulties in probing the composition and properties at the interface. The study of solute retention as a function of both bulk mobile phase properties and chemical modifications of surfaces is, however, likely to improve understanding of these critical interfaces. With this knowledge, further developments in bonded phase chemistry are expected to yield stationary phases with enhanced reversed-phase and ion-exchange properties.

5.2 Estimation of Acid Dissociation Values of Basic Analytes in Aqueous-Organic Mixtures using NMR Spectroscopy

The unexpected lack of retention for the basic solute lidocaine on the PFPP column demonstrated the need to further our knowledge regarding the degree of solute ionization in various HPLC mobile phases. This need prompted investigations aimed at determining the extent of protonation of basic solutes in various acetonitrile-water mixtures using NMR spectroscopy. The variation of chemical shifts for protons in the

vicinity of a basic nitrogen atom as a function of the medium pH can be related through the Henderson-Hasselbalch equation to calculate analyte acid dissociation constants. These values can be used to predict solute ionization across a range of solvent compositions and pH conditions.

Until now, the use of NMR spectroscopy to determine acid dissociation in solvent mixtures used for HPLC had been limited. In the current studies, NMR spectroscopy provided a non-invasive and direct probe of solute acid-base equilibria and their dependence on both the pH and organic cosolvent mole fraction. Knowledge of such equilibria enhances our ability to further define the fundamental mechanisms of retention in chromatographic processes. Specifically, we used the results of the NMR study to explain the lack of ion-exchange behavior of lidocaine on the PFPP stationary phase at ${}^{w}_{w}$pH 4, 90 v/v% acetonitrile reported in previous studies. Whereas lidocaine is largely ionized at pH 4 before addition of acetonitrile but is not substantially ionized after addition of acetonitrile to high mole fractions, its retention via ion-exchange mechanisms is minimal in high acetonitrile content mixtures. This finding highlights the conclusion that one should not assume that bases are protonated in high acetonitrile content solvents based on reported aqueous pK_a values even if the aqueous pH is adjusted to less than two pH units lower than the literature pK_a value, as is commonly practiced.

Measurements of pH values both before and after the addition of organic cosolvent demonstrated that pH changes as a function of the amount of cosolvent and the initial aqueous pH value. The use of a pH scale based on the measurement following addition of organic component (${}^{s}_{w}$pH) was shown to better reflect the thermodynamic

reality of the environment about the analyte as compared to pH measurements made in aqueous solvents before addition of organic. For control of interactions that depend on analyte degree of ionization, it is critical to measure the pH following the addition of organic and to recognize that solute pK_a values change significantly with the addition of organic modifier.

5.3 Mobile Phase Ion Concentration and Temperature Dependence of Retention and Selectivity using Fluorinated Stationary Phases

Strategies for rapid method development can be established through knowledge of the dominant molecular interactions that contribute to retention and selectivity and the parameters that control such interactions. In this final study it was demonstrated that retention and selectivity of basic analytes on a fluorinated stationary phase are strongly dependent on mobile phase ion concentration. Only two experiments were required for optimization of this parameter due to the demonstrated linear dependence of retention (log k') on the reciprocal of mobile phase ion concentration. Facile method development in terms of both selectivity and run time was demonstrated for a set of ephedrine alkaloids that are difficult to retain and separate using modern alkyl stationary phases. The study further substantiated earlier conclusions that ion-exchange mechanisms dominate retention of basic solutes on the PFPP phase and show a practical advantage of understanding the underlying mechanisms that contribute to retention and selectivity.

Temperature was shown to be an effective parameter for the manipulation of retention and selectivity for the alkaloid solutes as well. Retention at high organic modifier percentages on the fluorinated phase was shown to increase with increasing

temperature in contrast to the decrease in retention commonly observed in chromatographic processes dominated by nonionic interactions.[7,8] This observation may partially be explained by a lower solvation strength of the mobile phase at higher temperatures that consequently results in more loosely solvated ions at higher temperatures.[9] Decreased shielding of the ion due to the relatively poor solvation renders the ion more prone to coulombic interactions. The increased retention of a quaternary amine solute with increasing temperature refutes reports that the effect is solely due to changes in hydrophobic retention based on altered ionization states.[10-12] According to McCalley, the pK_a values of basic solutes decrease as absolute temperature increases.[10] Since selectivity in ion-exchange retention has been demonstrated in these studies to be a function of the degree of ionization of the solutes, temperature will undoubtedly have a pronounced effect where ion-exchange mechanisms are present. The use of elevated temperature in HPLC is currently an active area of research. Further studies using NMR spectroscopy are likely to be of value in determining the effects of temperature on analyte and surface silanol acid dissociation constants. Based on the conclusions of this study, ion-exchange interactions may become more prevalent even on modern alkyl stationary phases at elevated temperatures.

5.4 Final comments

Many chromatographers interested in the fundamental mechanisms governing retention and selectivity view retention as a function of interactions of solutes directly with the ligands used to modify the silica surface. A major conclusion from this work is

that solute interactions with the bulk solvent and with the solvent molecules and projected electric fields associated with the interfacial layer make substantial contributions to retention. Direct interactions with bonded phase ligands play a minor role in retention when using nonpolar alkyl (e.g. C18) phases, but probably are more important with the PFPP phase. In this interpretation, an often-ignored role of the bonded phase is to preferentially interact with the components of the mobile phase to influence the physical properties of the interface. This solvent composition at the interface will depend on the polarity of the ligand, specific molecular interactions provided by the ligand functional groups and the composition of the mobile phase. Such an interpretation should lead to alternative approaches to stationary phase design and method development strategies.

Although this study focused on the PFPP phase, some other commonly used stationary phases exhibit ion-exchange interactions but the importance of these interactions is not well understood. It is hoped that the results from this study will prompt other investigations to explain alternative and perhaps unexpected selectivity and retention observed on many of these phases.

Dorsey *et. al.*, examined the electroendoosmotic flow (EOF) characteristics of aqueous-organic solvents in capillary electrophoresis experiments.[13] A notable finding from this study was that the EOF increased dramatically from 80% to 100% acetonitrile. Since the EOF is generated at the surface of the silica capillary the finding suggests that acetonitrile plays a significant role in determining the properties of the electrical double-layer. In addition, the authors report that the presence of ionic additives in the solvent mixtures attenuates the increase of EOF in high percentages of acetonitrile. Each of these

findings is in agreement with the results of our chromatographic investigations. In both cases the properties of the solid-liquid interfaces responsible for observed EOF and chromatographic retention are not well understood and deserve further study. Further chromatographic and electrophoretic experiments that probe both the bulk solvent and surface modification effects on interfacial properties as well as further spectroscopic studies are likely to strengthen our knowledge of this critical realm.

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VITA

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After receiving his B.S. degree from the State University of New York College at Plattsburgh in 1989, David gained employment within the pharmaceutical industry where he was involved in analytical method development using various forms of chromatography and electrophoresis for 8 years. For the past 8 years, working directly in the chromatography industry, Dave has focused his efforts on the design, development and application of HPLC stationary phases. Of special interest is the understanding of molecular interactions that contribute to retention and selectivity in chromatographic processes. In September of 2000 he began pursuing his graduate work toward a Ph.D. in Analytical Chemistry at The Pennsylvania State University while managing an industrial analytical laboratory and raising three children.