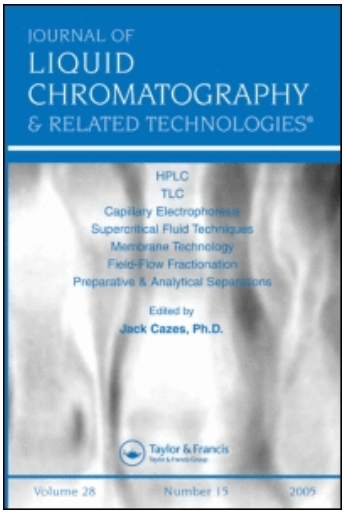

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Effects of Trifluoroacetic Acid Concentrations in Mobile Phases on HPLC Retention of Zwitterionic and Weakly Basic Triazole Derivatives

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Abstract: Minor changes in the concentration of trifluoroacetic acid (TFA), a popular acidic HPLC mobile phase modifier, resulted in dramatic affects on hydrophobic retention. Two 1,2,4-triazole carboxylic acids and the corresponding decarboxylated 1,2,4-triazoles were chromatographed using a reversed-phase column with 0.01% to 0.4% TFA in the mobile phases. Retention time shifts and reversed elution orders were observed. At low TFA concentrations, the decarboxylated 1,2,4-triazoles eluted earlier, whereas at high TFA concentrations the two 1,2,4-triazole carboxylic acids eluted earlier. An increased capacity factor, when TFA concentrations exceeded 0.05%, exhibited an increased hydrophobicity as a result of TFA-associated chaotropic effects. Formic acid, acetic acid, or phosphoric acid were not as effective as TFA and attenuated the retention shifts significantly such that the reversed order of elution was no longer observed. The use of a combination of TFA and triethylamine (TEA) generated similar chromatographic profiles to those obtained when TFA was used alone. In conclusion, ideal HPLC separations of zwitterionic and weakly basic compounds could sometimes be obtained simply by optimizing TFA concentration in mobile phases.

Keywords: Trifluoroacetic acid (TFA), TFA concentration, Zwitterions, Solvation, Chaotropic effect, Retention time shift, Elution order, 1,2,4-Triazoles

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INTRODUCTION

Protonation, ion-pairing, and LC-MS compatibility are often the rationales for selecting trifluoroacetic acid (TFA) as the mobile phase modifier in HPLC analyses of basic, amine-containing pharmaceuticals and their synthetic intermediates. The benefits of using TFA are so obvious that it has now become the primary acidic modifier in some method development laboratories.

The effect of TFA on retention of basic compounds in reversed-phase chromatography has been attributed primarily to its influence on solvation of the basic solutes under acidic conditions. The degree of solvation, and, subsequently, the degree of retention on reversed-phase columns were investigated, using TFA, phosphoric acid, perchloric acid, and a range of acids as mobile phase modifiers.[1–7] These studies, in which many simple, strongly basic amines were used as model compounds, demonstrated that TFA and other acids had a strong effect on protonation, solvation, and hydrophobic retention. The concentration of these acids in HPLC mobile phases as well as the type of acidic modifier influenced the retention of solutes. Generally, higher concentrations of the acidic modifiers resulted in stronger retention.[1–3,8] This correlation was considered to be the effect of desolvation or chaotropic effect.[9,10] Increased concentration of chaotropic anions, such as perchlorate, hexafluorophophorate, and tetrafluoroborate, exhibited a positive influence on chromatographic parameters including loading capacity, peak efficiency, and peak symmetry.[11]

The degree of ionization, protonation, and solvation of basic compounds under acidic conditions reflected their intrinsic basicity or pK_a values. These pK_a values, however, changed in the presence of organic solvents, like the commonly used solvents acetonitrile and methanol, which led to different solute retention profiles in reversed-phase HPLC.[12–25] The addition of organic solvents to the mobile phase changed the pK_a values of acidic modifiers as well with increased pK_a values reported for many weak acids including commonly used acids in HPLC.[26–32]

Many pharmaceutical compounds are basic, and their impurities, as well as some of the degradation products possess structural, physical and chemical properties that are closely related to the active pharmaceutical ingredient (API). At times separating impurities and degradation products from the main component is challenging. The two pairs of 1,2,4-triazoles shown in Figure 1 were used in the development of a new investigational drug. They are weakly basic and have similarities in structures, chemical properties, and reactivity. Minor differences exist between the pK_a values for each pair of triazoles, although one of the triazoles is zwitterionic. Separation of these triazoles by reversed-phase HPLC was readily achieved, but we undertook this study to elucidate the effects of TFA, as a mobile phase modifier, on the retention of these solutes. We found that varying the TFA concentration led to significant changes in the chromatographic profiles of these compounds.

