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Role of ion pairing in anionic additive effects on the separation of cationic drugs in reversed-phase liquid chromatography

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Abstract

Mobile phase additives can significantly affect the separation of cationic drugs in reversed-phase liquid chromatography (RPLC). Although there are many applications for anionic additives in RPLC separations, the retention mechanism of basic drugs in the presence of inorganic and highly hydrophilic anionic species in the mobile phase is not at all well understood. Two major retention mechanisms by which anionic additives can influence the retention of cations are: (1) ion pair formation in the mobile phase with subsequent retention of the neutral ion pair; (2) pre-sorption of anionic additives on the stationary phase followed by "dynamic ion-exchange" or "electrostatic interaction" with the analytes. Because the use of ion pair chromatography in the separation of proteins, peptides, and basic drugs is rapidly increasing, understanding the retention mechanism involved is becoming more important, especially for the smaller commonly used hydrophilic anionic additives (e.g., formate $HCOO^-$, chloride Cl^-, trifluoroacetate CF_3COO^-, perchlorate ClO_4^-, and hexafluorophosphate PF_6^-). In this work, we compared various anionic additives in light of their effects on the retention of basic drugs. As did many others we found that the addition of anionic additives (Cl^-, CF_3COO^-, ClO_4^-, PF_6^-) profoundly influences the retention of basic drugs. In order to explain the data and differentiate the mechanisms by which the anionic additives perturb the chromatography, we used ion pair formation constants independently measured by capillary electrophoresis (CE) under the mobile phase conditions (pH, solvent composition) identical to those used in chromatography. Agreement between the predicted and experimental chromatographic data under various conditions was evaluated. Under specific circumstances (e.g., pH, stationary phase, and nature of anionic additive), we conclude that the ion pair mechanism is more important than the dynamic ion-exchange and at other conditions it remains a significant contribution.

Keywords: RPLC; LC/MS; Basic pharmaceuticals; Anionic additive; Retention mechanism; Capillary electrophoresis; Ion pair formation constant

1. Introduction

Basic compounds constitute the largest single class of analytes in reversed-phase pharmaceutical separations. In previous studies, we showed that reversed-phase and ion-exchange interactions are the major modes of interaction in RPLC separation of basic drugs [1–3]. Due to presence of ionizable silanol groups on silica based stationary phases, certain strategies are often used to minimize peak tailing and achieve better resolution for basic drug separations [4].

The most frequently used approaches to controlling the retention of bases include mobile phase modification by either cationic or anionic additives. We have demonstrated that the use of cationic additives (e.g., the counterions hexylamine, octylamine, etc.) improves the peak shape and alters selectivity by blocking silanol groups on the stationary phase [1–3]. Similarly that one can use anionic additives for adjusting the retention of basic compounds and improving the resolution is also well known [5–13].

The usual anionic additives are surfactants with substantially hydrophobic chains, such as the alkyl sulfonates [5,8]. However, these amphiphilic additives tend to stick very strongly to the stationary phase and lead to difficulty in recovering the initial column properties. This inhibits the use of

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these types of anionic additives. These long chain additives are generally not used when mass spectrometers are used as the detectors.

Since the use of ion pair chromatography in protein, peptide, and drug separations is increasing, studies of the effects of anionic additives on the retention of basic drugs have been reported for several small hydrophilic anionic additives (e.g., $H_2PO_4^-$, CF_3COO^-, ClO_4^-, and PF_6^-) [9–16].

It has been demonstrated that the retention of basic drugs in the presence of anionic additives follows the order $H_2PO_4^- < HCOO^- < CH_3SO_3^- < Cl^- < NO_3^- < CF_3COO^- < BF_4^- < ClO_4^- < PF_6^-$. This is the inverse of the order of these anions to cause salting-out (i.e., the "Hofmeister effect": $H_2PO_4^- > SO_4^- > CH_3COO > Cl^- > Br^- > NO_3^- > ClO_4^-$) [12,17]. Furthermore we believe this same series governs the effect that a change in anion concentration has on retention. Thus an increase in the concentration of $H_2PO_4^-$ has a smaller effect than does an increase in concentration of ClO_4^- or PF_6^-. Related to our previous discussions, we believe that the anions' hydration free energies are the keys to understanding all of these effects [18,19]. A highly hydrated anion such as $H_2PO_4^-$ (Gibbs free energy change on hydration of the gas phase ion $\Delta G^\circ = -437$ kJ/mole [19]) is more reluctant to form an ion pair in water than a moderately well hydrated anions such acetate ($\Delta G^\circ = -373$ kJ/mole [19]) or chloride ($\Delta G^\circ = -347$ kJ/mole [19]). In contrast, a "poorly" hydrated anion such as ClO_4^- ($\Delta G^\circ = -214$ kJ/mole [19]) forms ion pairs in water much more easily and also tends to be sorbed more extensively by a nonpolar stationary phase.

LoBrutto and coworkers studied the effect of both pH and the concentration of different anionic additives on the retention of small basic drugs [9–11]. According to their studies, different trends in retention were obtained using phosphate, trifluoroacetate, and perchlorate. The effect of "chaotropic" anionic additives, such as perchlorate and trifluoroacetate, was attributed to their making desolvation of cationic analytes in water easier thereby enhancing the cations' hydrophobicity [10]. A rather non-specific "ion association" model was proposed to explain the experimental data [9,11]. The nature of the desolvation parameter, which the authors proposed, was not clear in terms of its relationship to the ion association (ion pairing) stability constant. According to their fitting results, dihydrogen phosphate produces much more stable ion-associated complexes compared to other anions (e.g., trifluoroacetate, perchlorate); however, no satisfactory explanation was offered for this observation which is quite contradictory to the usual order of ion pair formation [11]. Furthermore, the study of the effect of additive concentration and pH on the retention was not straightforward because these two variables were altered simultaneously [10]. Although in acidic solution the pH variations did not change the charge state of the basic analyte, they could alter the degree of protonation of silanol groups on the stationary phase and modify the strength of the silanophilic interactions. Also, ionic strength has a complicated effect on retention. As LoBrutto et al. pointed out [10], studies at constant ionic strength are needed.

In addition to the effect that small anionic additives have on retention and selectivity, McCalley has shown that peak shape and plate count can depend very strongly on the nature of the buffer [20]. He has shown that peak shapes of basic compounds at low pH were better with a phosphate buffer as compared to a formate buffer. He demonstrated that sample capacity of column depended on the nature of the buffer and that the degree of column overload was better when a phosphate buffer was used as compared to a formate buffer with the same amount of sample [21]. Further, he showed that addition of chloride salt to a dilute phosphate buffer could significantly improve retention and sample capacity. Although the difference in behavior of the phosphate and formate buffers was attributed to the higher ionic strength of the phosphate buffer, the possible role of ion pairing when chloride salt was added to augment the ionic strength of a diluted phosphate buffer was left open.

Gritti and Guiochon recently studied the effects of pH, concentration and type of buffer, and ionic strength on the adsorption isotherms and overloaded band profiles of cations on different silica based RPLC packings [14,15,22–24]. They have shown that the use of a buffer or a neutral salt (potassium chloride), an increase in ionic strength introduced by potassium chloride, a change of the nature of the buffer (phosphate, acetate, phthalate, succinate, formate, and citrate), and an increase in the concentration of buffer not only affected the retention and adsorption capacity of basic compounds significantly, but also modified the analytes' adsorption isotherms. Ion pair formation or ion-associated complex between the cations and the anionic additives was invoked to explain the experimental observations.

Huber and Premstaller studied the utility of different anionic additives in protein analysis by LC/MS [25]. Although trifluoroacetate can provide better peak shape, they showed that formic acid gave better signal detectability compared to trifluoroacetate. This was attributed to the suppression of ion formation in the gas phase by trifluoroacetate.

