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Influence of inorganic mobile phase additives on the retention, efficiency and peak symmetry of protonated basic compounds in reversed-phase liquid chromatography

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Abstract

Inorganic eluent additives affect the retention of protonated basic analytes in reversed-phase HPLC. This influence is attributed to the disruption of the analyte solvation–desolvation equilibria in the mobile phase, also known as "chaotropic effect". With an increase of counteranion concentration analyte retention increases with concomitant decrease in the tailing factor. Different inorganic counteranions at equimolar concentrations affect protonated basic analyte retention and peak symmetry to varying degrees. The effect of the concentrations of four different inorganic mobile phase additives (KPF_6, $NaClO_4$, $NaBF_4$, NaH_2PO_4) on the analyte retention, peak symmetry, and efficiency on a C8-bonded silica column has been studied. The analytes used in this study included phenols, toluene, benzyl amines, beta-blockers and ophthalmic drugs. The following trend in increase of basic analyte retention factor and decrease of tailing factor was found: $PF_6^- > ClO_4^- \sim BF_4^- > H_2PO_4^-$. With the increase of the counteranion concentration greater analyte loading could be achieved and consequently an increase in the apparent efficiency was observed until the maximum plate number for the column was achieved. At the highest concentration of counteranions, the peak efficiency for most of the basic compounds studied was similar to that of the neutral markers. In contrast, the neutral markers, such as phenols, showed no significant changes in retention, efficiency or loading capacity as counteranion concentration was increased.

Keywords: Chaotropicity; Basic analytes; Loading capacity; Hexafluorophosphate; Perchlorate; Peak symmetry; Efficiency

1. Introduction

Reversed-phase HPLC behavior of basic compounds has attracted significant interest since it was estimated that approximately 80% of drugs include basic functional groups [1]. Reversed-phase HPLC separation of organic bases of different pK_a values is of particular importance for the pharmaceutical industry [2,3]. However, the separation of basic compounds in reversed-phase HPLC usually is a challenging task due to their extreme changes in retention as a function of pH. Additionally, in some pH regions, basic compounds are prone to peak tailing, which causes poor peak efficiency [4]. Peak tailing can arise from several sources including sample overload [5,6], analyte interaction with residual silanols [7,8], slow detector response [9–11], extra-column effects [12,13] and slow adsorption–desorption kinetics [14].

The retention of ionogenic bases in liquid chromatography is strongly dependent upon the pH of the mobile phase. At pH values two units above the basic analyte pK_a, the analyte is in its predominately neutral form (>99%) and will show the greatest hydrophobic retention on alkyl bonded stationary phases. Sometimes separations at these pHs are not feasible

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either due to long analysis times or the optimal mobile phase pH may exceed the pH stability limit of the column. At a mobile phase pH two units less than the analyte pK_a the analyte is in its predominately ionized form (>99%) which will result in a significant reduction in retention and shorter analysis times. However, protonated basic compounds can have additional secondary interactions including ionic interactions with ionic [7,15,16] or polar [17–20] adsorption sites on the column. This may lead to increased retention, band tailing and column-to-column irreproducibility.

From classical column theory [21–23] efficiency is a column specific parameter and should be independent of the type and retention time of the analyte. A better packed column, smaller particles, minimum injection volume and sample load, lower mobile phase viscosity and higher operation temperature may contribute to the efficiency improvement [24,25]. This is true for an ideal situation in the absence of extra-column effects and any type of secondary equilibria. Analyte specific solvation–desolvation or ionization processes usually have a dramatic effect on the apparent peak efficiency for that particular compound [26].

One of the origins of peak tailing in chromatography can be attributed to energetic surface heterogeneity with overloading of highly energetic adsorption sites [5,17,19,27,28]. Moreover, possible ion-exchange types of interactions with these sites could lead to slow sorption–desorption of solute molecules from the strong sites compared to the weak sites leading to a further increase in band tailing [29]. It also has been shown by McCalley and coworkers that sample loading may have an effect on peak efficiency [5,30]. Thus, a decrease in sample size has led to the improvement in the efficiency of basic compounds.

Single solute adsorption equilibrium isotherms of three basic drugs (buspirone, doxepin, and diltiazem) analyzed with acetonitrile/water at pH = 3 were shown to exhibit a bi-Langmuir behavior [31]. The adsorption of the drugs was assumed to occur on two distinct kinds of sites with different adsorption energies, such that the low energy sites accounted for the hydrophobic interactions between the solutes and the alkyl chains and the high energy sites accounted for the ion-exchange interactions between the accessible active silanols and the protonated bases. Caffeine was also shown to exhibit a bi-Langmuir behavior on a Kromasil C8 column [32].

Guiochon and co-workers [28,33] also have studied tailing and multi-component adsorption isotherms and they have shown that kinetic tailing due to slow desorption from strong sites may exist in addition to non-linear tailing. The rates of mass transfer kinetics would be different on the strong and weak sites. Kinetic and non-linear tailing for basic analytes can both contribute to tailing leading to asymmetrical peaks. Therefore, flow rate and sample loading studies can be performed to determine the origin of the tailing.

Analysis of protonated basic compounds is sometimes plagued by interactions with accessible residual silanols. These secondary interactions could be reduced by suppressing interaction of the protonated basic compound with the ionized silanol active sites with the employment of ion-pair reagents. The adoption of ion-pair reagents to improve the efficiency of ionic compounds has long been established. The ion-pair reagent contributes to the peak shape improvement by diminishing the ion-exchange interaction between the ionic compounds and the silanols and shielding the active silanol sites on the stationary phase [34]. Silanol interactions can be reduced by using a high buffer concentration and choosing buffer cations that are strongly held by the silanols ($Na^+ < K^+ < NH_4^+ <$ triethylammonium < dimethyloctylammonium), therefore, blocking these active sites [35].

The pK_a of normal silanols on a silica surface has been estimated to be between 5 and 7, and is dependent on the environment and metal impurities of the silica matrix [7]. The presence of metal impurities in the silica matrix will lead to a reduction in the pK_a of the residual silanol groups. Generally, operating at a mobile phase pH below 3 the majority of silanols should be in their neutral form and will allow for minimization of silanol interactions with a protonated basic compound.