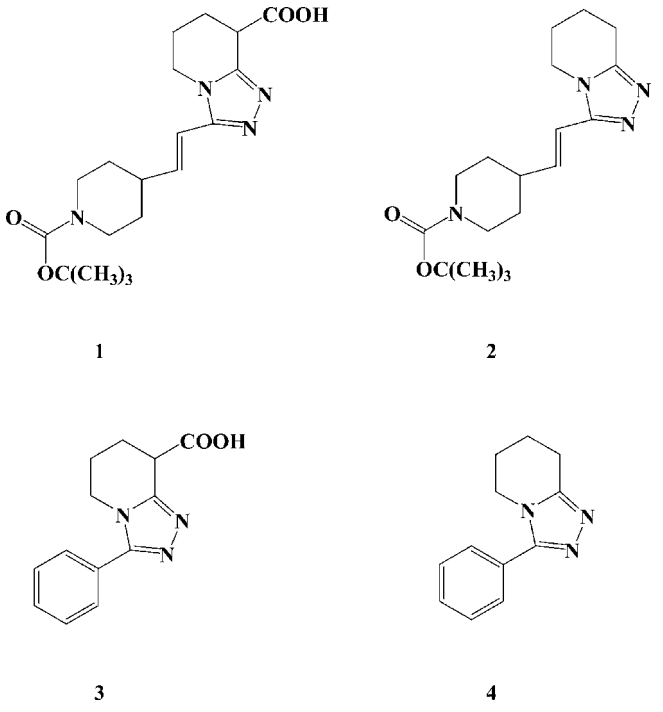

Figure 1. Structures of the triazoles included in the study.

EXPERIMENTAL

Chemicals and Reagents

Trifluoroacetic acid and 88% formic acid were purchased from J. T. Baker (Phillipsburg, NJ, USA), glacial acetic acid and 85% phosphoric acid were from Fisher Scientific (Fair Lawn, NJ, USA), and HPLC grade acetonitrile was from Burdick & Jackson (Muskegon, MI, USA). Water for preparation of HPLC mobile phases was purified using a Milli-Q Gradient system manufactured by Millipore (Bedford, MA, USA).

Triazole Derivatives

The 1,2,4-triazole carboxylic acid **1** was prepared by careful saponification of the corresponding ethyl ester.[33,34] The 1,2,4-triazole **2** was a process associated impurity, which was not isolated. The 3-phenyl-1,2,4-triazoles **3** and **4** were prepared from benzoic acid hydrazide and ethyl 2-oxo-piperidine-3-carboxylate or δ-valerolactam, respectively, by modification of Bonanomi and Biaocchi's protocol.[35] The pK_a values for **1**, **3**, and **4** were determined

by Robertson Microlit Laboratories, Inc. (Madison, NJ, USA), and reported as a weighted mean of replicate measurements.

Instrumentation

The Agilent 1100 HPLC system consisted of a quaternary pump equipped with a degasser, an auto-sampler, and a photodiode array (PDA) detector. The Waters system had a 2690 Alliance separation module and a 996 PDA detector. Column temperature was controlled at 25°C. Symmetry C_{18} (5 μm, 3.9 × 250 mm) and Symmetry Shield RP_8 (3.5 μm, 4.6 × 150 mm) columns were purchased from Waters (Medford, MA, USA), Luna C_{18} (2) (3 μm, 4.6 × 150 mm) was purchased from Phenomenex (Torrance, CA, USA), and Zorbax SB-C_{18} (3.5 μm, 4.6 × 150 mm) was purchased from Agilent Technologies (Palo Alto, CA, USA). A Model 8100 pH meter purchased from VWR (West Chester, PA, USA) was calibrated against standard pH solutions prior to HPLC mobile phase measurements.