As discussed, ion pair chromatography has attracted considerable attention in both pharmaceutical and biological separations. Even though it is well recognized that anionic additives have a significant effect on retention and peak shape, especially for cationic analytes, the retention mechanism of analytes in the presence of mobile phase additives has been hotly debated for several decades [5,7,8,26–31].

Horváth et al. systematically studied the effect of anionic additives [5]. They pointed out that there are two possible retention mechanisms that could account for the increase in retention of cations in the presence of anionic additives. The first is "dynamic ion-exchange" in the stationary phase. In this model, the anionic additive first sorbs to the stationary phase and creates a charged surface in the stationary phase. Subsequently, the analyte ion-exchanges (or electrostatically

interacts) with the charged stationary phase. In the alternative model, i.e., ion pair formation in the mobile phase, ion pairs between the cationic analyte and the added anion form in the mobile phase and then transfer into the stationary phase. Based on their results which involved highly hydrophobic alkyl sulfates and hexylsulfonate, Horváth et al. concluded that the retention processes was dominated by ion pair formation in the mobile phase with a lesser contribution from ion-exchange under certain conditions [5].

Knox and Hartwick studied the effect of highly hydrophobic alkyl sulfates of varying carbon number on the retention of cations [8]. The amount of mobile phase additive sorbed into the stationary phase was measured. They concluded that the pre-sorption of the additive to the stationary and subsequent dynamic ion-exchange accounted for the increase in the retention of cations and that ion pair formation in the mobile phase was not important. That is, dynamic ion-exchange is dominant. Although the studies of Knox and coworkers are quite persuasive, their conclusions are based on the studies of highly hydrophobic additives that have a very high propensity to sorb to the stationary phase, and their conclusions might not extend to less hydrophobic additives. It is hard to imagine that the same scenario applies to small, less hydrophobic organic and inorganic anionic additives, especially when some surface silanol groups are ionized.

According to results obtained under certain conditions, Bidlingmeyer et al. and Stranahan and Deming proposed a "broader" mechanistic model, which is usually referred as the "ion interaction" model [7,27]. Although the model could rationalize some previously unexplained phenomena, it was not satisfactory in interpreting other results [7,27].

In addition to the stoichiometric (ion-exchange) models, non-stoichiometric electrical double-layer models have been used to interpret the effect of added ionic materials on retention data [26,28–33]. Chen et al. reviewed the applications of such models for ion pair chromatography. A surface adsorption model proposed by Ståhlberg [33], a liquid partition model developed by Weber and Orr [31], a surface adsorption, diffuse layer ion-exchange model introduced by Cantwell [29], and a surface ion-exchange, diffuse layer ion-exchange model suggested by Deelder and Berg [28] were compared. The electrical double-layer models relate the change in analyte retention upon addition of salt to changes in the electric potential at the surface rather than to the conceptually simpler stoichiometric competition model involving ion-exchange equilibrium constants. The double-layer model is particularly effective in explaining the fact that under some conditions the negative retention factors can be obtained when the surface charge imparted by a sorbed eluent additive has the same sign as the probe analyte.

In a series of publications [34–37], Cecchi et al. proposed a model for the retention of different type of analytes in the presence of various mobile phase additives. The authors concluded that dominant mechanism varied with the experimental conditions.

Based on the above discussion, it was clear to us that no universal retention mechanism is able to explain all the observations under all conditions. That is, the retention mechanism is very much conditionally dependent. The dynamic ion-exchange (either stoichiometric or double-layer) retention mechanism that dominates for highly hydrophobic additives certainly is not necessarily correct for small hydrophilic additives. Considering the increasing importance of the application of these additives in both chromatography and mass spectrometry [9–15,25], we believe that an understanding of the retention process involved in the use of the more common, hydrophilic additives would certainly be important.

This paper aims to achieve an understanding of the fundamental aspect of the basic drug retention in the presence of the commonly used small hydrophilic anionic additives.

2. Theory

Fig. 1 illustrates the dynamic ion exchange in the stationary phase and ion pair formation in the mobile phase mechanisms. In contrast to previous discussions of anionic additive effects, we include the participation of ion-exchange sites due to the presence of ionized silanol groups. We treat such sites based on the two-site model [3]. We believe that for the relatively more hydrophilic anionic additives, especially in the presence of ionized silanol groups, the additive's propensity to sorb to the stationary phase is small. Thus, our initial premise is that ion pair formation in the mobile phase with the subsequent retention of the neutral ion pair is responsible for the increase in a cation's retention as a salt is added.

Under these assumptions, the retention of analyte can be described by the following equation [5]

$$\begin{aligned} k' &= \beta \frac{[A^+ : X^-]_s + [A^+]_s}{[A]_m + [A^+ : X^-]_m} \\ &= \beta \frac{K_{D,ip} K_{ip} [X^-] + K_{D,0}}{1 + K_{ip}[X^-]_m} \\ &= \frac{k'_{max} K_{ip} [X^-]_m + k'_0}{1 + K_{ip}[X^-]_m} \end{aligned} \quad (1)$$

where β is the phase ratio, K_{ip} is the ion pair formation constant between the analyte A^+ and anionic additive X^-, $K_{D,ip}$ is the distribution constant of the ion pair between the stationary and mobile phases, $K_{D,0}$ is the distribution constant of the non-ion paired analyte between the stationary and mobile phases, $[X^-]_m$ is the concentration of anionic additive, k' is the retention factor in the presence of anionic additive, k'_0 is the retention factor in the absence of anionic additive, k'_{max} is the limiting retention factor of the fully ion paired analyte at very high concentration of additive, and the subscripts m/s denote mobile and stationary phases.

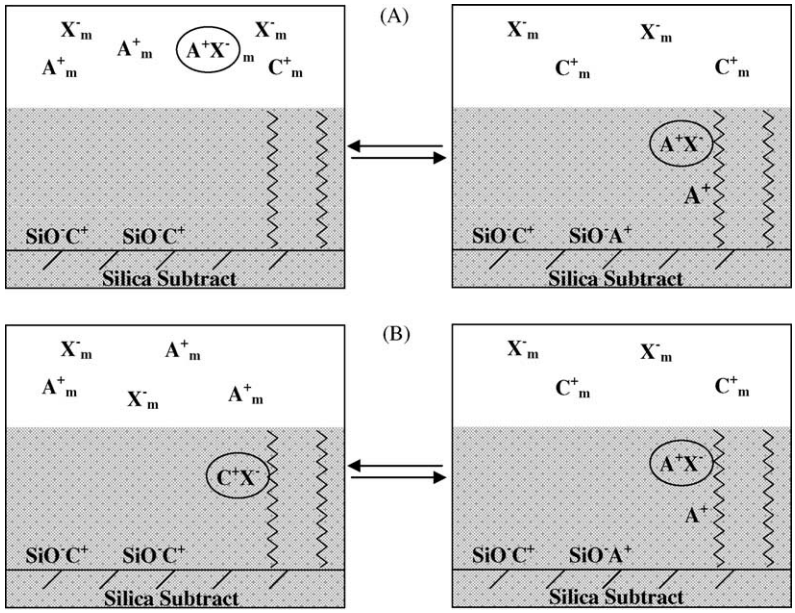

Fig. 1. Cartoon illustrations of interactions between cationic solute A^+ and bonded silica phase according to: (A) "ion pair formation in the mobile phase" and (B) "dynamic ion-exchange in the stationary phase" mechanisms in the presence of a mobile phase additive X^-C^+. Three types of interactions are included: pure ion-exchange site from ionised silanol groups; pure reversed-phase site; and reversed-phase site for the ion pair (A), or ion-exchange site from the sorbed anions (B).

