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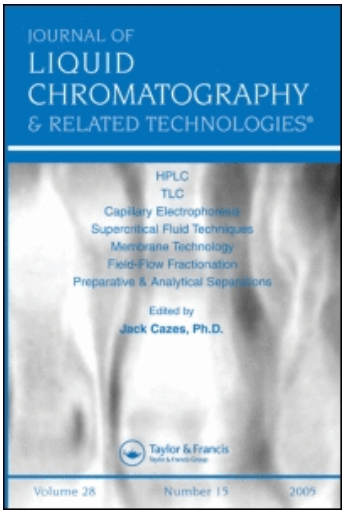

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Some Insights on Retention and Selectivity for Hydrophilic Interaction Chromatography

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Abstract: Using a pure silica stationary phase along with a simple acetonitrile–phosphoric acid aqueous mobile phase provides a powerful and versatile tool for the analysis of a broad range of polar compounds, such as amino acids and pharmaceutical compounds. The retention of solutes is influenced by the nature of the stationary phase, the organic solvent, and the aqueous mobile phase components. The retention mechanism is predominantly electrostatic interactions occurring between the solute and stationary phase. Selectivity for cationic or zwitterionic solutes is largely due to differences in their ion exchange capabilities, and, consequently, influenced by the pK_a and lipophilicity of the solutes. Steric hindrance in the proximity of the protonated site of the solutes may also influence selectivity and retention.

Keywords: Amino acids, Hydrophilic interaction chromatography, Polar compounds

INTRODUCTION

Reversed phase liquid chromatography (RPLC) refers to chromatography where the mobile phase is more polar than the stationary phase. This mode commonly utilizes aqueous buffer modified with an organic solvent

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such as methanol or acetonitrile, with a silica based C_{18} or C_8 stationary phase. The proliferation of different types of silica based stationary phases, their high efficiencies, the low UV cutoff of the commonly used mobile phase components, and the ability to run gradient programs covering a wide polarity range are some of the reasons why RPLC dominates the field of separation science. However, very polar solutes such as carboxylic acids, amines, and zwitterions may exhibit poor retention and selectivity in this mode. Retention can be enhanced to a limited extent through the use of a highly aqueous mobile phase in conjunction with a polar endcapped or polar embedded stationary phase. A second option is the use of ion pairing reagents in the mobile phase. This option, however, is often hampered by poor baselines, system peaks, and irreversible modification of the column.[1,2] A third option is to derivatize the polar solute with a hydrophobic moiety. For example, hydrophilic peptides and amino acids can be derivatized with 9-fluorenylmethyl chloroformate (FMOC) to enhance retention and sensitivity.[3,4] However, derivatization requires additional steps in sample preparation and is not amenable to polar compounds that do not possess functionalities that can be derivatized in a facile manner. A fourth option is to utilize chromatographic modes other than reversed phase.

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Normal phase liquid chromatography (NPLC), where the stationary phase is more polar than the mobile phase, is a complementary separation mode. It can be used for polar solutes that are poorly retained, for non-polar solutes that are strongly retained, or solutes that are labile or possess poor solubility in a RPLC mode. Retention occurs through polar interactions, such as hydrogen bonding and dipole interactions, between the solute and the stationary phase. The mobile phase is usually a mixture of a non-polar solvent such as heptane and a more polar solvent such as isopropanol. Commonly utilized stationary phases include cyano, diol, and silica. However, many of the highly polar solutes that are poorly retained in RPLC, are too strongly retained in NPLC. Additionally, many polar solutes exhibit poor solubility in non-aqueous mobile phases.

An alternative separation mode to RPLC or conventional NPLC for the analysis of polar solutes is hydrophilic interaction chromatography (HILIC). This mode utilizes a stationary phase that is more polar than the mobile phase and thus can be categorized as NPLC. However, it uses a more polar mobile phase than conventional NPLC. The mobile phase is usually a mixture of a polar organic solvent such as acetonitrile or methanol at high concentrations plus aqueous buffer at low concentrations. The stationary phase is generally unmodified, aminopropyl bonded, or sulfobetaine bonded silica. Solute retention appears to be multimodal, involving hydrogen bonding or coulombic interactions with the stationary phase or with tightly bonded water on the stationary phase, when using organic modifier enriched mobile phases.[5] Hence, solute retention increases with

increasing polarity of the solute.[6] The HILIC mode offers a number of advantages over RPLC and conventional NPLC for the analysis of polar compounds. Substantive retention and selectivity is observed, derivitization is not required, and no baseline artifacts are observed in contrast to ion pair chromatography.[2,7,8] Gradient programming can be utilized.

The HILIC mode has been used in the analysis of carbohydrates, nucleic acids, peptides,[6,9–12] and polar pharmaceutical compounds.[1,13–17] The separation mechanism for the HILIC mode has been discussed,[1,5,6,18,19] indicating that ion exchange and the pH of the mobile phase influence retention. In this study, the factors influencing retention and selectivity of polar compounds are further investigated in order to gain further insight for the retention of polar molecules under HILIC conditions. The probe molecules used were groups of structurally related polar compounds such as pyridine derivatives, amino acids, and aniline derivatives. The positional isomers and slightly different functional groups found in the selected probe molecules were intended to provide subtle details of factors influencing retention and selectivity. These chosen probe molecules generally exhibit poor retention/selectivity characteristics by both RPLC and conventional NPLC modes.

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EXPERIMENTAL

Reagents

Amino acids, aniline derivatives, and substituted pyridine compounds were of analytical reagent grade and were obtained from Sigma-Aldrich (Milwaukee, WI). The evaluated drug substance-(1R,2S,5S,6S)-2-amino-6-fluoro-4-oxobicyclo[3.1.0] hexane-2,6-dicarboxylic acid (Compound A) and its intermediates were prepared by Process Research at Merck Research Labs. Sodium formate and sodium perchlorate (HPLC grade) were obtained from Fisher Scientific Inc. (Fairlawn, NJ). Methanol, acetonitrile (both HPLC grade), acetic acid (99.99%), formic acid (88%), and phosphoric acid (99.999%) were all obtained from Sigma-Aldrich (Milwaukee, WI). Highly purified water (Hydro Service and Supplies, Garfield, NJ) was used throughout for the preparation of buffer and reagents. All samples were prepared in 100% acetonitrile or $10 \sim 20\%$ v/v 0.1% phosphoric acid aqueous and $90 \sim 80\%$ v/v acetonitrile mobile phase.