HPLC Methods

The HPLC methods employed a Waters' Symmetry C_{18} column, unless otherwise specified in the figure captions, 5–10 μL injection, 220 nm detection, and mobile phase A and B containing water and acetonitrile, respectively. The linear gradient to separate compounds **1** and **2** ran from 5% to 80% B in 40 minutes; the linear gradient for compounds **3** and **4** ran from 5% to 35% B in 20 minutes. TFA and other mobile phase modifiers were added, in units of v/v%, to both mobile phase A and B. A mixture of equal volumes of water and acetonitrile was used as the sample solvent, and the sample concentration was between 0.3 and 0.7 mg/mL. The pH values were obtained by measuring mobile phase A containing various concentrations of TFA.

RESULTS AND DISCUSSION

pH of Mobile Phase A at Various TFA Concentrations

Predictably, the pH of the mobile phase was inversely proportional to the TFA concentration; increasing the concentration of TFA resulted in a lower pH (Table 1). For example, the pH of mobile phase A was 2.84 at a TFA concentration of 0.01%; increasing the TFA concentration to 0.4% lowered the pH to 1.50. It was not possible to determine the apparent pH during chromatography but the pH of mobile phase A provided information on the direction and extent

Table 1. pH values of mobile phase A with various TFA concentrations

TFA concentration (%)	pH
0.01	2.84
0.05	2.30
0.10	2.03
0.15	1.88
0.20	1.76
0.30	1.62
0.40	1.50

The pH values were obtained by measuring the pH of mobile phase A (water and TFA) at room temperature.

of the changes in pH, and enabled one to predict the degree of protonation of analytes and their possible chromatographic behaviors.

As derivatized triazoles in Table 2 showed, compounds **1**, **3**, and **4**, are more basic than the parent compound, 1*H*-1,2,4-triazole, and are weak bases (Table 2). Because the 1,2,4-triazole **2**, was not isolated and was not available for pK_a measurements, the pK_a value for **2** was assumed to be similar to the pK_a of the 1,2,4-triazole carboxylic acid, **1**. At TFA concentrations of 0.01% to 0.15% the pH of mobile phase A was 2.84 to 1.88. Within this pH range, the 1,2,4-triazole ring in **1** was nearly completely protonated: i.e., 98% protonation at pH 2.84 and 99.8% protonation at pH 1.88. However, the carboxylic acid in **1** was only partially dissociated within this pH range; i.e., about 36% of the carboxyl groups of **1** were ionized at pH 2.84, and approximately 6% at pH 1.88. Overall, the 1,2,4-triazole carboxylic

Table 2. pK_a values of 1,2,4-triazoles

Compound	pK_a
1	4.54, 3.10
3	3.86, 2.28
4	3.90
1*H*-1,2,4-triazole	2.27

The pK_a values for the 1,2,4-triazoles **1**, **3**, and **4** were determined by extrapolation using methanol as a co-solvent. The pK_a value of 1*H*-1,2,4-triazole is from the *CRC Handbook of Chemistry and Physics*, 8–46, 82nd edition, 2001–2002.

acid **1** existed as a partially protonated, positively charged zwitterion within the pH range of 1.88 to 2.84. In contrast, in the absence of a carboxy group, **2** was protonated at all times in this pH range; i.e., 98% at pH 2.84 and 99.8% at pH 1.88. Assuming that hydrophobic retention and elution at constant temperature were not influenced by solvation of an analyte in the presence of acetonitrile, then compound **1** would elute faster as the TFA concentration was increased from 0.01% to 0.15%. In contrast, the retention time of compound **2** would not be affected over this pH range. The triazole ring of **3** was calculated to be 91% protonated at pH 2.84, and 99.6% protonated at pH 1.50. In contrast, approximately 78% of the carboxyl groups were ionized at pH 2.84, and 14% were ionized at pH 1.50. Thus, overall, **3** behaved as a nearly neutral zwitterion at lower TFA concentration, and became predominantly positively charged at higher TFA concentrations. The decarboxylated analogue, **4**, was 92% protonated at 0.01% TFA and was completely protonated at TFA concentrations above 0.4%. Again, with the same assumption as before, increasing TFA concentrations were expected to show a gradual decrease in the retention shift for **3** but no shift for **4.**