Similarly, the retention factor for analyte A^+ according to dynamic ion-exchange mechanism can be derived as [5,8]:

$$\begin{aligned} k' &= \beta \frac{[A^+ : X^-]_s + [A^+]_s}{[A^+]_m} \\ &= \beta \frac{K_{D,CX} K_{IEX,AX}[X^-] + K_{D,0}}{1 + K_{D,CX}[X^-]_m[C^+]_m} \\ &= \frac{K_{D,CX} k'_{max}[X^-] + k'_0}{1 + K_{D,CX}[X^-]_m[C^+]_m} \end{aligned} \quad (2)$$

where $K_{D,CX}$ is the distribution constant for the additive between the stationary and mobile phases, $K_{IEX,AX}$ is the equilibrium constant for the ion-exchange process of the analyte in the mobile phase and the sorbed anionic additive in the stationary phase, C^+ is the counterion (e.g., Na^+) associated with the anionic additive, and $k'_{max}$is the limiting retention factor observed at very strong sorption of the additive to the stationary phase.

At constant counterion concentration, Eq. (2) can be rewritten as:

$$\begin{aligned} k' &= \beta \frac{K_{D,CX} K_{IEX,AX}[X^-] + K_{D,0}}{1 + K_{D,CX}[X^-]_m[C^+]_m} \\ &= \frac{K_{D,CX} k'_{max}[X^-]_m + k'_0}{1 + K'[X^-]_m} \end{aligned} \quad (3)$$

where K' equals $K_{D,CX}[C^+]_m$.

Mathematically Eqs. (1) and (3) reduce to the same algebraic form

$$k' = \frac{B_1 + B_2[X^-]_m}{1 + B_3[X^-]_m} \quad (4)$$

where B_1, B_2, and B_3 are constants independent of $[X^-]_m$ under certain conditions.

As pointed by Knox and Hartwick [8], we cannot differentiate these two retention mechanisms by the study of retention as a function of the concentration of additive. Indeed, the initial and final states in the two mechanistic pictures are the same thus the mechanisms differ only in the sequence of steps, and consequently, *they are thermodynamically indistinguishable*.

If both ion pair formation and dynamic ion-exchange take place simultaneously, the following equation is obtained [5,8]

$$\begin{aligned} k' &= \frac{K[X^-]_m + k'_0}{(1 + K_{ip}[X^-]_m)(1 + K_{D,XC}[X^-]_m[C^+]_m)} \\ &= \frac{K_1 K_2[X^-]_m + k'_0}{(1 + K_{ip}[X^-]_m)(1 + K_{D,XC}[X^-]_m[C^+]_m)} \end{aligned} \quad (5)$$

where K is the product of K_1 and K_2.

Under extreme cases where $K_{D,CX}$ equals 0 or K_{ip} equals 0, Eq. (5) reduces to Eqs. (1) ($K_1 = K_{ip}$, $K_2 = K_{D,ip}$) and (2) ($K_1 = K_{D,CX}$, $K_2 = K_{IEX,AX}$), respectively.

It is K in Eq. (5) that cannot be chemically interpreted based solely on retention data. *Nevertheless, it is clear that K_1 and K_2 in Eq. (5) have very different chemical signifi-*

cances in the ion pair formation and dynamic ion-exchange mechanisms as shown by Eqs. (1) *and* (3). Furthermore, mere compliance of the data (i.e., plots of k' versus $[X^-]_m$) to Eqs. (1) or (3) provides no evidence for the validity of one versus the other mechanism. Lack of compliance of the data to the equations would certainly invalidate both as mechanisms. However, if independent experiments were carried out to measure K_{ip} or $K_{D,CX}$, new insight could be obtained about the nature of the controlling mechanism. Specifically, if one could show that K_1 were equal to K_{ip}, one would have a great deal more confidence that ion pairing is responsible for the variation in retention with the concentration of additive.

According to the two-site model of silanophilic interactions [3], k'_0 in Eqs. (1), (2), and (5) can be rewritten as

$$k'_0 = k'_{RP} + k'_{IEX} \tag{6}$$

where k'_{RP} is from the reversed-phase interactions, k'_{IEX} is the ion-exchange (silanophilic) retention from ionized silanol groups.

At low concentration of the counterion (i.e., species C^+), the following equation can be used to describe the two-site model [38]

$$k'_0 = k'_{RP} + k'_{IEX} = k'_{RP} + \frac{k'_{IEX,0}}{1 + K_C[C^+]_m} \tag{7}$$

where $k'_{IEX,0}$ is ion-exchange retention in the absence of counterion and K_C is the distribution constant for the counterion.

If we apply Eq. (7) to Eq. (1), we obtain Eq. (8)

$$k' = \frac{k'_{max}K_{ip}[X^-]_m + k'_0}{1 + K_{ip}[X^-]_m} = \frac{k'_{max}K_{ip}[X^-]_m + \{k'_{RP} + k'_{IEX,0}/(1 + K_C[X^+]_m)\}}{1 + K_{ip}[X^-]_m} \tag{8}$$

At constant counterion concentration, k'_{IEX} is independent of $[X^-]_m$, and k'_0 in Eq. (1) is constant.

To the best of our knowledge, Eq. (8) is the first time an equation that includes both effects due to ion pairing by an additive anion and competition by an additive cation on the retention of a cationic analyte has appeared. Some hypothetical examples of the dependence of retention on the concentration of added salts based on Eq. (8) are given in Fig. 2. We see that the different contributions from the two competitive processes, ion pairing and counterion displacement, can give very different pictures of the behavior of retention as a function of the concentration of added salts. The retention can increase, decrease, or effectively remain constant as the concentration of the additive is increased. These results clearly show that it is very important to understand that due to these opposing effects, *it is possible that an added salt could have no or little effect on retention even though both individual effects (i.e., ion pairing and competition for ionized silanols) are strong*. Clearly studies of cationic displacing agents as described in previous work [3] should be carried out with salts based on weak ion pairing anions such as formate, acetate and chloride (see below).

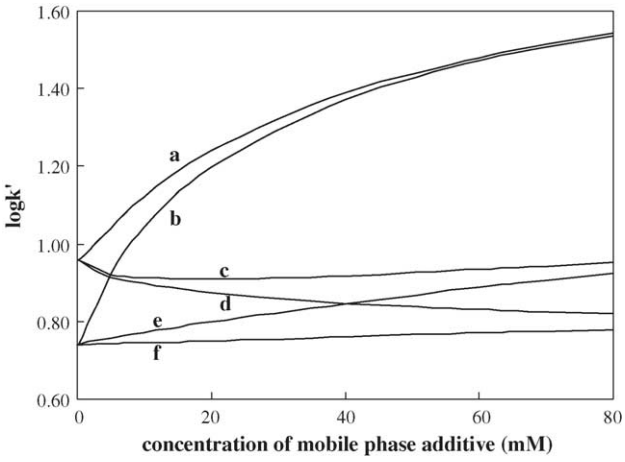

Fig. 2. Effect of mobile phase additive concentration on retention (see Eq. (8)). Values of k'_{max}, K_{ip}, k'_{RP}, $k'_{IEX,0}$, K_C are realistic based on experimental results. Plot legends: (a) high K_C–high K_{ip}; (b) low K_C–high K_{ip}; (c) high K_C–medium K_{ip}; (d) high K_C–low K_{ip}; (e) low K_C–medium K_{ip}; (f) low K_C–low K_{ip}.