However, analysis at these low pHs may cause early elution of these highly solvated protonated basic compounds unless mobile phase additives are employed. In previous papers, we described the retention behavior of several basic compounds as a function of the concentration of chaotropic mobile phase additives (ClO_4^-, PF_6^-, BF_4^-, $H_2PO_4^-$, $CF_3CO_2^-$) at a low pH [36,37]. The increasing order of retention was shown to agree with the theory of chaotropicity [38]. The increase in retention is related to the disruption of the solvation shell of the organic analyte upon interaction with inorganic mobile phase additive. This disruption in analyte solvation occurs at low concentrations of counteranions (below 20 mM) and is specific for the type of counteranions employed. For example, at an equimolar concentration of inorganic mobile phase additive the retention of the protonated basic analyte increases the most when employing hexafluorophosphate and the least when employing dihydrogenphosphate anion.

In this low pH region (pH < 3), the addition of chaotropic anions leads to a concomitant increase in retention as well as peak symmetry. This was first observed when the chaotropic approach was implemented for the analysis of substituted pyridines, aromatic amines and ophthalmic pharmaceutical compounds [39]. Later Roberts et al. also observed similar effects [40] during the analysis of primary, secondary, and tertiary benzyl amines and antidepressants.

The effect of four mobile phase additives (PF_6^-, ClO_4^-, BF_4^-, $H_2PO_4^-$) on basic analyte retention, peak symmetry, and efficiency on a C8-bonded silica column will be discussed. A low mobile phase pH was chosen to ensure that the basic analytes at the various organic compositions were in their fully protonated state and to minimize electrostatic interactions with the stationary phase. The analytes used in this study include phenols, toluene, benzyl amines, beta-blockers and ophthalmic drugs (Fig. 1). The influence of extra-column effects, sample loading (increasing analyte concentration at

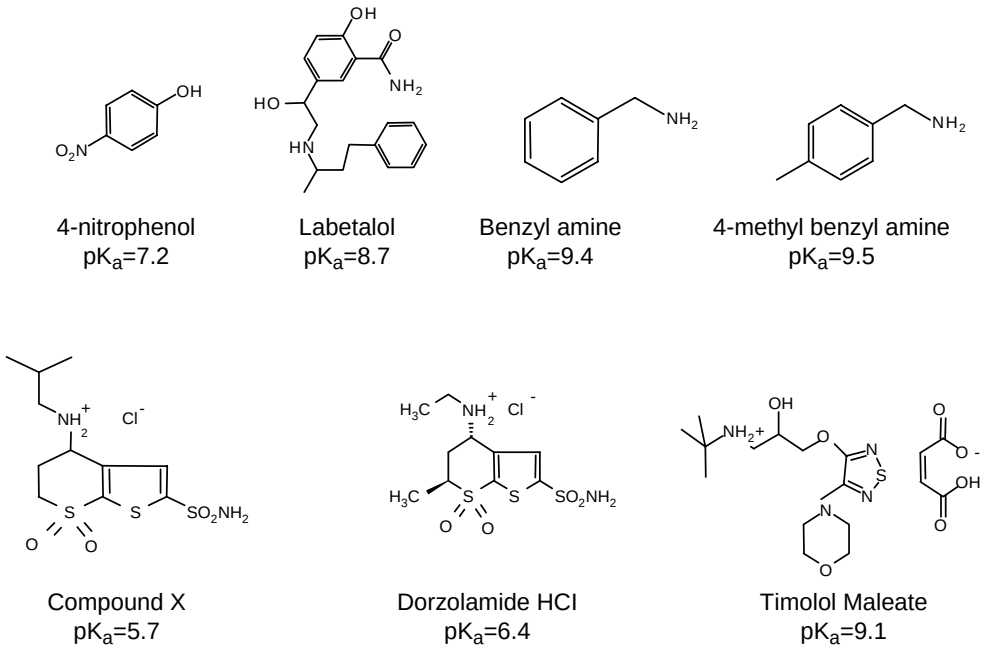

Fig. 1. Structure and pK_a of selected test compounds.

constant injection volume and increasing injection volume at constant analyte concentration) and flow rate on the peak efficiency and peak asymmetry will be discussed.

2. Experimental

2.1. Apparatus

The chromatographic system used throughout the entire study was an Agilent 1100 HPLC system equipped with a diode array detector. Chromatograms were processed using a PE Nelson version 3.1 data acquisition system (Cupertino, CA, USA). The column employed in this study was Zorbax Eclipse XDB-C8, 4.6 mm × 150 mm i.d., particle size 5 μm column. The Eclipse XDB-C8 column has a nominal surface area of 180 m^2/g and a pore size of 80 Å. Mobile phase pH was measured with a Fischer Scientific Accumet pH meter 15 (Denver Instrument, USA). The pH meter was calibrated with buffer solutions of pH 1.00, 2.00 and 4.00.

2.2. Chemicals

Acetonitrile (HPLC grade, +99.93%), was obtained from Sigma–Aldrich (St. Louis, MO, USA). Ortho-phosphoric acid (85%) and perchloric acid (70%) were obtained from Fluka (GMBH CH-9471 Buchs). Sodium phosphate, dibasic, anhydrous (99.5%) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Sodium dihydrogenphosphate (99.999%) and Potassium hexafluorophosphate (+99.9%), and sodium hexafluoroborate (+99%) were obtained from Aldrich (Milwaukee, WI, USA). All aqueous mobile phases were filtered using a Whatman nylon 66, 0.45 μm membrane filter (Fisherlane).

The analytes benzylamine, 2-methyl benzylamine, 3-methyl benzylamine, 4-methyl benzylamine, phenol, 2-nitrophenol, 4-nitrophenol, toluene, Labetalol, Alprenolol, and Propanolol hydrochloride were obtained from Aldrich (Milwaukee, WI, USA). The three ophthalmic compounds, Dorzolamide HCl, Timolol maleate and Compound X, were obtained from the sample repository at Merck Research Laboratories.