Effects of TFA Concentration on Retention Times

The TFA concentration in the mobile phases had a significant affect on the retention time and elution order of **1** and **2** as shown in Figure 2. When the TFA concentration was 0.01%, the 1,2,4-triazole, **2** eluted first at 17.8 minutes and the 1,2,4-triazole carboxylic acid, **1** eluted at 19.1 minutes. In contrast, when the TFA concentration was increased to 0.1%, the elution order reversed and now **1** eluted first at 18.8 minutes and **2** eluted at 19.3 minutes. Thus, simply increasing the TFA concentration from 0.01% to 0.1% resulted in a significant 1.5 minute change in the retention time of the 1,2,4-triazole, **2**, whereas, there was a much less dramatic change of 0.3 minutes in the retention time of the 1,2,4-triazole carboxylic acid **1**. Interestingly, **1** and **2** co-eluted at 18.4 minutes when the mobile phases contained 0.05% TFA. We observed a similar chromatographic profile for the simple 1,2,4-triazoles **3** and **4** as shown in Figure 3. In this case, there was no separation of the two compounds when the mobile phases contained 0.12% to 0.2% TFA. These examples clearly showed that for the weakly basic compounds examined an arbitrarily selected TFA concentration in the mobile phases could produce misleading results for process control analysis and could result in an inaccurate impurity profile.

The results of detailed experiments designed to probe the observed shifts in retention times associated with changes in TFA concentration were shown in Figure 4. The profiles of the capacity factors, k', for **1–4** were plotted against the TFA concentration in the mobile phases. The intersection of the curves in each graph represented the concentration of TFA at which

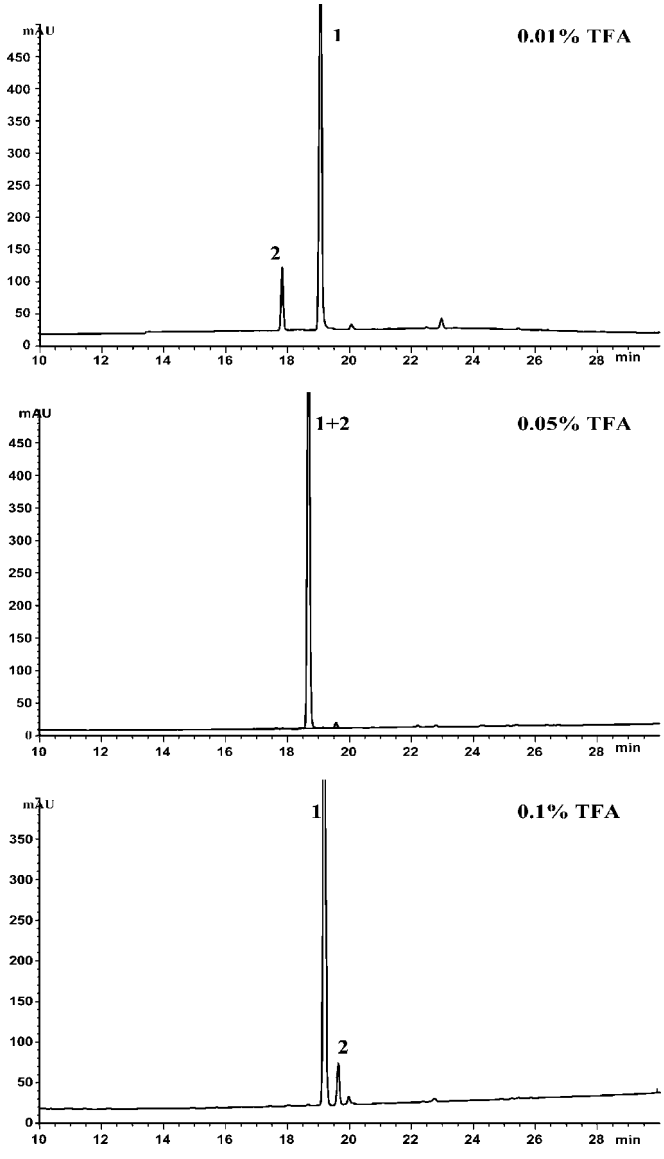

Figure 2. TFA concentration dependent HPLC separations for compounds 1 and 2. See Experimental section for details of the HPLC method. The column was operated at 25°C.

both compounds co-eluted, i.e., 0.05% for compounds **1** and **2** and 0.12% for compounds **3** and **4**. The curves for triazoles **1**, **3**, and **4** were characterized by a rapid downward shift in capacity factor at lower TFA concentrations and a steady upward trend at higher TFA concentrations. In contrast, compound **2**