To differentiate between the ion pair and dynamic ion-exchange models above, we measured K_{ip} by an independent technique namely capillary electrophoresis [18]. Since K_{ip} measured by CE does not involve a stationary phase, we believe that if the K_{ip} so measured gave good fits of chromatographic data to Eq. (1) then we could conclude that ion pairing is the dominant mechanism. It is very unlikely that $K_{D,CX}$ would equal K_{ip}. Based on the above discussion, we used CE data to decide between the two mechanisms.

In addition to the CE studies, we also attempted to determine the role of dynamic ion-exchange and the amount of adsorbed additive anion by a method introduced by Knox and Hartwick [8]. They showed that the retention of analytes of the same charge type as the sorbed additive decreases significantly as the amount of sorbed anion is increased. Thus we expected that if the hydrophilic anionic additives were to sorb significantly to the stationary phase then the anionic analytes would be excluded from the stationary phase [7,8]. In this case, anionic analytes should be more excluded when more anionic additive was sorbed by increasing its mobile phase concentration or by changing to an additive type that is more strongly sorbed.

3. Experimental

3.1. Instruments

All chromatographic experiments were carried out with a Hewlett-Packard 1090 chromatographic system equipped with a binary pump, a helium sparger, an autosampler, a thermostatted-column compartment, and a diode array detector (Agilent Technologies, Hewlett-Packard, Wilmington,

DE, USA). Data were collected and processed using Hewlett-Packard Chemstation software.

3.2. Analytical columns

The Stable Bond (SB) C_{18} column with dimensions of 50 mm × 4.6 mm i.d. was donated by Agilent Technologies Inc. (Wilmington, DE). The average particle size was 5 μm and the average pore diameter was 80 Å.

3.3. Reagents

All chemicals were reagent grade or better. Cationic drugs were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile was from Burdick & Jackson (Muskegon, MI, USA). HPLC water was obtained from a Barnstead Nanopure deionizing system (Dubuque, IA, USA) and run through an "organic-free" cartridge followed by a 0.2 μm particle filter. The solution was then degassed under helium. All solvents were filtered through a 0.2 μm filter (Lida Manufacturing, Kenosha, WI, USA) before use. Other chemicals used in this study were purchased from Aldrich (Aldrich, Milwaukee, WI, USA).

3.4. Chromatographic conditions

All chromatographic measurements were made at a flow rate of 1 mL/min, and detection was set at 254 nm. The column temperature was controlled to 35.0 °C with the Hewlett-Packard 1090 oven. The dead time was determined by injecting uracil. The buffers at pH 4.8 were prepared from acetic acid and sodium acetate. Formic acid (0.05%, v/v) was used for the pH 2.8 buffers. Mobile phases with different additives were prepared by adding the sodium salts of each anion to the buffers. Sodium chloride was used to adjust the ionic strength for some of the experiments. The concentrations of buffer and sodium salts were reported with respect to the volume of the aqueous–organic mixture. The samples of the anionic probe solutes (about 0.2 mM) were prepared in water and the drug samples (about 0.2 mM) are prepared in the mobile phase. The injection amount was set at 1 μL. Preliminary experiments indicated that under these conditions neither the volume nor sample concentration had an appreciable effect on the observed retention times.

4. Results and discussion

4.1. Effect of anionic additives type on the separation of cationic drug at pH 4.8

It has been shown that different anionic additives give very different retentions for cationic analytes [9–12]. Due to differences in the ionization constants (pK_a values) of the Bronsted acids corresponding to the different anions, differ-

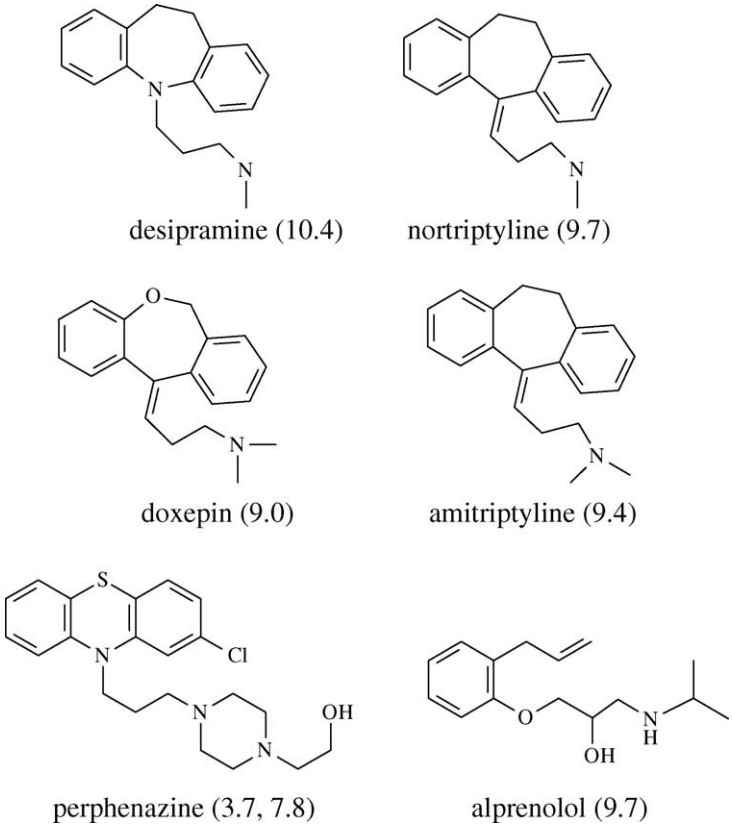

Fig. 3. Structures and pK_as of the basic drugs.

ent amounts of the anionic additives would be needed if the mobile phases were to be buffered to the same pH by use of the individual acids without use of an independent buffer. Thus an acetate buffer with 20 mM sodium acetate at pH 4.8 was used to establish the pH in all cases. To examine the effect of the type and concentration of each additive at fixed pH and ionic strength we varied only the amount of the sodium salt of the anion of interest. Acetate was chosen as the buffer system because it is a weak "ion pairing" agent and weak displacing species in anion chromatography [17,39].

The structures and pK_as of the cationic drugs used are given in Fig. 3. Fig. 4 shows the effect of the type of additive on the separation of the basic drugs. For the purpose of comparison, two neutral analytes are also included. Although only minor trends in the retention of neutral compounds are observed, the retention of all cations varied significantly with the type of additive. The retention factors of all drugs decrease in the order $PF_6^- > ClO_4^- > CF_3COO^- > Cl^-$. This trend agrees with the literature [9–12].

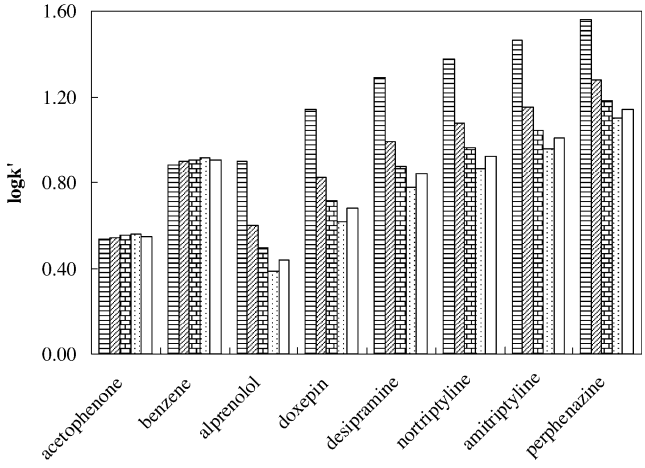

Fig. 4. Effect of anionic additive type on the retention of basic analytes. Conditions: 35/65 acetonitrile/buffer, 20 mM sodium acetate buffer at pH 4.8 (buffer only) and 20 mM NaX. Plot legends: from left to right, PF_6^-; ClO_4^-; CF_3COO^-; Cl^-; buffer only.

