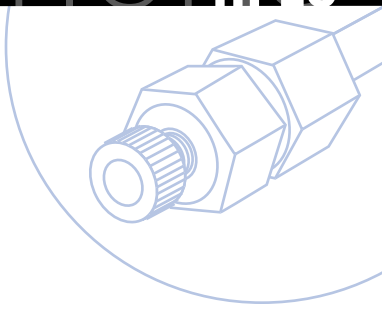

Deleterious Effects of Formic Acid without Salt Additives on the HILIC Analysis of Basic Compounds

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Abstract
Formic acid is an often-used mobile phase additive for adjusting pH in reversed phase liquid chromatography (RPLC), especially when using mass spectrometric (MS) detection. This practice has been carried over to hydrophilic interaction liquid chromatography (HILIC) separations. However, the mechanisms of action and the relative importance of buffer cation and anion are much different in HILIC than RPLC. For this reason buffer selection in HILIC mode requires consideration of buffer, analyte and chromatographic sorbent chemical properties to make an appropriate choice. Proper choice of buffer can make the difference between success and failure with HILIC. In this paper, the behavior of formic acid with and without the addition of various salts on the HILIC separation of basic analytes is explored. Recommendations for buffer choice for HILIC analysis of basic compounds are proposed.

Introduction
A usual requirement of an analytical method is robustness, which is typically defined as the ability of the method to provide accurate and precise results despite minor variations in equipment and conditions. Historically, HILIC has suffered from a reputation for poor robustness. However, with judicious choice of conditions, where analyte, buffer and chromatographic sorbent physical properties, such as surface silanol activity, are considered, robust HILIC methods can be developed. The HILIC retention mechanism primarily involves partitioning of polar analytes between a water enriched layer of solvent near the sorbent surface and the relatively more hydrophobic bulk eluent. Several other physical processes also play a major role in determining retention and selectivity in HILIC such as ion exchange, hydrogen-bonding, dipole-dipole, and others. A recent review by Hemstrom and Irgum (1) provides more detail regarding HILIC retention mechanisms. HILIC is generally performed using mobile phases containing high concentrations (> 70 v/v %) of acetonitrile. Organic solvents have a pronounced effect on buffer and analyte pK_a, especially as the concentration exceeds 50 v/v %. Several papers have detailed the effect of various organic solvents on analyte pK_a (2,3). In general, the pK_a of weak bases decrease with increasing organic while the pK_a of weak acids increase. The pK_a shifts can be quite significant in the high organic environment used for HILIC. For example, weak bases with aqueous pK_a less than ~4 typically will not be protonated in HILIC mobile phases when 0.1 v/v % formic acid is used. The pK_a of the base is decreased in HILIC mobile phase while the pK_a of the formic acid is increased. The increased pK_a of formic acid leads to an increase in mobile phase pH. The combination of these opposing changes in pK_a results in 0.1 v/v % formic acid being too weak to protonate bases with pK_a < ~4. Therefore, formic acid can provide acceptable chromatographic performance for weak bases with aqueous pK_a < ~4. However, basic compounds with aqueous pK_a greater than ~4 can be protonated under HILIC conditions with formic acid. Protonated bases can participate in cation exchange interactions with residual silanols on the sorbent surface. Poorly moderated ion exchange interactions can lead to poor chromatographic performance. Therefore, when choosing a buffer for the analysis of basic compounds the pK_a of the analytes of interest as well as the nature and pK_a of the buffer must be considered.

Experimental Conditions
The chromatographic system consisted of an Agilent 1100 series binary pump (Palo Alto, California), on-line solvent degasser, autosampler, column temperature module and either an Agilent 1100 diode array detector or an Applied Biosystems API3000 tandem mass spectrometer with TurboIonSpray® electrospray ionization interface (ESI). For UV analyses the system was controlled with HP Chemstation software, and for tandem MS analyses the system was controlled using Analyst 1.41 software.

Chromatographic separations were performed on a Phenomenex Luna® 3 µm HILIC, 100 x 2.0 mm ID column (Torrance, California).