Chromatographic Conditions

Analyses were performed with HP 1100 (Agilent Technologies, Palo Alto, CA) HPLC systems equipped with either quaternary or binary pumps.

The systems also included UV or photodiode array detectors. Data were collected using TurboChrom Navigator (Perkin Elmer, San Jose, CA).

HPLC separations were performed on Atlantis HILIC Silica columns (150×4.6 mm, 3 μm particle size, Waters, Milford, MA) at room temperature with detection at 200 nm or 210 nm. Low wavelength 200 nm was used for the drug substance and its impurities as they possessed weak chromophores. The flow rate was 1.0 mL/min and injection volume was 10 μL. Acetonitrile or methanol was used as the organic mobile phase. The aqueous mobile phase contained phosphoric acid, acetic acid, or perchloric acid. Two hours preequilibration time was required when using new columns or when changing mobile phase eluent in order to ensure consistent retention times.

Software for Calculation of log P and Ab Initio Molecular Modeling

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ACD Lab 6.0 log P dB and pK_a dB software (Advanced Chemistry Development, Inc., Toronto, Canada) was used to calculate the pK_a and log P values of some compounds. P is the partition coefficient of a compound defined by the ratio of the concentration of the compound in octanol to the concentration of the compound in water. Log P is the logarithm of the partition coefficient.

Molecular energies were calculated by using PC Spartan 04 (Wavefunction, Inc., Irvine, CA) software optimized with B3LYP/6-31G*.

RESULTS AND DISCUSSION

Dominant Interactions in HILIC Mode

Chromatographic retention for a solute is dependent upon the strength of its interactions with both the mobile and stationary phase. Increased retention is correlated to strong interactions with the stationary phase. Polar compounds are more strongly retained in the HILIC mode, while non-polar compounds are more strongly retained in the RPLC mode. There are a number of critical dissimilarities between RPLC and HILIC relating to the interaction of the solute with the mobile phase and the stationary phase that account for observed differences in retention and selectivity.

Under the aqueous rich conditions associated with the reversed phase mode, the solute is enclosed within a hydration sphere that must be removed prior to undergoing hydrophobic interactions with the apolar stationary phase. In acetonitrile rich mobile phases, individual water molecules interact with individual acetonitrile molecules through

hydrogen bonding and with minimal disruption of the weak dipole interactions of acetonitrile.[20] Similarly, in methanol rich mobile phases, there is very little water unassociated with methanol and the major constituents are water-methanol and methanol-methanol associates.[21] In both acetonitrile and methanol rich mobile phases, there would be very few water aggregates present. As a consequence, in the HILIC mode with an organic rich mobile phase a hydration cage is unlikely to exist and interactions between the solute and the organic solvent are enhanced. Thus solvation is very different for HILIC versus RPLC.

Organic solvents also have a significant effect on the protolytic properties of the stationary phase, ionizable mobile phase components, and ionizable solutes. When the percentage of organic solvent in the mobile phase increases, the mobile phase dielectric constant decreases and the activity coefficients of charged solutes decrease. As a result, there is a concomitant change in the pH of the mobile phase and the pK_a of the solute molecules.[22–32] Consequently, the measured pH of the aqueous mobile phase component or that of the mobile phase mixture using a potentiometric system does not reflect the true hydgrogen ion activity of the mobile phase mixture if the system has been calibrated using aqueous standards.[26,28]

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The dissociation of neutral or anionic acids results in the generation of ions with increased charge. However, the extent of dissociation and the activity of these ions are reduced with the reduction in the dielectric constant of the solvent. These effects result in an increase in the pK_a. In contrast, for the dissociation of a cationic acid (e.g ammonium ion), there is no change in the number of charges and a lowering of the solvent's dielectric constant has a smaller effect on the pK_a. Dissociation depends only on the solvation of the species resulting in a slight decrease in pK_a.[26] As an illustration, the pK_a value of phosphoric acid and acetic acid increase from 2.11 and 4.77 in pure water to 3.21 and 5.54, respectively, in 50% methanol while the pK_a of the ammonium ion decreases from 9.24 to 8.76.[26] Similar effects were reported with acetonitrile where the pK_a of phosphoric acid and acetic acid were found to increase 3.29 and 6.16 in 60% acetonitrile, respectively, while the pK_a of the ammonium ion decreased to 8.88.[30]

The nature of the stationary phase affects the type of interactions that the solute can undergo. Two different types of silanols with different acidities have been identified. Their pK_a have been determined to be 3.5 to 4.6 and 6.2 to 6.8 in 60% methanol.[33] The composition of these silanol sites are 45% of the more acidic silanols to 55% of the less acidic silanols.[33] Thus, a significant portion of the more acidic silanols will be deprotonated in the presence of 0.01 M phosphoric acid or acetic acid with a larger proportion deprotonated with acetic acid. These silanols can undergo electrostatic interactions such as ion exchange with cationic solutes.

The above considerations present a model for solute retention in the HILIC mode using a silica stationary phase. The cationic solute interacts with the stationary phase through ion exchange with deprotonated silanols. The strength of this interaction is dependent upon the charge, pK_a of the solute, and the pH of the mobile phase.

Retention of Pyridine under HILIC Conditions

The retention characteristics of pyridine (pK_a 5.17 in water and 4.44 in 81.4% acetonitrile/18.6% water[31]) under HILIC conditions were investigated. The retention of pyridine was first determined as a function of percent acetonitrile using 0.1% phosphoric acid as the aqueous modifier. Under these conditions and based on the pK_as of pyridine, phosphoric acid, and the more acidic silanol groups, there is a significant amount of pyridinium ions and deprotonated silanols present.