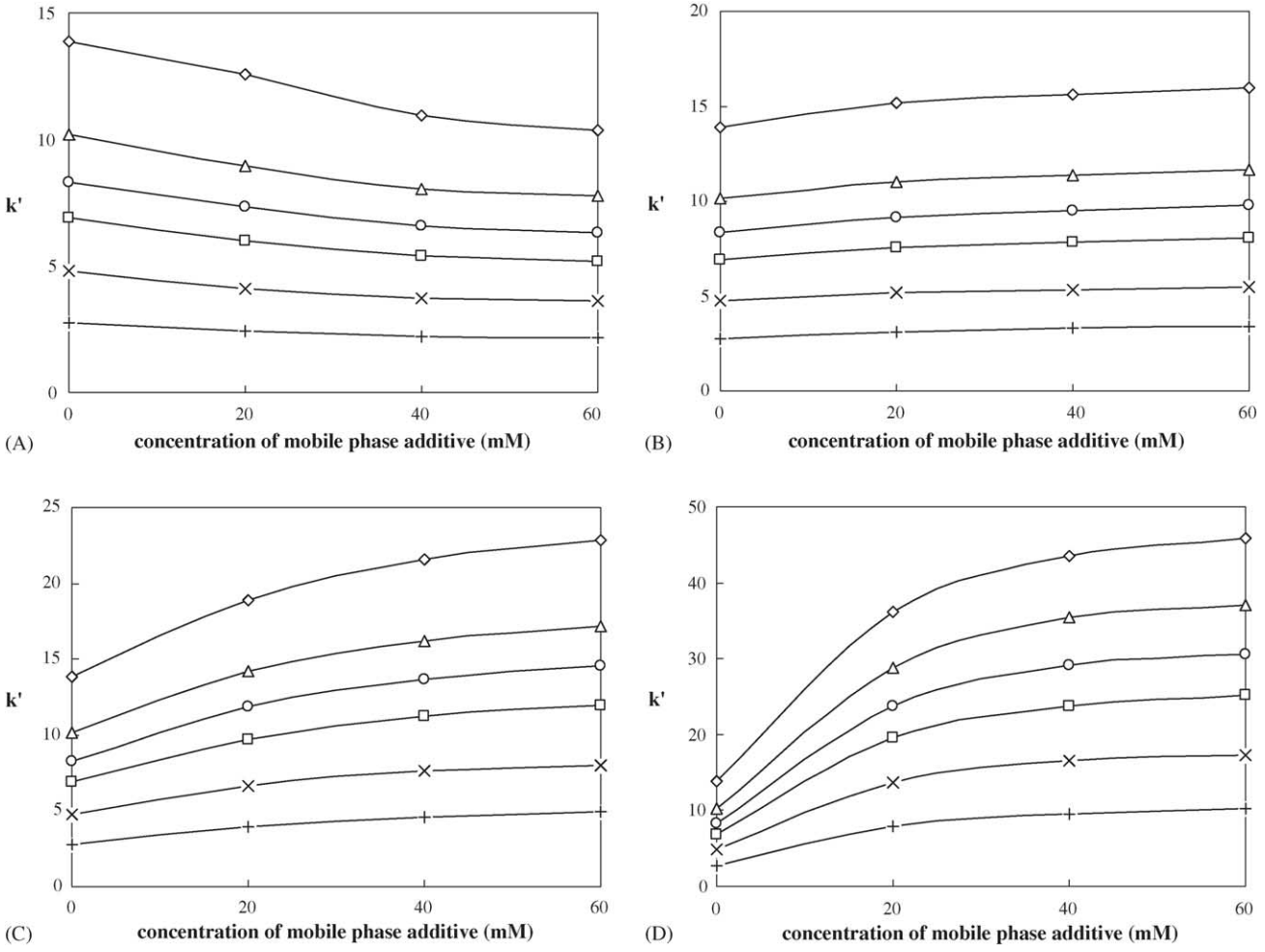

Fig. 5. Effect of mobile phase additive concentration on retention of basic analytes. Conditions: 35/65 acetonitrile/buffer, 20 mM sodium acetate buffer at pH 4.8 and 0–60 mM NaX. Plot legends: (+) alprenolol, (×) doxepin, (□) desipramine, (○) nortriptyline, (△) amitriptyline, (◇) perphenazine. (A) Cl^-; (B) CF_3COO^-; (C) ClO_4^-; (D) PF_6^-.

The effect of different additive concentrations on retention is shown in Fig. 5. Obviously, the concentration effects vary and we see patterns that conform to the range of predictions seen in Fig. 2 based on Eq. (8). Of special interest is the case for chloride. Even though an increase in concentration of the additives for trifluoroacetate, perchlorate, and hexafluorophosphate enhances retention for all drugs, their retention factors actually decrease upon increasing the concentration of chloride. Gritti and Guiochon also observed that the retention of a base decreased at neutral pH when phosphate and citrate buffers were used [15,24].

Since we did not maintain constant counterion concentration, the concentration of sodium also increased as we increased the additive concentration. The decrease in retention of basic drugs upon increase in sodium chloride concentration indicates that at pH 4.8, there is a significant contribution from ion-exchange interactions on ionized silanol groups on the phase used in this work.

We believe that two competitive processes are involved in the retention of cations as we increased the concentration of each mobile phase additive: a decrease in the interaction with ionized silanol groups caused by counterion competition effect due to the increased sodium concentration and an increase in the retention by one of the anionic effects described above. This competition depends on both the nature of the anionic additive and the silanophilicity of the stationary phase (see Fig. 2). For trifluoroacetate, perchlorate, and hexafluorophosphate, the second process dominates; while in the case of chloride, the cation displacement effect is marginally stronger.

Considering the results shown in Fig. 5A we believe that maintaining the counterion concentration constant, which was not done in previous studies [9–11], is essential for understanding the effect of anionic additives.

Addition of sodium chloride does not alter the pH of the mobile phase. We also conclude from Fig. 5A that chloride, as an additive, has a relatively small effect on the retention of basic drugs. Based on the above two advantages of this salt, we used sodium chloride to maintain a constant total ionic strength of 60 mM (excluding the ionic strength of the acetate buffer) in the following studies as we vary the concentration of anionic additives. In principle, we could have controlled