Chemicals
Nicotine and cotinine (1 mg/mL in methanol) were obtained from Cerilliant (Round Rock, Texas). Nornicotine, acyclovir, ganciclovir, toluene (void marker), and all buffers and salts (lithium chloride,

potassium chloride, sodium chloride, ammonium chloride, ammonium formate, ammonium acetate) were obtained from Sigma Aldrich (Bellefonte, Pennsylvania). Acetonitrile (ACN) was obtained from Honeywell Burdick and Jackson (Muskegon, Michigan) and formic acid from EMD Biosciences (Madison, Wisconsin). A nornicotine stock solution (5 mg/mL) was prepared by dissolving 25 mg nornicotine in 5 mL ACN. This was further diluted to give a nornicotine concentration of 1 mg/mL by adding 1 mL stock solution to 4 mL ACN. Standards (0.1 mg/mL each) were prepared by adding 100 µL of each 1 mg/mL stock solution to 700 µL ACN solution. An acyclovir and ganciclovir stock solution (0.5 mg/mL) was prepared by dissolving 5 mg of each compound in 10 mL of 0.1 v/v % ammonium hydroxide in water. This was further diluted to give acyclovir and ganciclovir concentrations of 2.5 µg/mL by adding 100 µL stock solution to 20 mL 90:10 ACN/100 mM ammonium formate buffer (pH 3.2). Standards (500 ng/mL) were prepared by adding 1 mL of 2.5 µg/mL stock solution to 4 mL 90:10 ACN/100 mM ammonium formate buffer (pH 3.2).

Chromatographic conditions

A stock solution of HILIC mobile phase was prepared by combining 1.8 L ACN, 200 mL of water and 2 mL of formic acid. The stock mobile phase (0.1 v/v % formic acid in 90/10 ACN/Water) was separated into 7 different 250 mL aliquots. Separate stock solutions (2.5 M) of each salt were prepared. To each 250 mL aliquot of stock mobile phase, 1 mL of a different stock salt solution was added giving an effective salt concentration of 10 mM. To the remaining 250 mL aliquot of stock mobile phase, 1 mL of water was added to ensure the elutropic strength of all mobile phases was equivalent. All separations were performed isocratically at 0.4 mL/min with column temperature controlled at 25 ˚C and a 1 µL injection volume. Nicotine and metabolites were detected using UV absorbance at 260 nm. Ganciclovir and acyclovir were detected using tandem MS in positive ion mode with mass transitions 256.2 ➙ 152.3 and 226.2 ➙ 152.2, respectively.

Results and Discussion

Results with 0.1 v/v % formic acid mobile phase

Basic analytes with aqueous pK_a > ~4 can be ionized under HILIC conditions with 0.1 v/v % formic acid and thus undergo cation exchange with the sorbent. With these analytes, formic acid typically does not provide the best chromatographic performance. One explanation for this behavior is that the hydronium cation generated by formic acid is not an effective competing cation for the ionized silanol groups. Therefore, the ion exchange component of the cationic basic analyte retention is more influential on overall separation performance. An additional factor may involve the combined effect the hydronium cation and formate anion have on the water layer near the sorbent surface. The effects different cations and anions have on the localized water structure at or near the surfaces of such diverse species as proteins, colloids and water soluble polymers have been well documented in the literature (4-7). Since the HILIC retention mechanism is highly dependant on the semi-immobilized water layer near the sorbent surface, it is expected that HILIC chromatographic performance will be affected by changes in localized water structure and ligand solvation brought about by different buffer cations and anions. Additionally, the ionic strength of the ~10 % dissociated formic acid is low compared to the buffers prepared from salts. The result is poor peak shape, slow column equilibration and overall poor reproducibility with formic acid without salt additives. Chromatograms showing column equilibration in HILIC mode using 0.1 v/v % formic acid with the basic analytes nicotine, cotinine and nornicotine are shown in Figure 1. The pK_a of these compounds are all > 4 and their structures and other physical properties are shown in Figure 2. As shown in the figure, retention times decrease over time and the analytes show generally poor peak shape when formic acid without salt additives is used. Such retention time instability likely has contributed to the belief that HILIC lacks the robustness of RPLC. The irreproducible behavior of nicotine and metabolites is contrasted with the good chromatographic performance of the weakly basic compounds acyclovir and ganciclovir shown in Figure 3. Both compounds have pK_a < 3 and show good chromatographic performance with formic acid without salt additives. While retention and selectivity for acyclovir and ganciclovir can be improved with the higher ionic strength 10 mM ammonium formate at pH 3.2, both mobile phase conditions provide good performance.