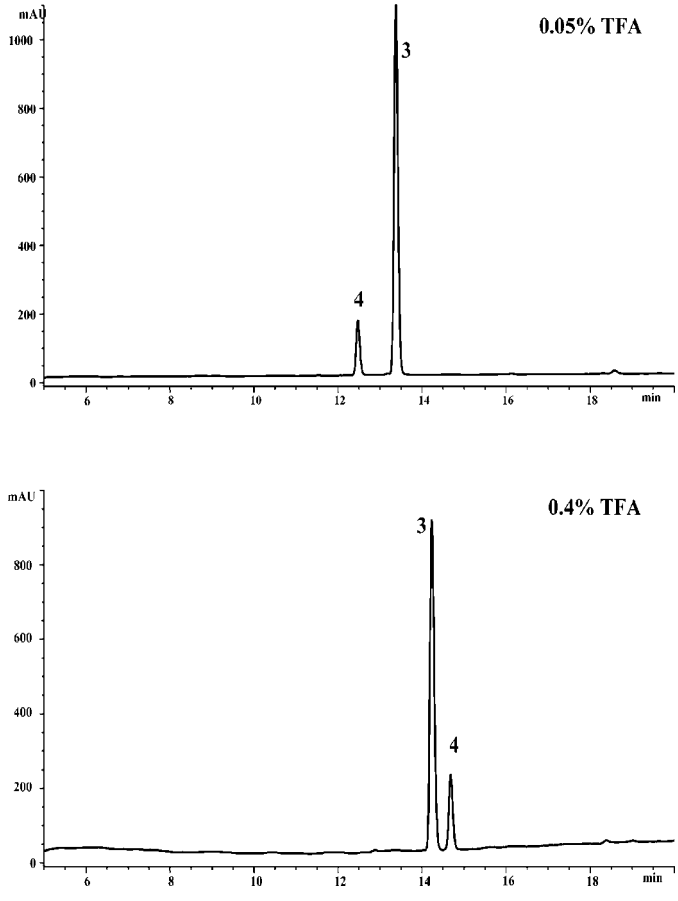

Figure 3. TFA concentration dependent separation for compounds 3 and 4. See Experimental section fopr details of the HPLC method. The column was operated at 25°C.

displayed a simple, positive correlation between the capacity factor and the TFA concentration in the mobile phases.

TFA Concentration; Acetonitrile and pK_a Values of the Analytes

It has been reported that the pK_a and pH values of formic, acetic, trichloroacetic, and phosphoric acids, all ionizable weak acids used as acidic modifiers in chromatography, were elevated by approximately 1 unit in solutions containing 40–50% methanol or acetonitrile[9–12] In contrast, the pK_a values for some basic analytes in solutions containing 40–50% methanol or acetonitrile are lowered by approximately one half unit.[14–17] These results have been attributed to a decrease in the dielectric constant of the medium upon addition of an

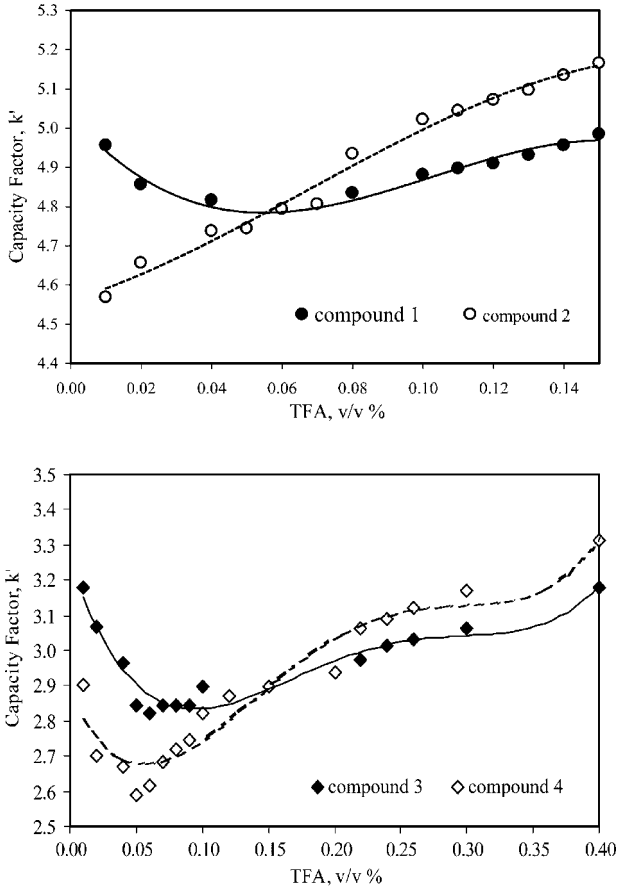

Figure 4. Plot of capacity factor, k', as a function of TFA concentration. See Experimental section for details of the HPLC methods.

organic solvent which resulted in reduced dissociation for the acids and the increased dissociation of the protonated bases. The chromatographic behavior shown in Figure 4 clearly illustrated such effects of the addition of an organic solvent on the pK_a values of weakly acidic and basic analytes.