Figure 1 depicts the retention trend. Pyridine is more strongly retained with increasing acetonitrile concentration. An increase in acetonitrile percentage decreases the dielectric constant of the mobile phase. The ion exchange interaction of pyridinium ion with deprotonated silanols are enhanced with the decreased polarity of the mobile phase resulting in increased retention for pyridine.

To demonstrate the strength of the ion exchange interaction, the retention of pyridine, nitrobenzene, benzyl alcohol, and benzoic acid was compared. Pyridine is the only one of these species capable of forming a positively charged ion that can then undergo ion exchange interactions with deprotonated silanols, and this is reflected in its strong retention (retention factor $k' = 4.1$) with a mobile phase containing 20:80 0.1% v/v phosphoric acid:acetonitrile. The other species are polar and

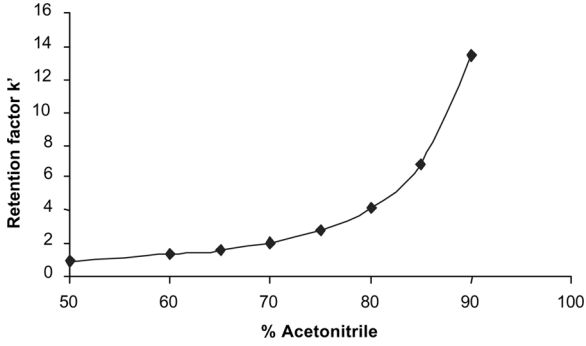

Figure 1. Retention factors of pyridine vs. percent of acetonitrile. Retention factor $k' = \frac{t_r - t_0}{t_0}$ (t_r is the retention time of the solute; t_0 is the mobile phase hold up time.)

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thus can undergo interactions such as hydrogen bonding and dipole-dipole interactions with the stationary phase. These species, however, were unretained under the same conditions. This observation provides strong evidence for the dominance of an ion exchange interaction occurring between pyridine and the stationary phase.

Next, the retention of pyridine was determined as a function of the type of aqueous mobile phase component. A mobile phase of 80% acetonitrile was used in conjunction with 0.1% (v/v) of either perchloric acid, phosphoric acid, or acetic acid. Retention factors of 0.8, 4.1, and 7.3 were observed with perchloric acid, phosphoric acid, and acetic acid, respectively. Due to its lower pK_a, perchloric acid is dissociated to a larger extent than the other acids. The lower pH of the perchloric acid mobile phase results in a smaller proportion of deprotonated silanols sites, reducing the capability for ion exchange interactions between pyridinium ions and deprotonated silanols. For phosphoric acid, the higher pH of the mobile phase results in greater deprotonation of silanol sites enhancing ion exchange interactions with the pyridinium. Therefore, a greater retention was achieved. For acetic acid, where the greatest retention was observed, the higher pK_a of acetic acid will lead to greater ion exchange interactions with the silanols as compared to phosphoric acid as a consequence of more deprotonated silanols being available. It can be argued that the higher pH of the mobile phase would also lead to a decrease in the number of pyridinium ions which should, with all other factors constant, decrease the ion exchange capability. However, the decrease in pyridinium ion appears to be countered by the increase in deprotonated silanols.

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Retention of Pyridine Derivatives under HILIC Conditions

The retention and selectivity of nine structurally related pyridine derivatives were investigated under HILIC mode (Figure 2, Table 1) to determine the effect of various functional groups on retention. The compounds included three pyridine alcohols, three pyridine carboxylic acid positional isomers, and three pyridine carboxamide positional isomers. A mobile phase of acetonitrile with 0.1% phosphoric acid as the aqueous component was utilized (Figure 3).

The first eluted solute is 2-pyridine carboxamide, which has a pK_a of 1.80 in water. This molecule likely exists as predominantly the neutral form under the chromatographic pH conditions of the mobile phase (pH is approximately 3.6 with 60% acetonitrile[29,31,32]) resulting in very little ion exchange between the solute and deprotonated silanols. 3-Pyridine carboxamide and 4 pyridine carboxamide have pK_as of 3.33 and 3.61, respectively, in water and are thus stronger bases with larger

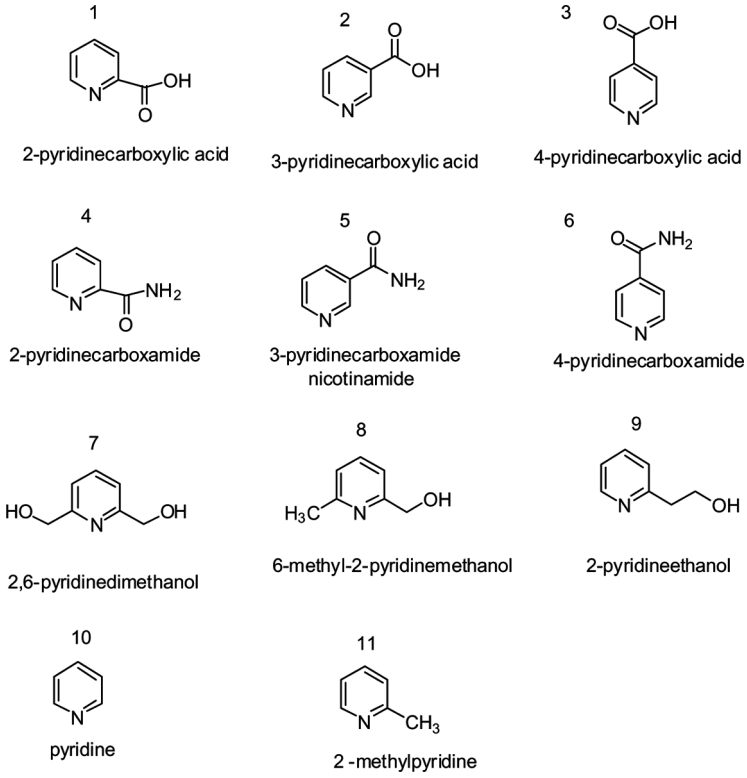

Figure 2. The structures of pyridine and its derivatives.