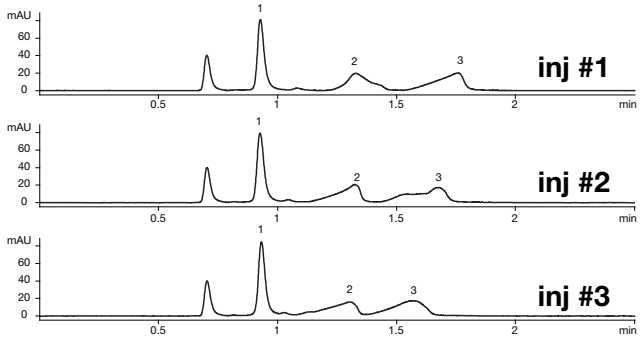

Figure 1: Retention time drift of cotinine (1), nicotine (2) and nornicotine (3) with 0.1 v/v % formic acid in 90/10 Acetonitrile/Water mobile phase. The very first peak in each chromatogram is the void marker toluene. Isocratic separation with Luna 3 µm HILIC 100 x 2.0 mm ID at 0.4 mL/min

Results with 0.1 v/v % formic acid with 10 mM salts in mobile phase

As discussed previously, for the higher pK_a basic functional groups of nicotine and its metabolites, formic acid without salt additives does not provide good chromatographic performance. However, peak shapes and retention time stability are greatly improved if a cation with a higher affinity for the ionized silanols, such as ammonium, is added to the mobile phase. A series of experiments demonstrating the effect of the cation counter ion on peak shape and retention time reproducibility was performed and the results shown in Figure 4. As shown in the figure, the addition of 10 mM of Li^+, Na^+, K^+ or NH_4^+ chloride improved peak shape and retention time stability (not shown) in all cases. The retention time increases observed with the addition of different cations correlates with the inverse of the hydrated radius of the cation and the inverse of the "normal" lyotropic series. The weak silicate ion exchange groups on the surface of the silica-based sorbent have been shown to behave quite differently than the strong sulfonate-based cation exchangers (8). These seemingly anomalous results are likely due to differences in affinity of silicate vs. sulfonate cation exchangers (8). The different cations experiments demonstrating the effect of the cation counter ion on peak shape and retention time reproducibility was performed and the results shown in Figure 4. As shown in the figure, the addition of 10 mM of Li^+, Na^+, K^+ or NH_4^+ chloride improved peak shape and retention time stability (not shown) in all cases. The retention time increases observed with the addition of different cations correlates with the inverse of the hydrated radius of the cation and the inverse of the "normal" lyotropic series. The weak silicate ion exchange groups on the surface of the silica-based sorbent have been shown to behave quite differently than the strong sulfonate-based cation exchangers (8). These seemingly anomalous results are likely due to differences in affinity of silicate vs. sulfonate cation exchangers (8). The different cations and the associated anion also likely affect the hydrated layer near the sorbent surface by affecting the localized water structure, dielectric constant, and zeta potential.