As discussed earlier, the triazole acid **1** would be partially protonated and overall would behave as a positively charged zwitterion within the pH range of 2.84 to 1.88, in the absence of any organic solvent. However, under the current separation conditions in the presence of acetonitrile, the triazole acid **1** likely shifted from a neutral to a partially positively charged zwitterion as the TFA concentration increased from 0.01% to 0.05%. At concentrations above 0.05% protonation continued. A concave profile for the capacity factor strongly supported such a protonation process. However, acetonitrile had negligible effects on protonation of triazole **2** since it was predominantly positively

charged. This resulted in a simple positive trend throughout the pH range. On the other hand, profiles of the capacity factor shift for compounds **3** and **4** were somewhat more complicated, exhibiting a steep initial decline followed by a gradual increase. Compound **3**, presumably a neutral zwitterion at a TFA concentration of 0.01%, was quickly protonated as the TFA concentration rose to 0.05%. Compound **4** existed as a slightly charged species at a TFA concentration of 0.01% and quickly became predominately protonated as the TFA concentration rose to 0.05%.

TFA Concentration and Chaotropic Effect

When the TFA concentration was $>0.05\%$, each of the triazoles showed a continuously prolonged retention with increasing TFA concentrations as shown in Figure 4. Although the shifts of the capacity factor were not proportional to the incremental changes in TFA concentration, positive trends were generally observed. Notably, the rates of capacity factor shift were different near the intersecting points for each pair of triazoles, i.e., compounds **1** and **2** and compounds **3** and **4**. Compounds **2** and **4** showed increased shifting rates relative to the triazole acids **1** and **3** as the TFA concentrations were increased. This resulted in the reversed elution orders at higher mobile phase TFA concentrations.

Basic compounds were reported to exhibit a TFA concentration dependent retention that was affected by the chaotropic effect between basic analytes and acidic modifiers.[1,13,21–23] The trifluoroacetate counter ion was characterized as a chaotropic ion with less localized charge, high polarizability and a low level of hydration, that usually had a chromatographic effect on basic analytes.[5] When a basic analyte was dissolved in an aqueous solution with low concentrations of a chaotropic anion such as TFA, the analyte was protonated, presumably forming a chaotropic anion-associated complex or an ion pair through ionic interaction, and was solvated with water molecules through hydrogen bonding. When additional chaotropic anion was added to the solution, the elevated ionic strength disrupted the original hydration sheath of the ion-associated complex, reduced its solvation, and formed a new ion-associated complex with increased hydrophobicity, which in turn resulted in a prolonged retention on a reversed-phase HPLC.

The results shown in Figure 4 suggested such a chaotropic effect in separation of the triazole acids. The hydrophobicity of compounds **1** and **3** possibly as neutral zwitterions, as well as compound **4** in its slightly protonated form, was quickly being weakened by protonation, as the TFA concentration increased from 0.01%. At 0.05% TFA, the overall hydrophobicity of these compounds reached the weakest level and the downward retention shift ended. As the TFA concentration continued to increase, the protonated triazole derivatives became the predominant forms, and the hydrophobicity resulting from the chaotropic effect prevailed, leading to the observed

upward shifts in the capacity factors. As the TFA concentration rose higher, the chaotropic effect continued. The capacity factor shift of compound **2** exhibited clearer evidence of a chaotropic effect. Increasing the TFA concentration altered the chromatographic behavior and the upward shift of the capacity factor persisted throughout the range of TFA concentration. This was in contrast to our earlier assumption that no retention shift would occur. The presence of a carboxylic acid group reduced the chaotropic effect for compounds **1** and **3**. The negative charge within the zwitterions presumably repelled the negatively charged TFA chaotropic ion, and prevented, to some degree, the formation of an ion-associated complex. The TFA-induced hydrophobicity of these carboxylated triazoles was, therefore, weakened, and the rate of capacity factor shift was lessened, compared with that of the decarboxylated analogues.