Table 1. pK_a[34–36] values of pyridine, and its derivatives. pK_a values in bold represent those corresponding to the pyridine nitrogen

No.	Name of neutral compound	pK_a1	pK_a2	k′
1	2-Pyridinecarboxylic acid	1.01	**5.29**	0.4
2	3-Pyridinecarboxylic acid	2.07	**4.75**	1.5
3	4-Pyridinecarboxylic acid	1.84	**4.84**	1.9
4	2-Pyridinecarboxamide	**1.80**		2.1
5	3-Pyridinecarboxamide	**3.33**		2.4
6	4-Pyridinecarboxamide	**3.61**		2.6
7	2,6-Pyridinedimethanol	**4.79**		3.4
8	6-Methyl-2-Pyridinemethanol	**5.73**		3.6
9	2-Pyridineethanol	**5.17**		3.8
10	Pyridine	**5.17**		4.3
11	2-methylpyridine	**6.00**		4.3

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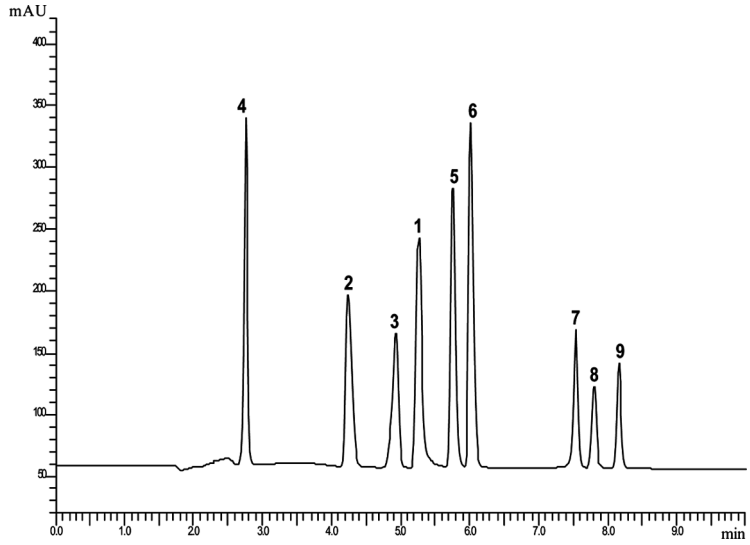

Figure 3. Separation of the pyridine derivatives. Chromatographic conditions: Atlantic HILIC Silica, 3 µm, 150 × 4.6 mm column, mobile phase A—0.1% phosphoric acid in D.I. water; B—Acetonitrile; gradient at 95% B to 60% B for 7 min, and then held at 60% B for 5 min. detection at 210 nm.

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proportions of pyridinium ions to interact with deprotonated silanols relative to 2-pyridine carboxamide. Retention of the carboxamides consequently appears to correlate with the basicity of the pyridine nitrogen reflecting an ion exchange interaction occurring between the pyridinium ions and deprotonated active silica sites.

All three of the pyridine carboxylic acid solutes elute after 2-pyridine carboxamide but before 3-pyridine carboxamide. Their relatively weaker interactions compared to 3-pyridine carboxamide can be attributed to the ability to exist as zwitterions at the pH of the mobile phase. In the zwitterion state, the negative charge of the decarboxylate group will reduce the ion exchange interactions between the protonated base and the deprotonated silica through electrostatic repulsion. The retention trend within the three pyridine carboxylic acid solutes, however, correlates with the pK_a of the pyridinium ions. Retention increases with increasing pK_a, reflecting an increase in the proportion of pyridinium ions.

The pyridine alcohols are more strongly retained than the carboxamide derivatives reflecting their higher pK_a values. However, the elution orders of the alcohols do not correlate with their pK_a values (Table 1). The first eluted alcohol is the 2,6-pyridinemethanol, which has the lowest pK_a value. However, 6-methyl-2-pyridinemethanol elutes before 2-pyridineethanol despite its higher pK_a. This anomaly can be explained in terms of

intramolecular hydrogen bonding occurring between the pyridine nitrogen and its hydroxyl proton. Ab initio molecular energy calculations optimized with B3LYP/6-31G* indicate that the hydrogen bond lengths for 6-methyl-2-pyridinemethanol and 2-pyridineethanol are 1.966A and 2.018A, respectively. Thus, 6-methyl-2-pyridinemethanol is more likely to form intramolecular hydrogen bonds in a relatively apolar medium, reducing its ability to ion exchange with deprotonated silanols. In addition, the methyl group on 6-methyl-2-pyridinemethanol may provide steric hindrance further reducing the ion exchange interactions between the pyridinium ion and the deprotonated silanols. The steric hindrance effect was further investigated by comparing the retentions of pyridine and 2-methylpyridine. The two solutes coeluted despite their differences in pK_a. It appears that the ion exchange interaction that should favor the more basic 2-methylpyridine (pK_a 6.00 in water) is offset by steric hindrance from the methyl group at the 2 position.

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Retention of Amino Acids under HILIC Conditions

The separation of amino acids by HILIC using bonded stationary phases has been previously reported.[6,9,11] In this study, the retention of five amino acids were investigated. The amino acids were separated on pure silica using acetonitrile/0.1% phosphoric acid (Table 2, Figures 4 and 5). Under the mobile phase conditions, histidine is doubly protonated and the presence of two protonated nitrogens provides the strongest ion exchange interaction with deprotonated silanols. Hence, it is the last eluted solute.

The other four amino acids are singly protonated with similar pK_a values for the α-ammonium moiety ($pK_a > 8.8$ in water). Therefore, they

Table 2. pK_a values[34–36] and relative hydrophobicity [37,38] of amino acids. pK_a values in bold represent the α-ammonium moiety

Compound name		*pKa			Hydrophobicity	Retention factor
		pK_a1	pK_a2	pK_a3		
Histidine (His)	a	1.8 (+2)	**6.04 (+1)**	**9.33 (0)**	−42	4.7
Asparagine	b	2.1 (+1)	**8.80 (0)**		−41	2.5
Aspartic acid	c	1.99 (+1)	3.90 (0)	**9.90 (−1)**	−18	1.9
Tyrosine	d	2.20 (+1)	**9.11 (0)**		49	1.7
Tryptophan	e	2.38 (+1)	**9.39 (0)**		84	1.5

*Protonated cations are designated by (+1), (+2), etc., after the pK_a value; neutral species by (0).