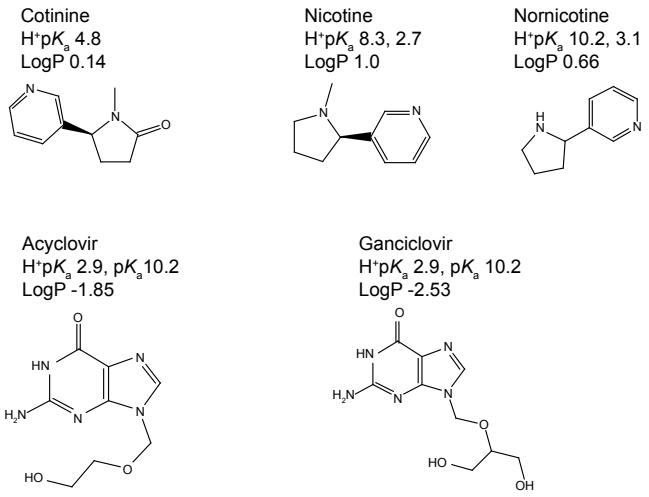

Figure 2: Structure and physical properties of the basic test probes cotinine, nicotine, nornicotine, ganciclovir and acyclovir.

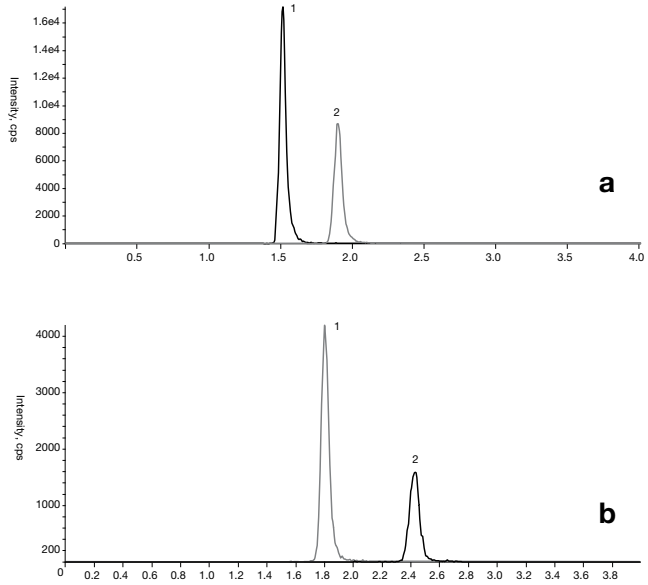

Figure 3: HILIC separation of the weakly basic (pK_a ~2.9) antiviral compounds acyclovir (1) and ganciclovir (2) using 90/10 Acetonitrile/Water with 0.1 v/v % formic acid (a) and 90/10 Acetonitrile/Water with 10 mM ammonium formate (b). Isocratic separation with Luna 3 µm HILIC 100 x 2.0 mm ID at 0.4 mL/min