2.3. Chromatographic conditions

The retention data were recorded at controlled temperature of 25 °C using isocratic conditions with a flow rate of 1 mL/min. For all the experiments, a mobile phase composition of 5/95 acetonitrile/aqueous was used for benzyl amines, 10/90 acetonitrile/aqueous for ophthalmic compounds, 25/75 acetonitrile/aqueous for phenolic compounds and beta-blockers, 50/50 acetonitrile/aqueous for toluene. These mobile phase compositions were chosen based on organic study experiments to avoid extra-column effects. All analyte solutions were diluted with the respective mobile phase composition that they were analyzed with. The concentration of each analyte was 0.1 mg/mL for phenolic compounds and toluene, 0.5 mg/mL for benzyl amines and beta-blockers. The concentration of ophthalmic drugs varied, 0.5 mg/mL for Compound X, 1 mg/mL for Dorzolamide HCl and 2 mg/mL for Timolol maleate and the injection volume used was 1 μL. The wavelength adopted for each type of analyte was 254 nm for ophthalmic drugs and toluene and 225 nm for phenols, benzyl amines and beta-blockers. Dif-

ferent wavelengths were employed for the loading study in order to keep peak signal in range. A test mixture of toluene and 4-nitrophenol was used as a system suitability check before and after each experiment to monitor the performance and stability of the column. Moreover, the first analyte analyzed in a sequence was rerun at the end of the sequence to ensure system reproducibility. Fresh solutions were prepared before each experiment and were analyzed with a cooled autosampler at 4 °C protected from light. Column void volume was determined to be 1.3 mL by the injection of deuterated acetonitrile in a neat acetonitrile eluent employing refractive index detection [41].

2.4. Counteranion studies

To study the effect of type and counteranion concentration, different counteranions were added only in the aqueous portion of the mobile phase. $HClO_4$ was used to adjust ClO_4^- concentration in water (pH < 2.5) from 2.7 to 68 mM. KPF_6 was used to adjust PF_6^- concentration in 0.1% (v/v) H_3PO_4 (pH = 2.2) from 1 to 50 mM. $NaBF_4$ was used to adjust BF_4^- concentration in 0.1% (v/v) H_3PO_4 (pH = 2.2) from 1 to 50 mM. Mixtures of dilute H_3PO_4 and/or NaH_2PO_4 were used to adjust $H_2PO_4^-$ concentration (pH < 2.8) from 10 to 50 mM. The pH of the aqueous portion of the mobile phase was measured for all studies.

2.5. Loading studies

Two types of loading studies were performed using the 0.1% (v/v) H_3PO_4 mobile phase in which the analyte load (μg) was modified by: (1) increasing the injection volume (0.1–100 μl) at a constant analyte concentration and (2) increasing analyte concentrations from 0.1 to 50 mg/mL using a constant injection volume (1 μl).

For the loading studies with varying perchlorate concentration (2.7–68 mM), a constant injection volume (1 μL) with increasing analyte concentrations from 0.1 to 50 mg/mL was used.

2.6. Flow rate studies

For the flow rate studies with 0.1% (v/v) H_3PO_4 and with 68 mM ClO_4^- mobile phases, the following flow rates were used (mL/min): 0.3, 0.5, 0.8, 1, 1.2, 1.5, and 2. The analyte loads used for these studies were: benzyl amine 0.4 μg, 2-methyl benzylamine 0.2 μg, 3-methyl benzylamine 0.2 μg, 4-methyl benzyl amine 0.2 μg, phenol 0.12 μg, propanolol, Labetalol and alprenolol 0.5 μg each, and Dorzolamide HCl 0.9 μg, toluene 0.8 μg.

2.7. Efficiency and asymmetry measurements

Peak efficiency was determined from peak widths at 50, 10, and 5% of the peak heights using the following formulas:

$$N_{h/2} = 5.545\left(\frac{t_R}{w_{h/2}}\right)^2 \qquad N_{10\%h} = 18.421\left(\frac{t_R}{w_{10\%h}}\right)^2$$

$$N_{5\%h} = 23.966\left(\frac{t_R}{w_{5\%h}}\right)^2$$

Coefficients for the formulas above were obtained from the basic definition of the efficiency as a square of the ratio of the retention time to the standard deviation for ideal peak ($N = (t_R/\sigma)^2$). Ideal peak is usually assumed to have Gaussian shape and could be described by height normalized Gaussian function:

$$f(x) = \exp\left(-\frac{1}{2\sigma^2}(x-\mu)^2\right) \tag{1}$$

Coefficients for the efficiency calculation could be obtained as a square of the ratio of Gaussian function on the corresponding height to its sigma value. For the 10% of the peak height, for example (0.1 of the total peak height) this will be:

$$\left[\frac{2\times\sqrt{-2\sigma^2\,\ln(0.1)}}{\sigma}\right]^2 = -8\ln(0.1) = 18.421 \tag{2}$$

Usual coefficient 16, which is often recommended to use for baseline efficiency calculations is only applicable if the distance between the intersections of the baseline and tangents to the peak inflection points is used. We were using numerical data for the peak curve itself (peak widths at 5, 10 and 50% peak height) obtained by the Turbochrom data acquisition system (Perkin-Elmer, Norwalk, CT). Peak asymmetry was determined from the tailing factor at 5% peak height obtained by the Turbochrom data acquisition system. The tailing factor equation that the software used was $T = W_{0.05}/2f$, where $W_{0.05}$ is the peak at 5% peak height, f the width (time) between the peak maximum and the front edge of the peak at 5% of the peak height.

2.8. Extra-column effects

A lower efficiency is usually observed for early eluting components since the extra-column effects lead to an apparent loss of peak efficiency [42]. This problem could be overcome by choosing an organic eluent composition where target component shows high retention factor and N is independent of further changes in the eluent composition (i.e., at decreasing organic eluent composition N is essentially constant). In this case, apparent efficiency should be independent of the eluent composition above a certain value of k'. Prior to studying the effects of analyte loading and flow rate and effects of type and concentration of counteranion on the peak efficiency and peak asymmetry, the optimal eluent composition for each analyte was determined. These organic eluent compositions were used for all studies in this paper. For the following sets of analytes, the optimal organic concentrations necessary to minimize extra-column effects on the peak efficiency were determined: benzylamines (5%, v/v,

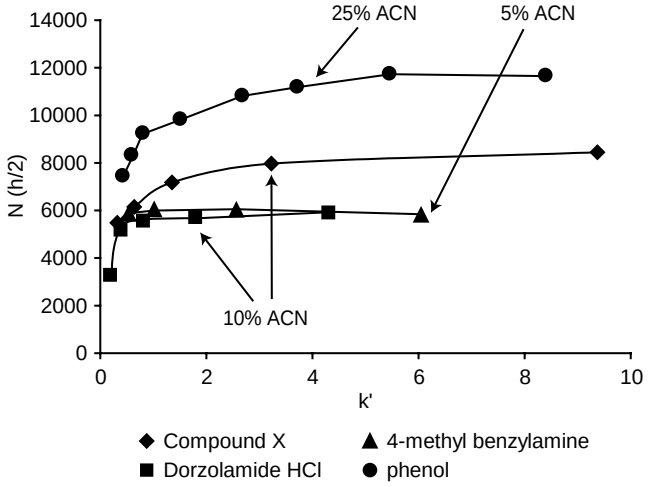

Fig. 2. $N_{(h/2)}$ for ophthalmic compounds, 4-methyl benzylamine, and phenol vs. retention factor at different organic compositions. Chromatographic conditions: 0.1% (v/v) H_3PO_4:acetonitrile eluent. Organic concentration was from 5 to 50% (v/v); flow rate: 1.0 mL/min; temperature: 25 °C; analyte load: 0.5–1 μg.