Effects of Acidic Modifiers

Representative chromatographic profiles using phosphoric acid, formic acid, acetic acid, and a combination of TFA and TEA as mobile phase modifiers are shown in Figure 5. Due to only partial protonation at higher pHs, both compounds **1** and **2** were retained on the column significantly longer at low concentrations of phosphoric acid, formic acid, and acetic acid in the mobile phases than at higher concentrations of these modifiers. We attributed this behavior to the absence of or an insufficient chaotropic effect of these three acids. Although dihydrogenphosphate, perchlorate, trifluoroacetate, as well as many other counter ions, were all considered to be chaotropic anions,[1,2,11] the chaotropic affect associated with dihydrogenphosphate was obviously insufficient to alter the hydrophobicities of compounds **1** and **2** under the experimental conditions.

The combination of TFA and TEA produced little chromatographic variation from the use of TFA alone. The analytes showed similar retention times at both low and high concentrations of TFA and TEA. We observed a reversal of elution order, which suggests possibly identical retention mechanisms. Triethylamine was traditionally used as a silanol-blocking additive to reduce peak tailing to chromatograph basic analytes, but our results showed a limited application of TEA for these particularly weakly basic triazoles. Identical results using TFA alone and using TFA and TEA has been discussed extensively in an investigation in which linear solvation energy relationship (LSER) was used to delineate retention mechanism.[5]

Chromatographic Profiles on Other Columns

Separations of **1** and **2** were also evaluated using other columns and 0.01% and 0.1% TFA in the mobile phases with the results shown in Figure 6. The

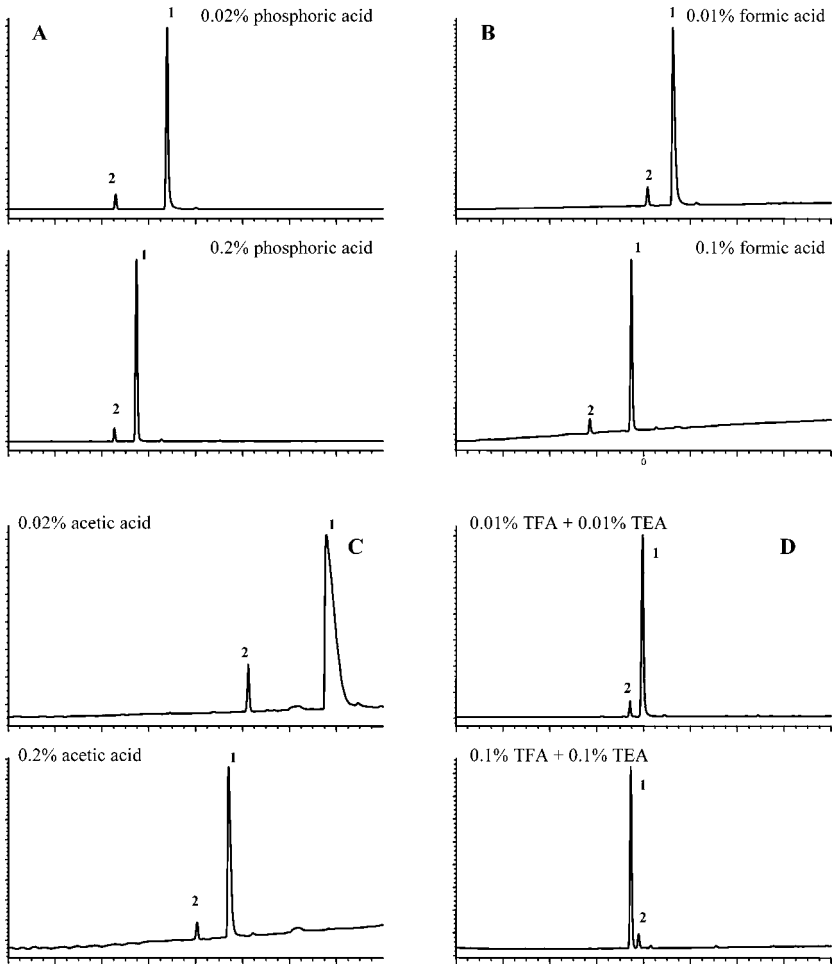

Figure 5. Chromatographic profiles of compounds 1 and 2 with different acidic modifiers. See Experimental section for details of the HPLC method. The column was operated at 25°C. Acidic modifiers added to the mobile phases were phosphoric acid (A), formic acid (B), acetic acid (C), and TFA + TERA (D). Each chromatogram was plotted vertically at full scale and from 12 to 28 minutes.