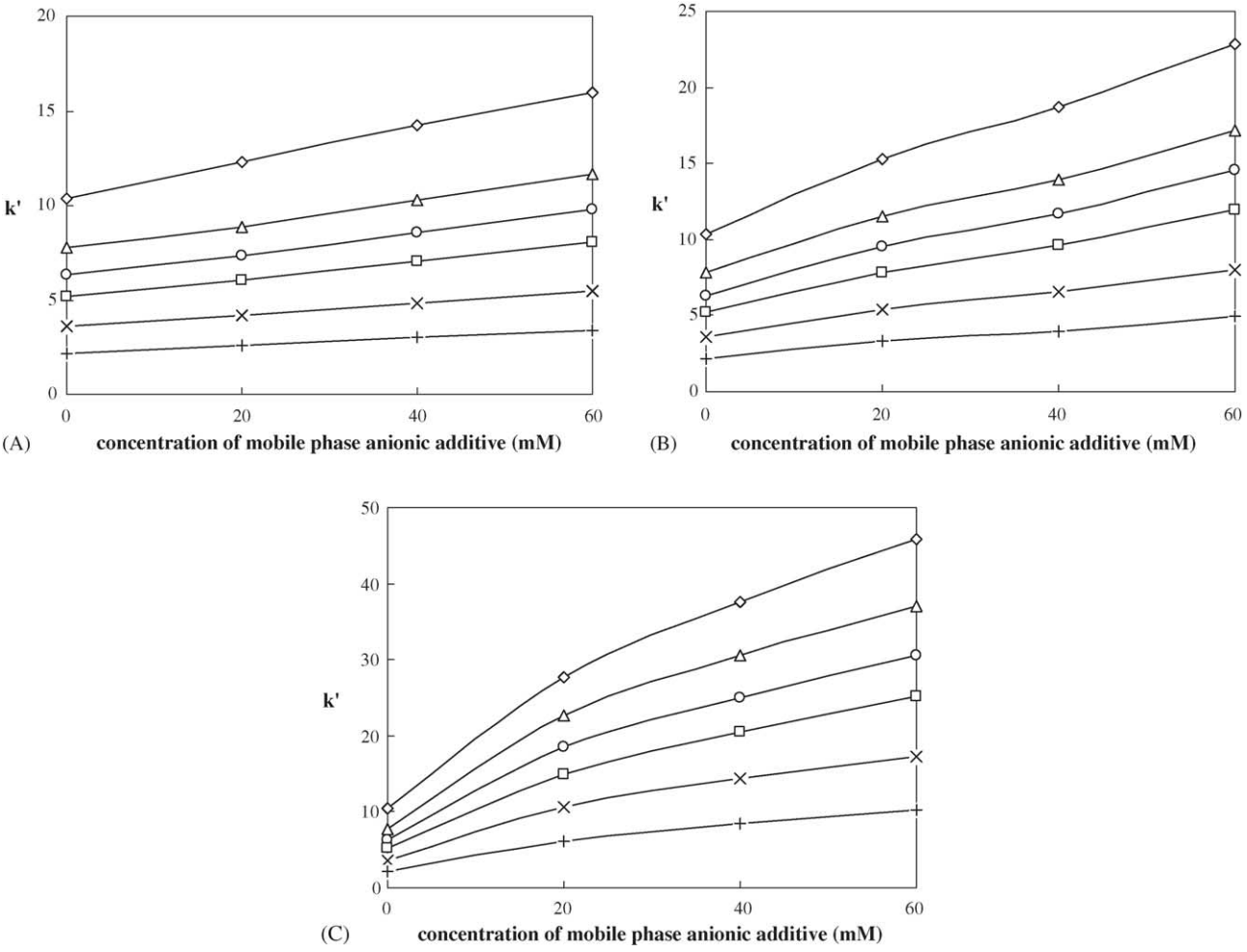

Fig. 6. Effect of anionic additive concentration on retention of basic analytes at a constant ionic strength of 60 mM maintained by sodium chloride (excluding the ionic strength from acetate buffer). Conditions: 35/65 acetonitrile/buffer, 20 mM sodium acetate buffer at pH 4.8 and 0–60 mM NaX. Plot legends: same as Fig. 5. (A) CF_3COO^-; (B) ClO_4^-; (C) PF_6^-.

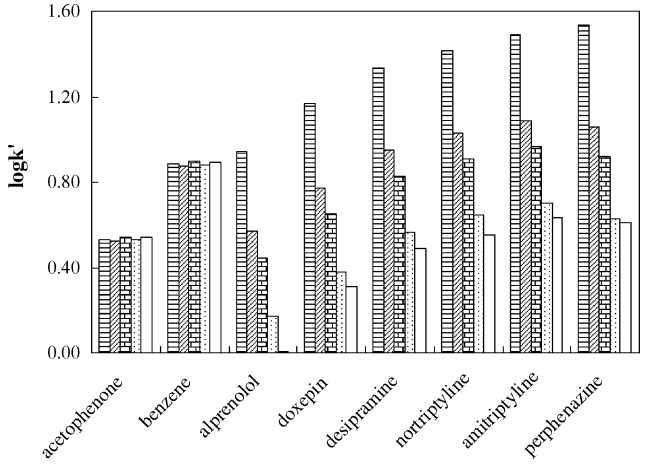

Fig. 7. Effect of anionic additive type on the retention of basic analytes. Conditions: 35/65 acetonitrile/buffer, 0.05% formic acid at pH 2.8 (buffer only) and 20 mM NaX. Plot legends: from left to right, PF_6^-; ClO_4^-; CF_3COO^-; Cl^-; buffer only.

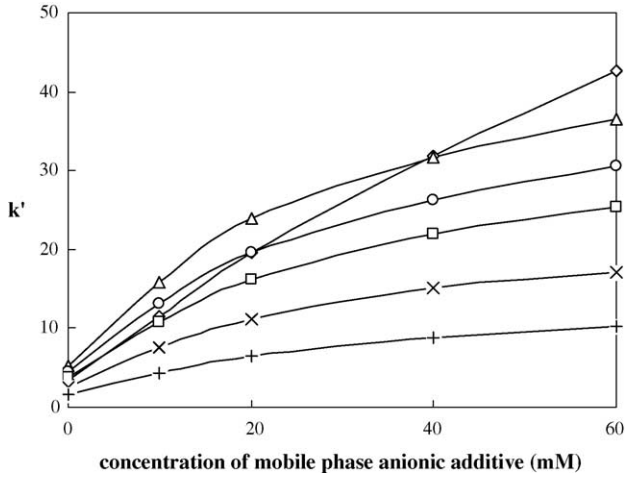

Fig. 9. Effect of anionic additive concentration on retention of basic analytes at a constant ionic strength of 60 mM maintained by sodium chloride (excluding the ionic strength from 0.05% formic acid). Conditions: 35/65 acetonitrile/buffer, 0.05% formic acid at pH 2.8 and 0–60 mM $NaPF_6$. Plot legends: same as Fig. 5.

the ionic strength by simultaneously varying the concentration of acetic acid and sodium acetate keeping them in the same ratio but we were afraid that inadvertent (but minor) variations in pH would be very troublesome.

Fig. 6 gives the effect of additive concentration at constant ionic strength. As expected, hexafluorophosphate has a much stronger effect than perchlorate and trifluoroacetate. Also, the curve in Fig. 6 at constant ion strength is steeper compared to that in Fig. 5.

According to Eqs. (1) and (3), when the concentration of anionic additive is high enough, k' reaches a maximum (k'_{max}) and the curve of k' versus $[X^-]_m$ flattens out at a "saturation" limit. We want to point out that the "saturation" limit phenomenon as mentioned by LoBrutto et al. [9], is quite different with and without ionic strength control.

4.2. Effect of anionic additives type on the separation of cationic drug at pH 2.8

To address the issue of the silanophilicity of the stationary phase, we performed a similar study at pH 2.8 where the higher acidity should block ionization of most of the surface silanols. Since formate has a relatively small effect on the retention of cations [12], we used 0.05% (v/v) formic acid to maintain a pH of 2.8. The effect of additive type on the retention of the bases is shown in Fig. 7. Again, the different additives have dramatically different effects on the retention of cations.