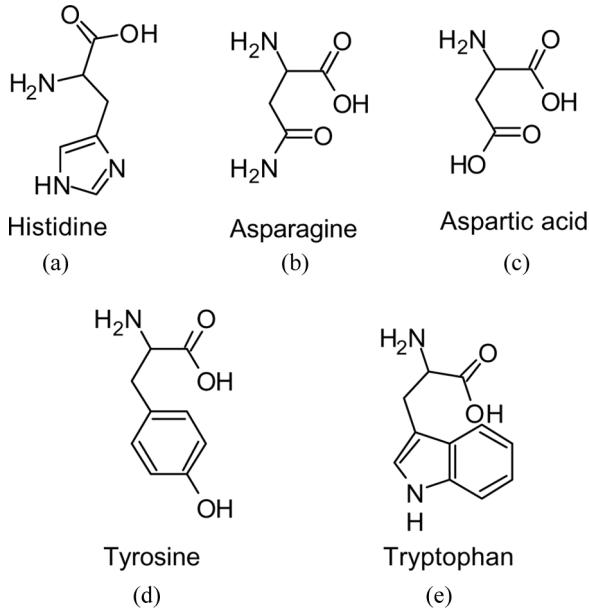

Figure 4. The structures of the amino acids.

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should all be fully protonated at the amine functionality and should undergo ion exchange interactions with deprotonated silanols to a similar extent, resulting in coelution if ion pairing was the only retention factor.

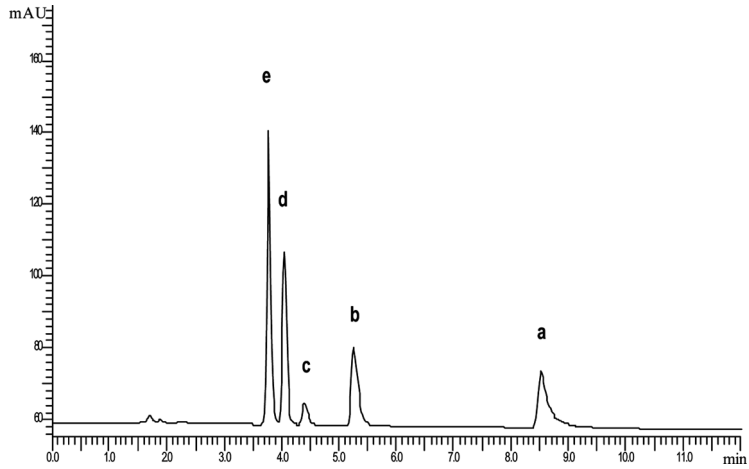

Figure 5. Separation of the amino acids. Chromatographic conditions are the same as in Figure 3, except the gradient was changed to 75% B to 45% B in 15 min.

However, their hydrophobicity increases in the sequence: asparagine < aspartic acid tyrosine < tryptophan[37] and retention is found to decrease with increasing hydrophobicity of the solute. This trend reflects the decreasing strength of polar interactions with the stationary phase with increasing hydrophobicity of the solute. The order of elution is opposite to that expected for RPLC.

Retention of Aniline Derivatives under HILIC Conditions

The retention of five aniline derivatives (Figure 6) using pure silica as the stationary phase and acetonitrile/ 0.1% phosphoric acid (Figure 7) as the mobile phase was investigated. All of the aniline derivatives possess similar pK_a values for the α ammonium moiety (pK_a: 4.48–5.04) (Table 3). Under the chromatographic conditions, 1,2-benzenediamine has the strongest retention due to the strong ion exchange capability and the absence of a negatively charged group that can lead to electrostatic repulsion. 2-Aminobenzoic acid is the least polar (highest log P) and thus would undergo weaker interactions with the stationary phase, as reflected by the fact that it is the least retained. 5-amino-1,3-Benzenedicarboxylic acid is eluted second as the two carboxylic acid groups provide significant electrostatic repulsion as compared to 3-aminobenzoic acid, which is eluted third.

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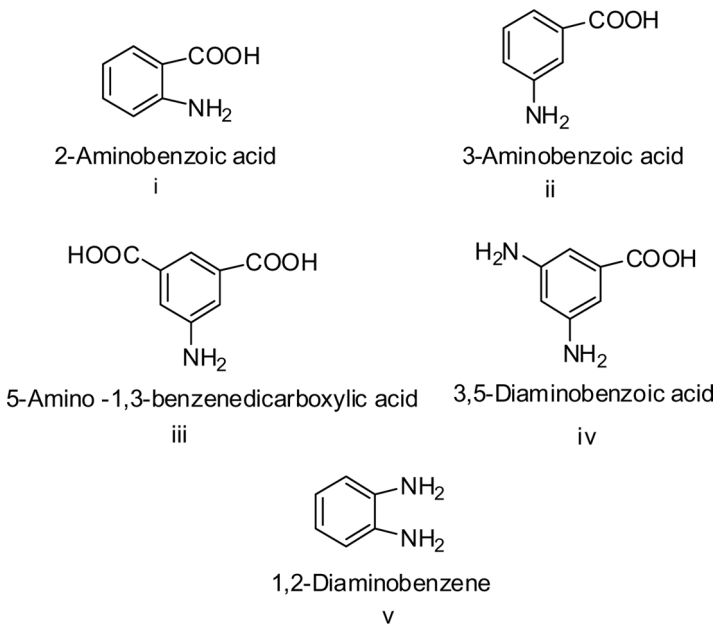

Figure 6. Structures of the aniline derivatives.