Symmetry Shield RP_8 column yielded essentially identical chromatographic profiles to those obtained with the Symmetry C_{18} column. The Zorbax SB-C18 column gave distorted peak shapes and no reversal of elution order using 0.01%TFA which may result from the uncapped stationary phase. As the TFA concentration was increased to 0.1%, peak shape and column efficiency improved. LoBrutto and co-workers attributed the improved column efficiency to the chaotropic effect.[11] The higher bonded phase coverage of the Luna C18(2) column apparently interacted more strongly

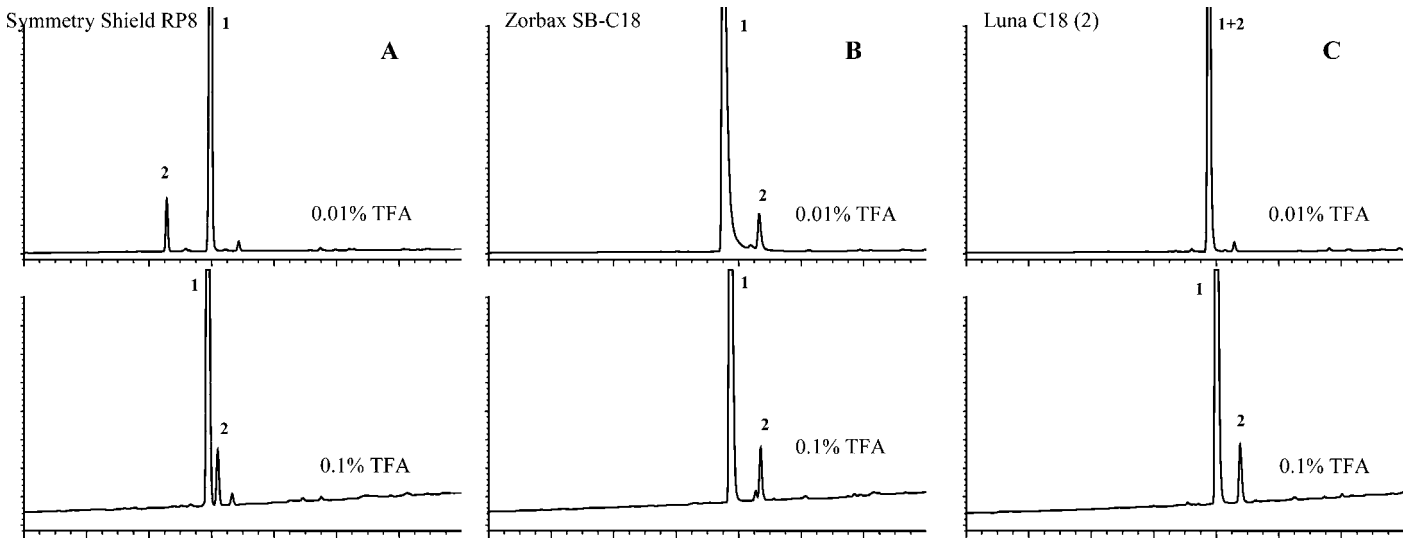

Figure 6. Chromatographic profiles of compounds **1** and **2** with different stationary phases. See Experimental section for details of the HPLC method. The columns were operated at 25°C and include Symmetry Shield RP-8, 3.5 μm, 4.6 × 150 mm (A); Zorbax SB-C-18, 3.5 μm, 4.6 × 150 mm (B); and Luna C-18 (2), 3 μm, 4.6 × 150 mm (C). Each of the chromatograms was plotted from 12 to 28 minutes on the x-axis and 0–200 mAU on the y-axis.

and selectively with **2** when 0.01% TFA was added to the mobile phase. Increasing the TFA concentration to 0.1% did resolve **1** and **2**.

CONCLUSIONS

Separations of weakly basic and zwitterionic compounds, such as the triazole derivatives in this study were obtained readily by optimizing the TFA concentration in the HPLC mobile phases. Minor alterations in the TFA concentration of the mobile phases could dramatically affect retention times and result in reversed elution order. The presence of acetonitrile in the mobile phase and changes in TFA concentration affected the hydrophobic behavior of the analytes, which in turn produced shifts in capacity factors. For this particular study these dramatic changes essentially reflected the influence of chaotropic anions.

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