Fig. 8 shows the effect of additive concentration on the retention of basic drugs at pH 2.8. Fig. 9 gives the effect of additive concentration at constant ionic strength. In contradis-

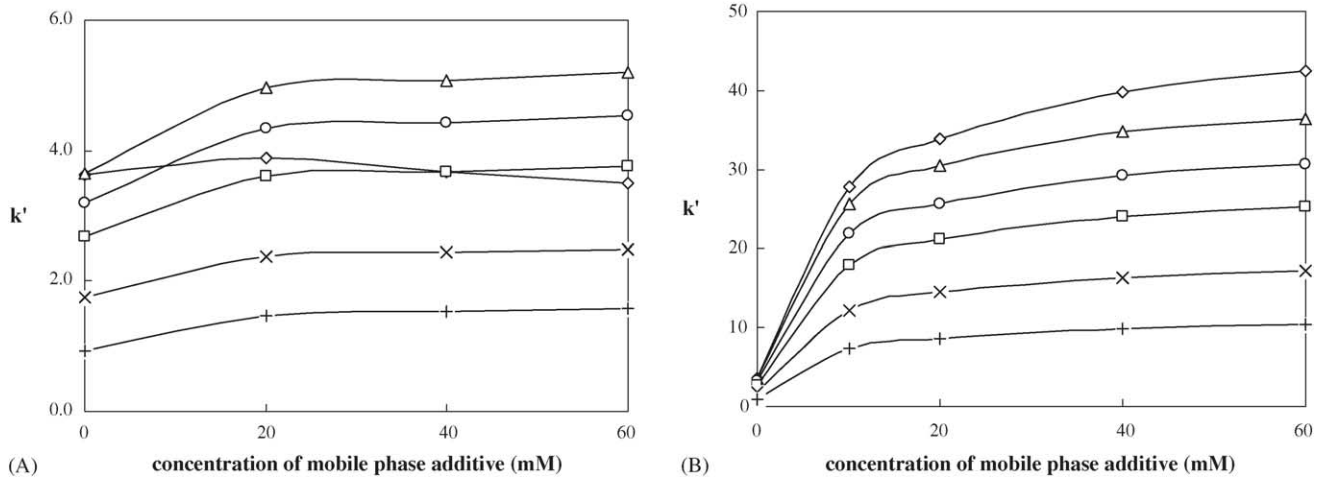

Fig. 8. Effect of mobile phase additive concentration on retention of basic analytes. Conditions: 35/65 acetonitrile/buffer, 0.05% formic acid at pH 2.8 and 0–60 mM NaX. Plot legends: same as Fig. 5. (A) Cl^-; (B) PF_6^-.

tinction to the behavior at pH 4.8, the retention now increases as the concentration of sodium chloride is increased. Since these analytes are all strong bases, they are all fully protonated at both pHs, the different results seen with sodium chloride at these two pH conditions confirm that there is a significant change in this stationary phase between pH 4.8 and 2.8. We believe that at pH 2.8 most, but not all, of the silanol groups are protonated and thus there is only a small ion-exchange contribution to retention. At pH 2.8, the counterion competition effect of increasing the sodium ion concentration can no longer overcome the retention enhancing anionic additive effect of the increased chloride concentration.

The above results for sodium chloride provide a clear picture showing the change in silanophilic effect of the stationary phase as we vary the pH of the mobile phase. We also believe that this change explains another difference: the type of anionic additive has a more pronounced effect on the retention of basic drugs at pH 2.8 than at 4.8. At pH 4.8, silanophilic interactions play a more important role, and therefore abate the differences due to the type of anionic additive. However, at pH 2.8, the anionic additive effect is stronger than the sodium displacement effect.

We want to point out that the above observations by no means prove that silanol groups on the current stationary phase are fully protonated at pH 2.8. As seen in Fig. 8A, the decrease in retention of the doubly protonated diamine perphenazine at increasing concentration of sodium chloride is a clear sign that there are still ionized silanol groups at pH conditions as low as 2.8. This again convinces us of the importance of maintaining the counterion concentration constant in studies of the effect of anion to minimize the complications from silanophilic interactions.

4.3. Retention model of cationic drugs in the presence of anionic additives

There has been a long-standing debate as to whether ion pair formation in the mobile phase or the dynamic ion-exchange in the stationary phase is involved in the retention of cationic analytes in the presence of anionic additives. If ion pair formation in the mobile phase controls the retention process, then ion pair formation constants obtained from fitting chromatographic retention data as a function of the anion concentration and that measured by CE should be comparable.

To test for ion pair formation in the mobile phase, we used CE to measure the ion pair formation constants of nortriptyline, amitriptyline, and perphenazine under solvent conditions identical to chromatographic mobile phase used here. The K_{ip} data are given in Table 1 [18].

As seen in Table 1, the ion pairing varies from trifluoroacetate to hexafluorophosphate. Although ion pair formation constant data for chloride ion is not available, our CE results clearly demonstrated that basic drugs and the chloride ions do form very weak ion pairs [18], this is consistent with the results we observed in RPLC (see Fig. 8A). The ion pairing ability of chloride is weaker than the other three anions used in the current study. However, the extent of ion pairing for all anions used here is not large. At a typical RPLC mobile phase additive concentration of 20 mM, the percentages of the analyte that are present in the mobile phase as ion pairs are about 15%, 6%, and 3% for hexafluorophosphate, perchlorate, and trifluoroacetate, respectively. The fraction of the bases present as a chloride pairs is even smaller.

Table 1
Effect of anionic additive type on ion pair formation constants[a]

Solute/additive		PF_6^-	ClO_4^-	CF_3COO^-
Amitriptyline	K_{ip}[b]	9.15	3.93	1.65
	s.d.[c]	0.39	0.37	0.16
Nortriptyline	K_{ip}	7.18	3.17	1.64
	s.d.	0.56	0.56	0.04
Perphenazine	K_{ip}	5.76	3.36	1.81
	s.d.	0.26	0.38	0.06

[a] CE running conditions same as Fig. 6.
[b] Ion pair formation constant (M^{-1}).
[c] Standard error of ion pair formation constant (M^{-1}).

Table 2 and Fig. 10 give the results of fitting retention data at pH 4.8 according to Eq. (1) by adjusting only one parameter, namely k'_{max}. All k' data were obtained at a constant ionic strength of 60 mM (excluding the ionic strength from acetate buffer). We fixed the value of k'_0 at the value of k' observed using the acetate buffer and 60 mM sodium chloride, and the value of K_{ip} was set equal to that measured by CE at this ionic strength.

Excellent agreement is obtained for both trifluoroacetate and perchlorate. The fitting results for hexafluorophosphate are not as good, but still the agreement strikes us as quite acceptable. Again, we want to point out that there is only one adjustable parameter used to fit our data to Eq. (1) and it is not the K_{ip} which controls the shape of the curve. This certainly leads to a significant higher reliability of fitting results compared to multi-parameter nonlinear fitting. Values of k'_{max} obtained show the reasonable trend $k'_{max}(PF_6^-) > k'_{max}(ClO_4^-) > k'_{max}(CF_3COO^-)$ which is the same as the order of ion pairing constants. *We believe that the good agreement in fitting through the use of the CE estimate of K_{ip}confirms the ion pair formation mechanism of the effect of anionic additives on the retention of cationic drugs*.

Although the CE data for K_{ip} were obtained at pH 4.8 with acetate buffer instead of formate at pH 2.8, we believe that pH has a relatively small effect on K_{ip} for the strongly basic drugs used in this study. To a good approximation, we can assume that under these two conditions with the same percentage of organic modifier, K_{ip} is similar.