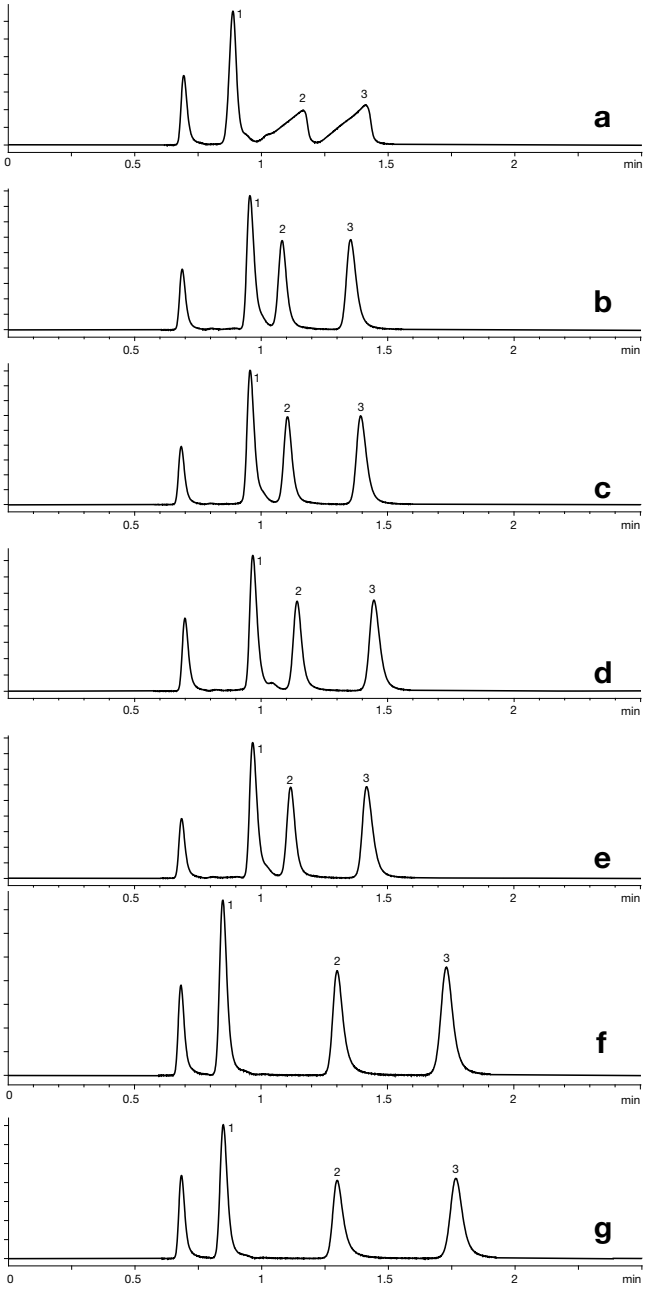

Figure 4: Effect of H^+(a), Li^+ (b), Na^+ (c), K^+ (d), NH_4^+ (e), NH_4 formate (f) and NH_4 acetate (g) on the chromatographic separation of cotinine (1), nicotine (2) and nornicotine (3). The very first peak in each chromatogram is the void marker toluene. Isocratic separation with Luna 3 µm HILIC 100 x 2.0 mm ID at 0.4 mL/min

ORDERING INFORMATION

Part No.	Description
00D-4449-B0	Luna 3 µ HILIC 100 x 2.0

Effect of anion on retention and selectivity

Anions have generally been shown to dominate the water structuring behavior of electrolytes, and therefore, the role of the anion in HILIC chromatographic performance cannot be ignored. In these examples all salts were chlorides with the exception of ammonium formate and acetate. Interestingly, addition of the ammonium formate and acetate salts showed a larger impact on retention than the ammonium chloride salt. Of particular interest is the decrease in retention for cotinine with addition of ammonium formate or acetate. The retention of both nicotine and nornicotine increased with the addition of the acetate salts while the retention of cotinine decreased. One explanation for this behavior is the increase in mobile phase pH (~0.5 pH units) with addition of acetate or formate. The pK_a of the pyridinyl functional group in cotinine is much higher than those in nicotine and nornicotine. Therefore, the observed increase in aqueous pH from 2.7 to ~3.2 with the addition of formate or acetate likely decreases the ionization of this compound. Decreasing ionization decreases the hydrophilicity and thus reduces retention in HILIC. The significantly lower pK_a (< 4) of the pyridinyl functional groups in nicotine and nornicotine explain why their retention does not decrease. On the contrary, these compounds show increased retention which may be related to the aforementioned changes on the hydrated layer.

Conclusions

Formic acid is often used for adjusting mobile phase pH in RPLC but it is not the best choice for HILIC separations of basic analytes. For analytes with aqueous pK_a greater than ~4 the ammonium salts of acetic and formic acid provide much better chromatographic performance than formic acid and are still compatible with MS detection. Various counter ions were shown to improve chromatographic performance of basic analytes over that observed with formic acid without salt additives. The mechanism of action responsible for the improved performance with added counter ions is likely a combination of improved competition for the silicate cation exchange groups on the surface, changes in the properties of the hydrated layer, and changes in the solvation environment of the ligand. These results show the mechanism of action and the relative importance of the buffer cation and anion exerts a much stronger influence on separation performance of basic compounds in HILIC than RPLC. Therefore, the choice of buffer cation and anion should be made considering the analyte type (acid or base) and pK_a as well as the buffer type and pK_a.

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