MeCN), ophthalmic compounds (10%, v/v, MeCN), phenolic compounds (25%, v/v, MeCN) and beta-blockers (25%, v/v, MeCN) and toluene (50%, v/v, MeCN). The effect of $N_{(h/2)}$ (determined at 50% peak height) versus retention factor at increasing organic composition is shown for phenol, 4-methylbenzyl amine and ophthalmic compounds (Fig. 2).

3. Results and discussion

Theoretically, a column can generate a certain maximum number of theoretical plates at the optimum flow rate (minimum on the Van Demmter curve). This number should be independent of the type of the analyte and mobile phase. In reality, any secondary processes, energetic surface heterogeneity, or restrictions in sorption–desorption kinetics in the column will result in the specific decrease of the efficiency for a particular compound. For early eluting compounds extra-column effects may also contribute to the decrease in the overall efficiency.

3.1. Influence of increased analyte loading on peak efficiency and peak asymmetry

It is sometimes necessary to inject large sample sizes to enable the detection of small impurities. However, using large sample sizes may contribute to broad and tailing peaks for the target analyte [5,6,30]. Column loading studies using a 0.1% (v/v) H_3PO_4 mobile phase, pH 2.1 were performed at loads from 0.1–50 μg for the basic and neutral compounds. At low pH, all of the basic compounds studied show significant tailing with increasing sample load. The variation of column performance (changes in peak symmetry and efficiency) with increasing sample mass (constant analyte concentration and increasing injection volume) is shown in Fig. 3A and B. The variation of column performance with increasing sample mass by increasing the analyte concentration at constant injection volume was also investigated and similar results were obtained.

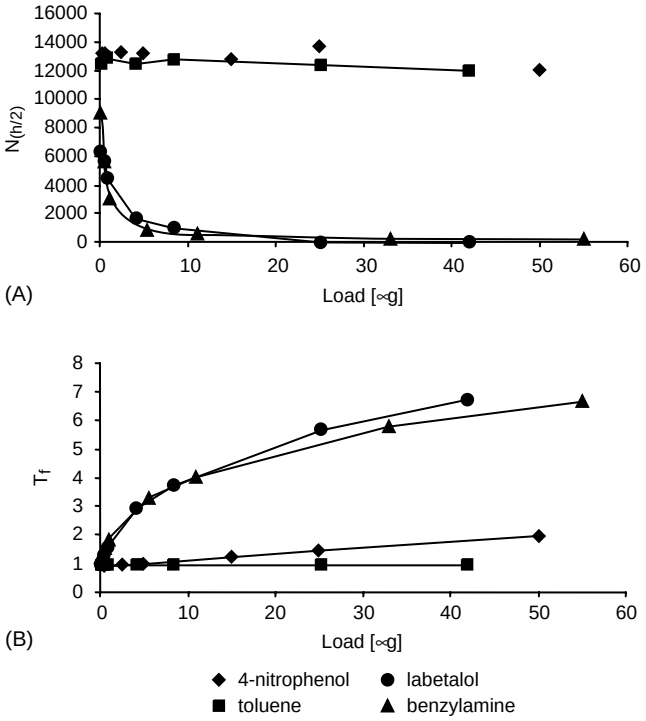

Fig. 3. Effect of analyte load on: (A) $N_{(h/2)}$ and (B) tailing factor. Chromatographic conditions: 0.1% (v/v) H_3PO_4:acetonitrile eluent; benzylamine (5% acetonitrile), toluene (50% acetonitrile), Labetalol and 4-nitrophenol (25% acetonitrile), flow rate: 1.0 mL/min; temperature: 25 °C; analyte load: 0.5–50 μg.

The efficiency and tailing factor of toluene was not affected by sample load. Phenolic compounds do show a slight increase in tailing factor and decrease in efficiency (determined at 5 and 10% peak height) with increase of sample load indicating some type of secondary interaction. This behavior observed for basic compounds could be attributed to two possible factors: secondary equilibria in the mobile phase (solvation–desolvation equilibria) and "overload" or exceeding the linear portion of the adsorption isotherm [43,44].

A chromatographic overlay for two basic compounds (Labetalol and Dorzolamide HCl) at analyte loads from 1 to 50 μg using a 10 mM dihydrogen phosphate mobile phase is shown in Figs. 4a and 5a. These overlays reveal a typical pattern where the peak tails for different analyte loads coincide, indicating a so called "thermodynamic overload" which occurs when analyte concentration exceeds the linear region on the adsorption isotherm and this isotherm curvature inevitably leads to right angled peaks [44–47].

In a simplified form, it is easy to show that peak maxima will be shifted towards lower retention volumes when analyte concentration corresponds to the non-linear isotherm region. Eq. (3) relates the analyte retention with its adsorp-

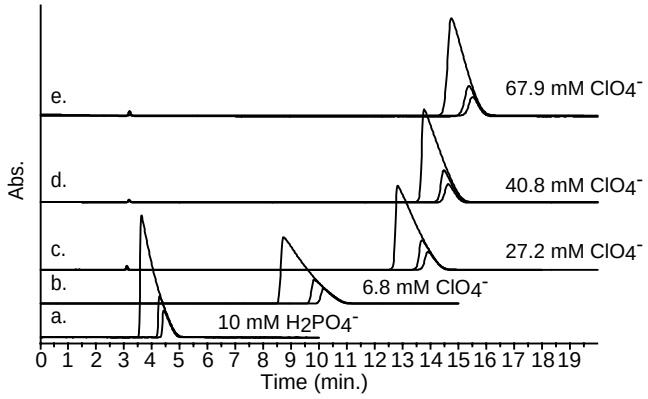

Fig. 4. Chromatographic overlays of Labetalol analyzed at different analyte concentrations using increasing mobile phase concentration of perchlorate anion. Chromatographic conditions: analyte load: 3.3, 6.5, 31.2 μg, (a) 75%:0.1% (v/v) H_3PO_4:25% acetonitrile; (b) 75%:0.05% (v/v) $HClO_4$:25% acetonitrile; (c) 75%:0.3% (v/v) $HClO_4$:25% acetonitrile; (d) 75%:0.4% (v/v) $HClO_4$:25% acetonitrile; (e) 75%:0.5% (v/v) $HClO_4$:25% acetonitrile.