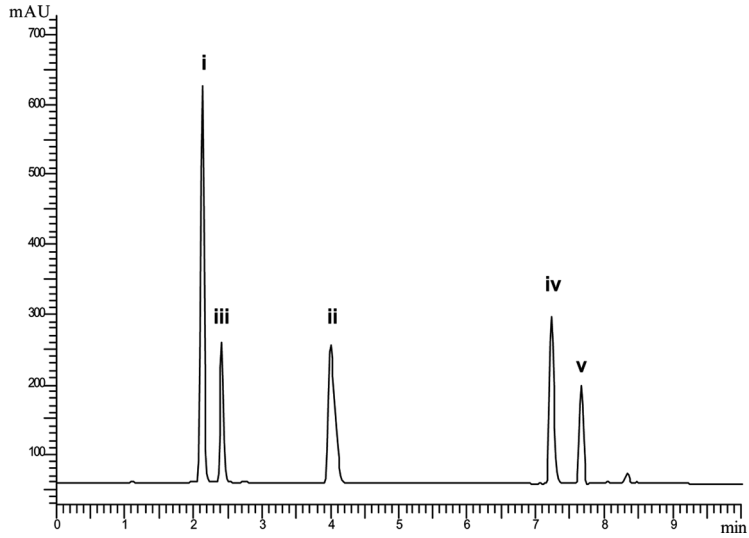

Figure 7. Retention of aniline derivatives. Chromatographic condition: Atlantic Hilic Silica, 3 μm, 150 × 4.6 mm column, mobile phase A—0.1% phosphoric acid in D.I. water; B—Acetonitrile; gradient at 95% B to 60% B in 7 min. and hold at 60% B for 3 min. detection at 210 nm.

3,5-Diaminobenzoic acid is the fourth solute eluted due to its high polarity (it has smallest log P, Table 3). It elutes before 1,2-benzenediamine as its carboxylic group does provide some electrostatic repulsion.

Application of HILIC for the Separation of a Pharmaceutical Compound

Figure 8 depicts the structures of a drug candidate (A), two process intermediates (B, C), and a synthetic impurity (D). Compound A,

Table 3. pK_a values and log P values[34–36] of the aniline derivatives. pK_a values in bold represent the α-ammonium moiety

No.	Compound name	pK_a1	pK_a2	pK_a3	log P
i	2-Aminobenzoic acid	2.17 (+1)	**4.85 (0)**		1.21
ii	3-Aminobenzoic acid	3.07 (+1)	**4.79 (0)**		0.78
iii	5-Amino-1, 3-benzene-dicarboxylic acid	2.4 (+1)	3.69 (0)	**4.91 (−1)**	0.72
iv	3,5-Diaminobenzoic acid	1.49 (+2)	**3.5 (+1)**	**5.04 (0)**	−0.29
v	1,2-Benezenediamine	**0.8 (+2)**	**4.48 (+1)**		0.05

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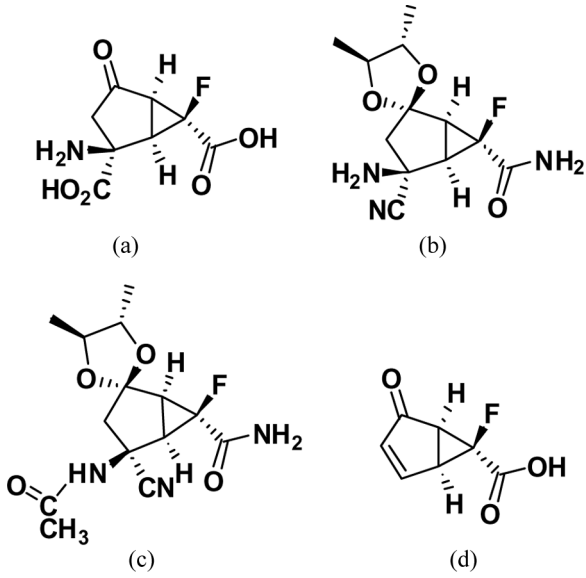

Figure 8. The structures of the amino acid drug candidate, intermediates, and impurity. a is the drug candidate, b and c are the intermediates and d is an impurity.

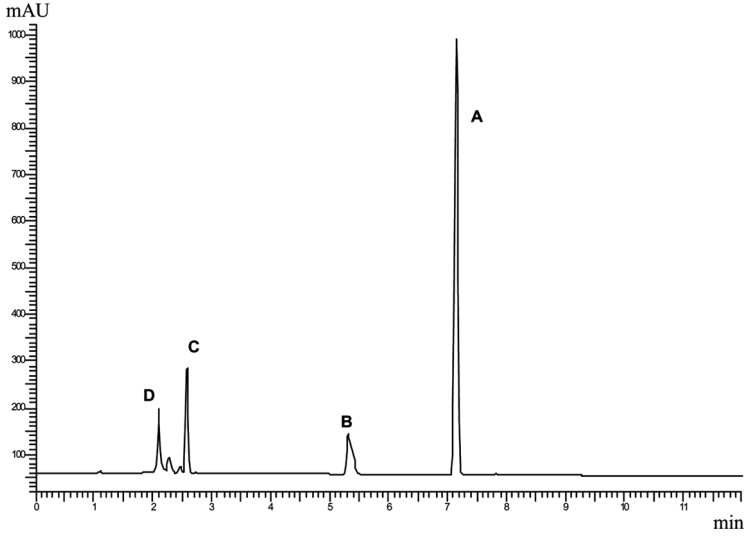

Figure 9. Retention of drug candidate A and its impurities. Chromatographic conditions were the same as in Figure 3 except $\alpha = 200$ nm.

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Table 4. pK_a and log P [34–36] of the amino acid drug candidate, intermediates

Compound	pK_a1	pK_a2	pK_a3	log P	Retention factor
A	7.24	2.34	1.63	−1.98	5.5
B	3.93			−1.01	3.8
C				−1.4	1.4
D	2.46			−1.13	0.91

(1R,2S,5S,6S)-2-amino-6-fluoro-4-oxobicyclo[3.1.0] hexane-2,6-dicarboxylic acid, is a polar amino acid, from a class of conformationally constrained, highly selective, and orally active group II metabotropic glutamate receptor (mGluR) agonists[39–42] targeted for the treatment of schizophrenia and depression, and other neurological diseases.[43,44] The high polarity of the solutes leads to poor retention under reversed phase conditions. However, retention and separation were afforded under HILIC conditions with a silica column and an acetonitrile/0.1% phosphoric acid mobile phase (Figure 9).