tion isotherm [46] and Eq. (4) is a basic form of the excess adsorption isotherm first introduced by Everett [43,48] and Riedo and Kovats [44].

$$V_R(c) = V_0 + S\frac{\mathrm{d}\Gamma(c)}{\mathrm{d}c} \tag{3}$$

$$\Gamma(x) = A\frac{(K-1)x(1-x)}{1+(K-1)x} \tag{4}$$

where V_0 is the total volume of the liquid phase in the column (void volume); S the total adsorbent surface area in the column, and K the analyte thermodynamic equilibrium constant, which could be represented as $\exp(\Delta G/RT)$. Coefficient A usually is analyte specific and represents molecular surface area as well as analyte molar volume and is related to the adsorbent capacity or the ability of the adsorbent to retain

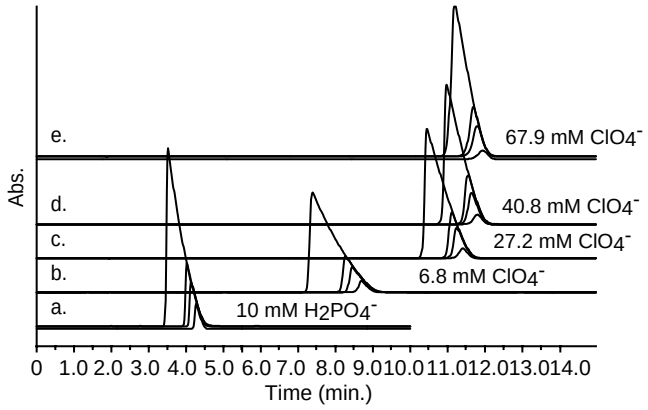

Fig. 5. Chromatographic overlays of Dorzolamide HCl analyzed at different analyte concentrations using increasing mobile phase concentration of perchlorate anion. Chromatographic conditions: Analyte load: 1.4, 5.2, 9.2, 48 μg, (a) 90%:0.1% (v/v) H_3PO_4:10% acetonitrile; (b) 90%:0.05% (v/v) $HClO_4$:10% acetonitrile; (c) 90%:0.3% (v/v) $HClO_4$:10% acetonitrile; (d) 90%:0.4% (v/v) $HClO_4$:10% acetonitrile; (e) 90%:0.5% (v/v) $HClO_4$:10% acetonitrile.

certain amount of the analyte on the surface. Eq. (4) represents a concave curve where the derivative decreases with an increase of the analyte concentration, and according to Eq. (3) this will lead to the decrease of the retention volume.

As shown in Fig. 4 for Labetalol, the load of 3 μg employing the 0.1% (v/v) H_3PO_4 mobile phase leads to the appearance of significant tailing. Tailing at low basic analyte loads has also been observed by others in the literature [5,6,30]. For Labetalol this corresponds to approximately a 10 μM concentration in the column effluent (peak width is 0.9 min, and flow is 1.0 mL/min, therefore, volume is 0.9 mL and from molecular weight and amount injected the number of moles is determined and concentration is calculated accordingly). At the same time, toluene which is a non-ionic analyte did not show any significant tailing at much higher concentrations (up to 42 μg). Phenol is a polar analyte that exhibited some tailing at high concentrations (>20 μg). As we discussed in Section 1, these effects (tailing) are usually associated with the presence of secondary retention sites or in other words energetic surface heterogeneity.

Assuming the additive interactions of the analyte with these different adsorption sites, one can construct the retention model as a superposition of two isotherms. Eq. (3) could be written in the form:

$$V_R(c) = V_0 + S_1\frac{\mathrm{d}\Gamma_1(c)}{\mathrm{d}c} + S_2\frac{\mathrm{d}\Gamma_2(c)}{\mathrm{d}c} \tag{5}$$

where S_1 and S_2 represent the adsorbent surface area occupied by corresponding adsorption sites, and Γ_1 and Γ_2 are corresponding adsorption isotherms of the analyte on these adsorption sites, respectively. The shape of the resulting peak is defined by the balance between the surface areas and initial slopes of the individual isotherms. The triangular peak shape indicates similar magnitude of the last two terms of Eq. (5) as opposed to a more exponential peak shape which would indicate a more significant difference in the magnitude of the last two terms.

Analyte loading experiments were also conducted with increasing concentration of perchlorate modifier in the mobile phase. Chromatographic overlays for two basic compounds (Labetalol and Dorzolamide HCl) at analyte loads from 1 to 50 μg at four increasing concentrations of perchlorate anion (10 mM dihydrogen phosphate (0 mM perchlorate), 6.8, 27.2, 40.8 and 68 mM perchlorate) are shown in Figs. 4 and 5.

The increase of the concentration of the counteranions in the mobile phase led to the decrease of the peak tail, while different loads still perfectly overlaid along the common tail profile. This indicates that the major contribution to the peak asymmetry is due to a pure thermodynamic tailing with a very small effect from kinetic broadening (Van Deemter type broadening). This decrease of the tailing could only be associated with the extended linearity of the adsorption isotherm, as a result of the increased overall adsorption capacity.

One possible explanation for this behavior could be attributed to two types of interactions of the analyte in the acetonitrile adsorbed layer. From the interpretation of isotherms

on the basis of the theory of excess adsorption it was shown that a significant accumulation of acetonitrile eluent component (10–14 Å) on top of the adsorbent surface of alkyl modified adsorbents (C1–C18 monomeric bonded phases) exists [49–53]. The existence of an adsorbed layer of considerable thickness with a composition different from that of the mobile phase suggests that the analyte and chaotropic anions partition from the bulk mobile phase into the adsorbed organic layer followed by their adsorption from that layer on to the surface of the bonded phase. Therefore, the two types of interactions of the analyte in the adsorbed organic layer would include:

(a) dispersive interactions of the analyte with adsorbed acetonitrile (common for all types of analytes; fairly straight isotherm; high adsorption capacity);
(b) ionic interactions if protonated basic analytes interact with active acidic sites present on the adsorbent surface ("residual silanols") or with inorganic anions embedded in the adsorbed acetonitrile layer (inorganic anions with high charge delocalization). These counteranions could be embedded in the organic adsorbed layer [50–53], introducing an electrostatic component in the retention mechanism of basic analytes. The higher the charge delocalization of the counteranion, the greater the probability that it could be embedded in the acetonitrile adsorbed layer. The presence of the triple bond in acetonitrile allows for dispersive interactions with these anions embedded in the acetonitrile layer. The greater the counteranion concentration, the higher the adsorption capacity for this type of interaction and the straighter the analyte isotherm resulting in a shorter tail. Excessive electrostatic interactions are relatively weak in the presence of significant amount of counteranions in the solution, and this would lead to the relatively low initial isotherm slope. Electrostatic interactions are relatively long-distance ones, which would explain relatively high adsorption capacity and non-exponential shape of the peak tail. This is presented as a valuable hypothesis for the explanation of observed effects and this hypothesis should be tested with additional experiments designed specifically for this purpose. Qualitatively the influence of these chaotropic counteranions on the peak efficiency and asymmetry can also be evaluated.

The effect of increasing perchlorate counteranion concentration at increased analyte loading on peak efficiency and tailing factor was studied for benzyl amine, Dorzolamide HCl and Labetalol (Fig. 6A and B). With an increase in counteranion concentration at all analyte loadings an increase in peak efficiency and decrease in peak tailing were observed. The contributions to an increase in the apparent peak efficiency and decrease in peak tailing may be attributed to:

(1) secondary equilibrium processes including faster solvation–desolvation equilibria of the protonated solvated basic analyte at constant analyte load and higher counter anion concentration in the mobile phase; and
(2) extended linearity of the adsorption isotherm with higher chaotropic counteranion concentration.

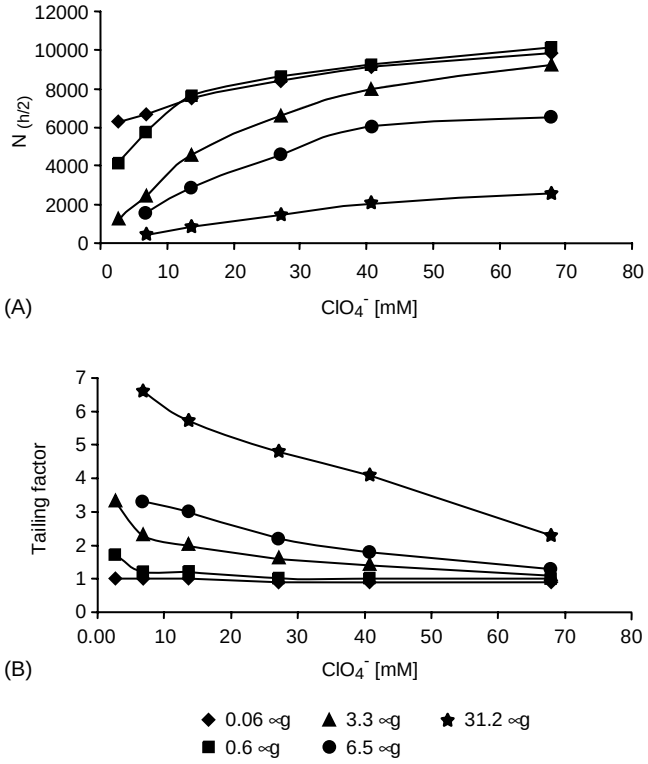

Fig. 6. Effect of analyte load and perchlorate counteranion concentration on Labetalol apparent efficiency and tailing factor. Chromatographic conditions: analyte load: 0.06–31.2 μg, 75% water:25% acetonitrile. Water adjusted with 0.025–0.5% (v/v) $HClO_4$. (A) $N_{(h/2)}$ vs. perchlorate concentration. (B) Tailing factor vs. perchlorate concentration.

It is also proposed that the addition of chaotropic counteranions in the *bulk mobile phase* could disrupt this solvation equilibrium by ion-association of the negatively charged counteranion and the positively charged basic analyte. As a result, the analysis of the basic analytes in their predominately desolvated state would lead to an apparent increase in the peak efficiency and peak symmetry.

At increased counteranion concentration, the desolvation equilibria is shifted to the more desolvated species (ion-associated complex) thus decreasing any secondary equilibria effects that may affect the peak efficiency. This leads to more favorable mass-transfer kinetics, resulting in an increase in the peak efficiency. However, at higher analyte loadings the amount of counteranion available to disrupt this equilibrium is minimized and consequently lower peak efficiencies were observed. Moreover, the increase of the perchlorate anion in the mobile phase also leads to an increase in the adsorbent capacity, which leads to a more linear adsorption isotherm further resulting in more symmetrical peaks.

Increasing the load of basic analytes in order to increase analyte sensitivity can lead to a decrease in apparent peak efficiency and increase in peak tailing. However, if an analysis

must be performed at a relatively high sample load the addition of a chaotropic additive may be employed to increase the apparent peak efficiency and symmetry. Much higher loading capacities could be obtained by operating columns with these mobile phase additives without substantial deterioration in efficiency.

3.2. Effect of type and chaotropic counteranion concentration on peak efficiency and peak asymmetry

Increasing the chaotropic counteranion concentration of perchlorate, hexafluorophosphate, and tetrafluoroborate in the mobile phase for almost all basic compounds studied led to an increase in the apparent efficiency of the system until the maximum plate number for the column was achieved. However, the effect of increasing dihydrogenphosphate counteranion concentration had a much smaller effect on increasing the apparent analyte efficiency and peak symmetry. In comparison to all anions studied, hexafluorophosphate is the most charge delocalized anion and dihydrogen phosphate is the least charge delocalized anion. The PF_6^- counteranion at low concentrations had the greatest effect on the improvement of the peak asymmetry compared to the other additives and no significant differences were observed when calculating the efficiency at different peak heights for beta-blockers and ophthalmic drugs.

Fig. 7A shows how the efficiency for the three ophthalmic drug compounds increases relatively fast when the concentration of counteranion BF_4^- was increased from 1 to 10 mM, then the efficiency of these basic compounds increases slowly until it achieves the maximum column efficiency (phenols, neutral markers). Also, with an increase of BF_4^- counteranion concentration, the tailing factor of basic compounds decreases and approaches the tailing factor of the neutral analytes, phenolic compounds, pK_as > 7(Fig. 7B).