Both impurity C and intermediate D lack an amino group to form a positive ion to undergo ion exchange interactions with the stationary phase and thus are eluted early. Impurity C elutes after intermediate D as a consequence of its greater polarity (logP of −1.13 for impurity D versus −1.40 for intermediate C; Table 4). Intermediate B may be less retained than drug substance A as it is even less polar (logP of −1.01 for intermediate B versus −1.98 for Compound A) leading to weaker polar interactions with the stationary phase.

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CONCLUSION

The HILIC mode offers an alternative approach for the analysis of polar basic compounds that are poorly retained under reversed phase conditions. The retention mechanism for cations using a bare silica stationary phase is predominantly ion exchange with deprotonated silanols. The strength of the ion exchange is dependent upon the extent of protonation of the solute and deprotonation of silanol sites of the stationary phase. This ion exchange may be weakened by electrostatic repulsion from negatively charged moieties on the solute or by steric hindrance.

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REFERENCES

1. Nawrocki, J. The silanol group and its role in liquid chromatography. J. Chromatogr. A. **1997**, *779*, 29–71.
2. Law, B. Use of silica with reversed-phase type eluents for the analysis of basic drugs and metabolites. Trends Anal. Chem. **1990**, *9*, 31–36.
3. Einarsson, S.; Josefsson, B.; Lagerkvist, S. Separation of basic drugs by high-performance liquid chromatography on a silica column using a methanol-ethylenediamine buffer. J. Chromatogr. **1983**, *280*, 609–618.
4. Roturier, J.M.; Bars, D.L.; Gripon, J.C. Separation and identification of hydrophilic peptides in dairy products using FMOC derivatization. J. Chromatogr. A. **1995**, *696*, 209–217.
5. Hemstrom, P.; Irgum, K. Hydrophilic interaction chromatography. J. Sep. Sci. **2006**, *29*, 1784–1821.
6. Alpert, A.J. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. J. Chromatogr. **1990**, *499*, 177–196.
7. Law, B. Analysis of basic compounds on a silica column with an aqueous methanol eluent: The use of quantitative structure-retention relationships in metabolite identification. J. Chromatogr. **1987**, *407*, 1–18.
8. Binder, S.R. In: *Analysis of Addictive and Misused Drugs*; Adamovics, J.A., Ed.; Marcel Dekker, Inc.: New York. 1995, 133.
9. Schlichtherle-Cerny, H.; Affolter, M.; Cerny, C. Hydrophilic interaction liquid chromatography coupled to electrospray mass spectrometry of small polar compounds in food analysis. Anal. Chem. **2003**, *75*, 2349–2354.
10. Churms, S.C. Recent progress in carbohydrate separation by high-performance liquid chromatography based on hydrophilic interaction. J. Chromatogr. A. **1996**, *720*, 75–91.
11. Oyler, A.R.; Armmstrong, B.L.; Cha, J.Y.; Zhou, M.X.; Yang, Q.; Robinson, R.I.; Dunphy, R.; Burinsky, D.J. Hydrophilic interaction chromatography on amino-silica phases complements reversed-phase high-performance liquid chromatography and capillary electrophoresis for peptide analysis. J. Chromatogr. A. **1996**, *724*, 378–383.
12. Tanaka, H.; Zhou, X.J.; Masayoshi, O. Characterization of a novel diol column for high-performance liquid chromatography. J. Chromatogr A. **2003**, *987*, 119–125.
13. Strege, M.A. Hydrophilic interaction chromatography-electrospray mass spectrometry analysis of polar compounds for natural product drug discovery. Anal. Chem. **1998**, *70*, 2439–2445.
14. Strege, M.A.; Stevenson, S.; Lawrence, S.M. Mixed-Mode Anion-Cation Exchange/Hydrophilic Interaction Liquid chromatography-electrospray mass spectrometry as an alternative to reversed phase for small molecule drug discovery. Anal. Chem. **2000**, *72*, 4629–4633.
15. Guo, Y.; Huang, A. A HILIC method for the analysis of tromethamine as the counter ion in an investigational pharmaceutical salt. J. Pharm. Biomed. Anal. **2003**, *31*, 1191–1201.