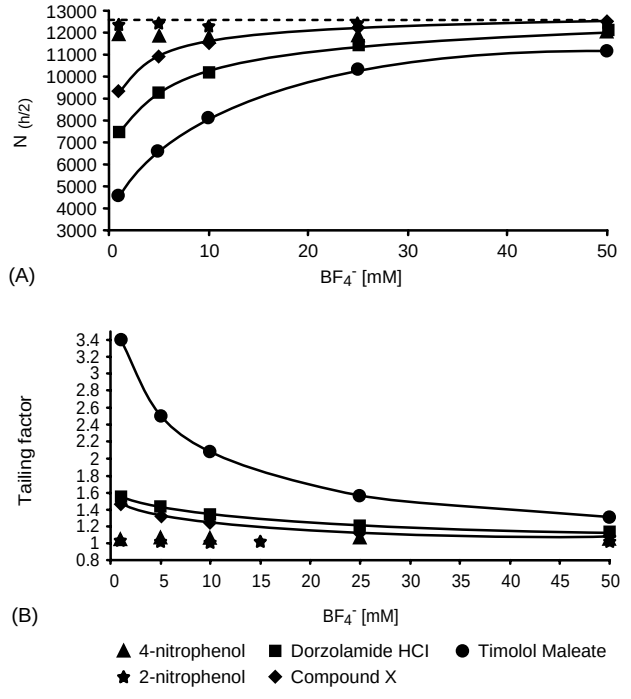

Fig. 7. Effect of tetrafluoroborate concentration on analyte apparent efficiency and tailing factor. Mobile phase: 0.1% (v/v) phosphoric acid + xBF$_4$ [1–50 mM]: acetonitrile, ophthalmic compounds (10% acetonitrile), phenols (25% acetonitrile), (A) $N_{(h/2)}$ vs. tetrafluoroborate concentration. (B) Tailing factor vs. tetrafluoroborate concentration.

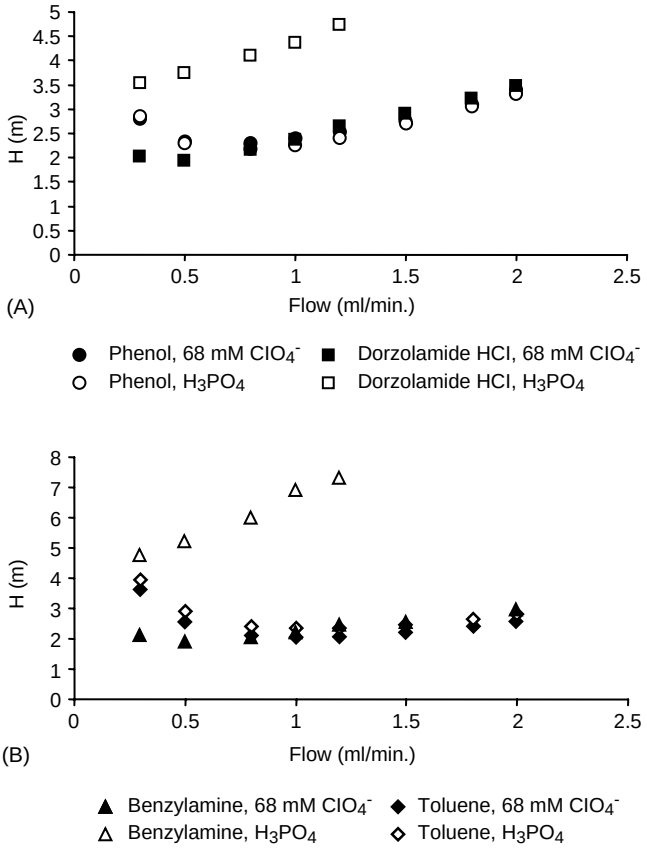

Fig. 8. Van Deemter plots of plate height (H) vs. flow rate. Mobile phases: (A) Dorzolamide HCl:90% water:10% acetonitrile. Water adjusted with either 0.1% (v/v) phosphoric acid or 0.5% (v/v) $HClO_4$, Phenol:75% water:25% acetonitrile. Water adjusted with either 0.1% (v/v) phosphoric acid or 0.5% (v/v) $HClO_4$. (B) Benzylamine:95% water:5% acetonitrile. Water adjusted with either 0.1% (v/v) phosphoric acid or 0.5% (v/v) $HClO_4$, toluene:50% water:50% acetonitrile. Water adjusted with either 0.1% (v/v) phosphoric acid or 0.5% (v/v) $HClO_4$.

At the highest concentration of counteranions (PF_6^-, ClO_4^-, BF_4^-), the number of plates for most of the basic compounds studied was similar to that of the neutral markers. In contrast, the neutral markers, phenols, showed no significant changes in retention and efficiency with increased counteranion concentration.

3.3. Effect of flow rate on peak efficiency and peak asymmetry with chaotropic mobile phase additives

Flow rate studies were performed to investigate the influence of the flow rate on the efficiency of the protonated basic analytes to elucidate if there was a contribution to de-

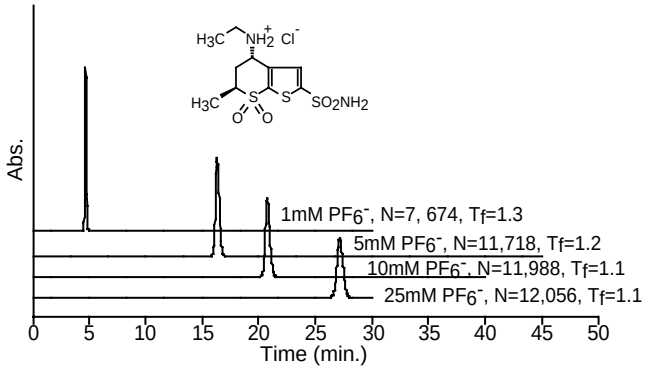

Fig. 9. Effect of hexafluorophosphate concentration on analyte retention, peak efficiency, $N_{(h/2)}$, and tailing factor. Chromatographic conditions: Mobile phase: 90%, 0.1% (v/v) phosphoric acid + *x*PF6 [1–25 mM]: 10% acetonitrile, flow rate: 1.0 mL/min; temperature: 25 °C; analyte load: 1 μg; wavelength: 254 nm.

crease in efficiency due to a kinetic origin (linear conditions). The analyte loads used were 0.1–1 μg for all analytes to minimize the effects on peak tailing from a thermodynamic origin (non-linear effects). If the existence of two different types of adsorption sites does exist they may have different equilibrium isotherms and therefore different rates of mass transfer kinetics (peak tailing of a kinetic origin). Flow rate studies using two different mobile phases, 0.1% (v/v) H_3PO_4/MeCN and 68 mM ClO_4^-/MeCN were conducted. For phenol and toluene using both mobile phase systems the classical Van Deemter profile is observed with a minimum at 0.5 and 1.0 mL/min respectively (Fig. 8A and B) and the peak efficiencies at 5, 10 and 50% peak height were similar. However, using the 0.1% (v/v) H_3PO_4/MeCN mobile phase, for benzyl amine and Dorzolamide HCl a minimum is not observed even at 0.2 mL/min (Fig. 8A and B). Both curves are shifted up and show a slight increase of the slope compared to neutral compounds which would indicate a larger mass transfer term (C) in the van Deemter equation. This suggests two types of sites with different adsorption energies exist. This would lead to slow interaction kinetics of the protonated bases with secondary adsorption sites with higher adsorption energy as well as faster kinetics of hydrophobic interactions with the alkyl bonded layer.

Similar effects were observed by McCalley for quninine, pyridine, and nortriptyline on an Inertsil ODS-3 column such that a clear optimum flow rate was not observed for any of the bases in the flow rate region studied (0.5–3.0 mL/min) and an increase in reduced plate height was observed with an increase in flow rate [24]. For the basic compounds, at each of the flow rates with the 0.1% (v/v) H_3PO_4 mobile phase the peak efficiencies determined at 50, 10 and 5% peak height decreased, respectively, indicating that the decrease in peak efficiency could be attributed to multiple processes leading to peak asymmetry.

A comparison of the Van Deemter plots constructed for benzyl amine and Dorzolamide HCl at low analyte loads using the 0.1% (v/v) H_3PO_4 mobile phase versus the 68 mM ClO_4^- mobile phase showed a significantly different Van Deemter profile (Fig. 8A and B). For the basic compounds, the Van Deemter profiles determined using the 68 mM ClO_4^- mobile phase exhibit similar profiles as the neutral compounds. All other basic analytes studied showed similar effects (beta-blocker, benzyl amines and ophthalmic compounds). Therefore, at high chaotropic counteranion concentration and low analyte load at all flow rates studied it was shown that the maximum column efficiency could be obtained for the protonated basic analytes.

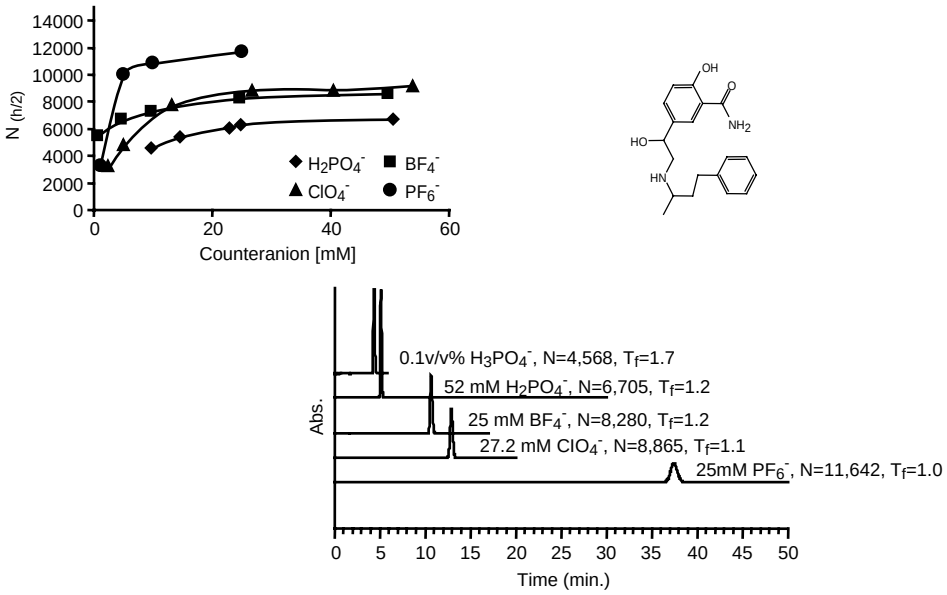

Fig. 10. Effect of counteranion type and concentration on analyte retention, peak efficiency, $N_{(h/2)}$, and tailing factor. Chromatographic conditions: Mobile phase: 75% aqueous:25% acetonitrile. Effective counteranion concentration for each mobile phase indicated in figure legend, flow rate: 1.0 mL/min; temperature: 25 °C; analyte load: 0.5 μg; wavelength: 225 nm.

3.4. Applications

Fig. 9 is a chromatographic overlay of Dorzolamide HCl analyzed at four increasing PF_6^- counteranion concentrations. As the concentration increased, peak tailing decreased, and peak efficiency and analyte retention increased. This method could be further optimized by increasing the organic concentration or flow rate so shorter elution times could be obtained. Fig. 10 shows the effect of different counteranions on basic analyte (labetalol) retention. Depending upon the desired selectivity between a neutral component and a charged basic analyte a particular chaotropic counteranion could be employed.

4. Conclusion

The use of chaotropic anions for a chromatographic separation may be beneficial as a method development strategy. It was determined that enhancement of loading capacity, retention, peak efficiency and peak symmetry could be obtained by the addition of chaotropic anions in the mobile phases and the degree of enhancement was also dependent upon the type and concentration of counteranion employed. These modifiers may replace the need for changing column type and/or addition of hydrophobic "ion-pairing" reagents. It should be emphasized that the current results have been performed on a particular type of hydrophobic stationary phase with an acetonitrile mobile phase at low pH.

The elucidation of where ion-interaction actually occurs is a subject of intense research currently being performed in our laboratories. If the process occurs in the mobile phase in which the dielectric constant (dielectric constant of water is 80) is high ion-pair formation is not favored and ion-interaction is the better term to use. There is a greater propensity to form ion-pairs in solvents of lower dielectric constant (<50), which are predominately organic. However, if interaction between protonated base occurs with adsorbed counteranion in the acetonitrile layer close to the surface, hence a predominately organic environment indeed ion-pair formation close to the surface may be possible leading to an ion-exchange type of mechanism. Further studies need to be conducted with inorganic mobile phase additives at low and high pH with various acetonitrile and methanol mobile phase eluents to further elucidate the mechanism of interaction of the additives with the stationary phase and with the protonated basic analytes.

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