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16. Li, R.; Huang, J. Chromatographic behavior of epirubicin and its analogues on high-purity silica in hydrophilic interaction chromatography. J. Chromatogr. A. **2004**, *1041*, 163–169.
17. Dallet, P.; Labat, L.; Kummer, E.; Dubost, J.P. Determination of urea, allantoin and lysine pyroglutamate in cosmetic samples by hydrophilic interaction chromatography. J. Chromatogr. B. **2000**, *742*, 447–452.
18. Bidlingmeyer, B.A.; Del Rios, J.K.; Korpi, J. Separation of organic amine compounds on silica gel with reversed-phase eluents. Anal. Chem. **1982**, *54*, 442–447.
19. Cox, G.B.; Stout, R.W. Study of the retention mechanism for basic compounds on silica under ''pseudo-reversed-phase'' conditions. J. Chromatogr. **1987**, *384*, 315–336.
20. Marcus, Y.; Migron, Y. Polarity, hydrogen bonding, and structure of mixtures of water and cyanomethane. J. Phys. Chem. **1991**, *95*, 400–406.
21. Katz, E.D.; Lochmuller, C.H.; Scott, R.P.W. Methanol-water association and its effect on solute retention in liquid chromatography. Anal. Chem. **1989**, *61*, 349–355.
22. McCalley, D.V. Influence of analyte stereochemistry and basicity on peak shape of basic compounds in high-performance liquid chromatography with reversed-phase columns, using pyridine and alkyl-substituted derivatives as probe compounds. J. Chromatogr. A. **1994**, *664*, 139–147.
23. Barbosa, J.; Beltran, J.; Sanz-Nebot, V. Ionization constants of pH reference materials in acetonitrile—water mixtures up to 70% (w/w). Anal. Chim. Acta. **1994**, *288*, 271–278.
24. Barbosa, J.; Sanz-Nebot, V. Preferential solvation in acetonitrile-water mixtures: Relationship between solvatochromic parameters and standard pH values. J. Chem. Soc. Faraday Trans. **1994**, *90*, 3287–3292.
25. Roses, M.; Canals, I.; Alleman, H.; Siigur, K.; Bosch, E. Retention of ionizable compounds on HPLC. 2. Effect of pH, ionic strength, and mobile phase composition on the retention of weak acids. Anal. Chem. **1996**, *68*, 4094–4100.
26. Bosch, E.; Bou, P.; Alleman, H.; Roses, M. Retention of Ionizable Compounds on HPLC. pH Scale in Methanol-Water and the pK and pH Values of Buffers. Anal. Chem. **1996**, *68*, 3651–3657.
27. Sykora, D.; Tesarova, E.; Popl, M. Interactions of basic compounds in reversed-phase high-performance liquid chromatography influence of sorbent character, mobile phase composition, and pH on retention of basic compounds, J. Chromatogr. A. **1997**, *758*, 37–51.
28. Canals, I.; Oumada, F.; Roses, M.; Bosch, E. Retention of ionizable compounds on HPLC. 6. pH measurements with the glass electrode in methanol–water mixtures. J. Chromatogr. A **2001**, *911*, 191–202.
29. LoBrutto, R.; Jones, A.; Kazakevich, Y.; McNair, H. Effect of the eluent pH and acidic modifiers in high-performance liquid chromatography retention of basic analytes. J. Chromatogr. A **2001**, *913*, 173–187.
30. Espinosa, S.; Bosch, E.; Roses, M. Retention of Ionizable Compounds on HPLC. 12. The Properties of liquid chromatography buffers in acetonitrile-water mobile phases that influence HPLC retention. Anal. Chem. **2002**, *74*, 3809–3818.

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31. Espinosa, S.; Bosch, E.; Roses, M. Retention of ionizable compounds on HPLC. 14 Acid-base pK values in acetonitrile-water mobile phases. J. Chromatogr. A. **2002**, *964*, 55–66.
32. Subirats, X.; Bosch, E.; Roses, M. Retention of ionizable compounds on HPLC. 15. Estimation of the pH variation of aqueous buffers with the change of the acetonitrile fraction of the mobile phase. J. Chromatogr. A. **2004**, *1059*, 33–42.
33. Mendez, A.; Bosch, E.; Roses, M.; Neue, U. Comparison of the acidity of residual silanol groups in several liquid chromatography columns. J. Chromatogr. A. **2003**, *986*, 33–44.
34. Lide, D.R., Ed. *CRC, Handbook of Chemistry and Physics*, 85th Ed.; CRC Press: Boca Raton, FL, 2004.
35. Dean, J.A., Ed. *Lange's Handbook of Chemistry*, 14th Ed.; McGraw-Hill: New York, 1992.
36. The predicted values of pK_a and log P were obtained using the ACD Lab 6.0 software (Advanced Chemistry Development, Inc., Toronto, Canada).
37. Sereda, T.J.; Mant, C.T.; Sonnichsen, F.D.; Hodges, R.S. Reversed-phase chromatography of synthetic amphipathic α-helical peptides as a model for ligand/receptor interactions Effect of changing hydrophobic environment on the relative hydrophilicity/hydrophobicity of amino acid side-chains. J. Chromatogr. A. **1994**, *676*, 139–153.
38. http://www.sigmaaldrich.com/Area_of Interest/Biochemicals/Peptide_Explorer/Key_Resources/Reference_Chart.html
39. Nakazato, A.; Kumagai, T.; Sakagami, K.; Yoshikawa, R.; Suzuki, Y.; Chaki, S.; Ito, H.; Taguchi, T.; Nakanishi, S.; Okuyama, S. Synthesis SARs, and pharmacological characterization of 2-amino-3- or 6-fluorobicyclo[3.1.0] hexane-2,6-dicarboxylic acid derivatives as potent, selective, and orally active group II metabotropic glutamate receptor agonists. J. Med. Chem. **2000**, *43*, 4893–4909.
40. Monn, J.; Valli, M.; Massey, S.; Wright, R.; Salhoff, C.; Johnson, B.; Howe, T.; Alt, C.; Rhodes, G.; Robey, R.; Griffey, K.; Tizzano, J.; Kallman, M.; Helton, D.; Schoepp, D. Design, synthesis, and pharmacological characterization of (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740): A potent, selective, and orally active group 2 metabotropic glutamate receptor agonist possessing anticonvulsant and anxiolytic properties. J. Med. Chem. **1997**, *40*, 528–537.
41. Helton, D.; Tizzano, J.; Monn, J.; Schoepp, D.; Kallman, M. Anxiolytic and Side-Effect Profile of LY354740: A potent, highly selective, orally active agonist for group II metabotropic glutamate receptors. J. Pharmacol. Exp. Ther. **1998**, *284*, 651–660.
42. Monn, J.; Valli, M.; Massey, S.; Hansen, M.; Kress, T.; Wepsiec, J.; Harkness, A.; Grutsch, J.; Wright, R.; Johnson, B.; Andis, S.; Kingston, A.; Tomlinson, R.; Lewis, R.; Griffey, K.; Tizano, J.; Schoepp, D. Synthesis, pharmacological characterization, and molecular modeling of heterobicyclic amino acids related to (+)-2-aminobicyclo[3.1.0] hexane-2,6-dicarboxylic acid (LY354740): Identification of two new potent, selective and systemically active agonists for group II metabotropic glutamate receptors. J. Med. Chem. **1999**, *42*, 1027–1040.

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43. Wickelgren, I. A new route to treating Schizophrenia? Science **1998**, *281*, 1264–1265.
44. Moghaddam, B.; Adams, B. Reversal of phencyclidine effects by a group II metabotropic glutamate receptor agonist in rats. Science. **1998**, *281*, 1349–1352.

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