Fig. 11 shows the fitting results for hexafluorophosphate at pH 2.8 using the K_{ip} measured at pH 4.8 (perphenazine is not included due to the change in protonation state since its second pK_a is 3.7). In contrast to the fitting results at pH 4.8, the errors are much bigger and the fitting is rather poor. This suggests the possibility that the anionic additive sorbs

Table 2
RPLC fitting results according to the ion pair formation in the mobile phase mechanism with K_{ip} measured by CE at pH 4.8[a]

Solute/fitting results	k'_{max}[b]	Concentration of sodium trifluoroacetate					
		60 mM		40 mM		20 mM	
		k'[c]	$\Delta k'$[d]	k'	$\Delta k'$	k'	$\Delta k'$
Nortriptyline	43.9	9.8	0.010	8.6	−0.006	7.3	−0.024
Amitriptyline	49.3	11.6	0.010	10.3	−0.005	8.9	−0.026
Perphenazine	67.3	16.0	0.001	14.3	0.002	12.3	−0.008
	k'_{max}	Concentration of sodium perchlorate					
		60 mM		40 mM		20 mM	
		k'	$\Delta k'$	k'	$\Delta k'$	k'	$\Delta k'$
Nortriptyline	56.9	14.5	0.010	11.7	−0.027	9.6	0.022
Amitriptyline	56.0	17.2	0.013	13.9	−0.030	11.5	0.019
Perphenazine	83.7	22.9	0.007	18.7	−0.019	15.3	0.017
	k'_{max}	Concentration of sodium hexafluorophosphate					
		60 mM		40 mM		20 mM	
		k'	$\Delta k'$	k'	$\Delta k'$	k'	$\Delta k'$
Nortriptyline	89.5	30.6	−0.027	25.0	0.004	18.5	0.095
Amitriptyline	92.5	37.0	−0.022	30.6	0.003	22.6	0.077
Perphenazine	153.8	45.9	−0.028	37.6	0.010	27.7	0.091

[a] Fitting results according to Eq. (1) based on k' results from Fig. 6 and K_{ip} from Table 1.
[b] Fitting results of k'_{max} in Eq. (1).
[c] Experimental data from RPLC at pH 4.8 (see Fig. 6).
[d] Error of predicted data $\Delta k' = \frac{k'_{experimental} - k'_{predicted}}{k'_{experimental}}$.

to the stationary phase; this is much more probable at pH 2.8 than at pH 4.8 because at the lower pH the stationary phase barely has any negative charge as it does at pH 4.8 and thus anion sorption should be much greater at the lower pH.

4.4. Exclusion of anionic probes in the presence of anionic additives

4.4.1. Studies at pH 2.8

To assess the extent to which the anionic additives are sorbed onto the stationary phase we measured the degree of Donnan exclusion of easily detected UV active probe anions. When anionic additives (such as chloride) sorb to the stationary phase, as proposed in the "dynamic ion-exchange" mechanism, the double-layer model shows that a negative surface potential will develop and this in turn will act to exclude probe anions.

Fig. 12 shows the extent of exclusion of probe anions at pH 2.8. As seen in Fig. 12A, one of the anionic probes (i.e., iodide) is actually retained when chloride is used as the anionic additive, indicating that very little chloride is adsorbed. The negative surface charge is so small that it is not able to exclude iodide. We conclude that chloride sorption is very weak and nearly non-existent.

The situation with hexafluorophosphate as the additive is quite different: the exclusion of the anionic probes now takes place to a considerable extent as compared to the case with chloride (see Fig. 12B). Exclusion of the probe anions decreases with increasing concentration of hexafluorophosphate (see Fig. 12B); however, the exclusion does not change as hexafluorophosphate is varied from 20 to 60 mM at constant total ionic strength (see Fig. 12C).

Comparison of Fig. 12B and C shows that ionic strength has a considerable impact on the effect that different concentrations of hexafluorophosphate have on anion exclusion. The decrease of anion exclusion from 20 to 60 mM hexafluorophosphate seems to be a pure ionic strength issue.

We believe this same ionic strength issue also contributes to the difference in cation retention with fixed and variable ionic strength as concentration of hexafluorophosphate is varied (see Figs. 8B and 9), which we could not ascribe solely to ion pair formation competition. While it is true that we need to vary the chloride concentration to hold ionic strength constant and higher ionic strength tends to diminish retention due to the competition from salts to ion pairing [5,40,41], ion pair competition by chloride cannot be very strong (see Fig. 8A). We believe that the difference in behavior seen in Fig. 12B and C is also operative in the cation retention data. It is clear that increased ionic strength decreased the Coulombic interactions between the cationic analyte and the sorbed hexafluorophosphate, therefore, decreased retention as shown in Fig. 9.

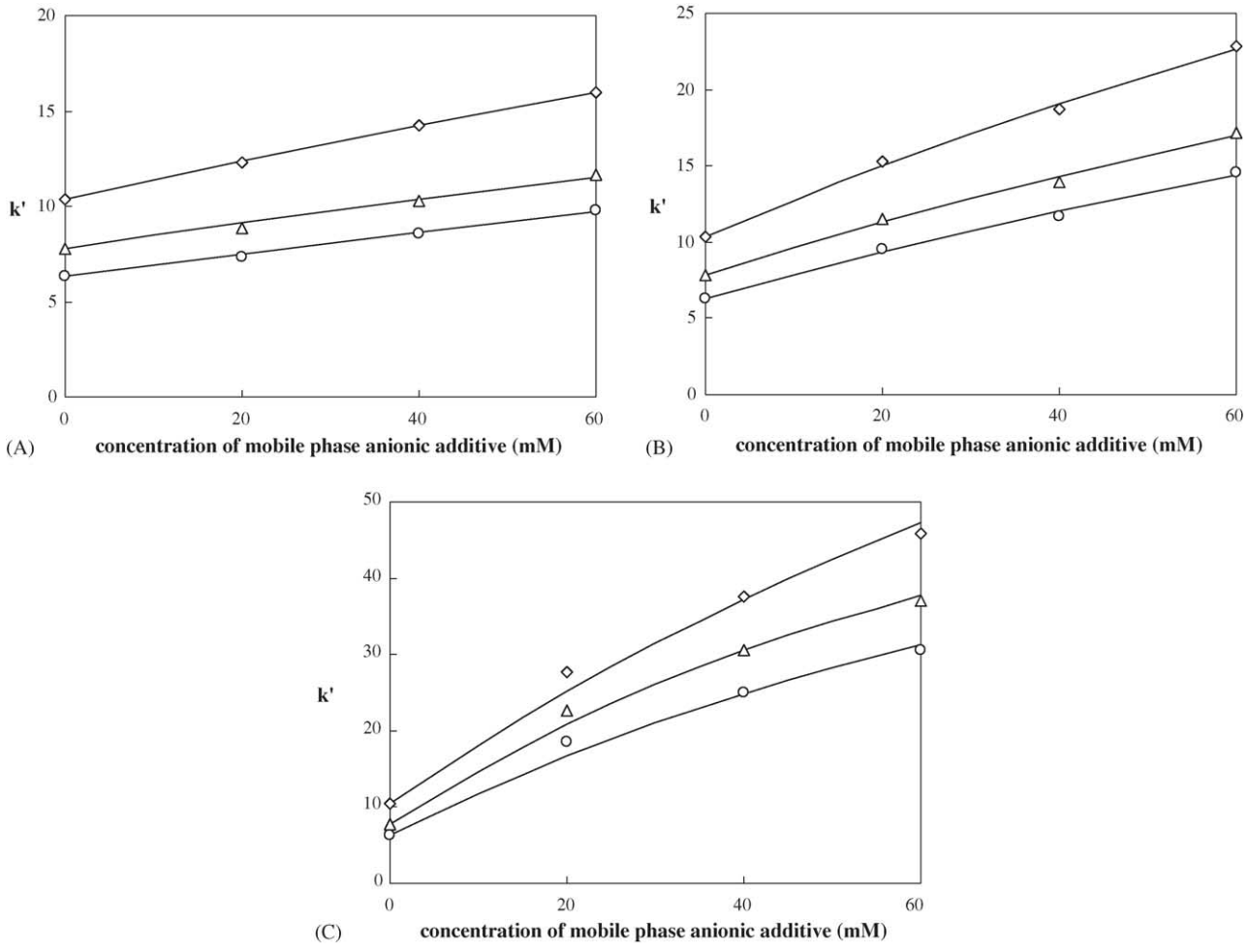

Fig. 10. Comparison of the experimental data with the predicted data based on fitting results in Table 2. Plot legends: lines, predicted data; symbols, experimental data (◯) nortriptyline, (△) amitriptyline, (◊) perphenazine. (A) CF_3COO^-; (B) ClO_4^-; (C) PF_6^-.

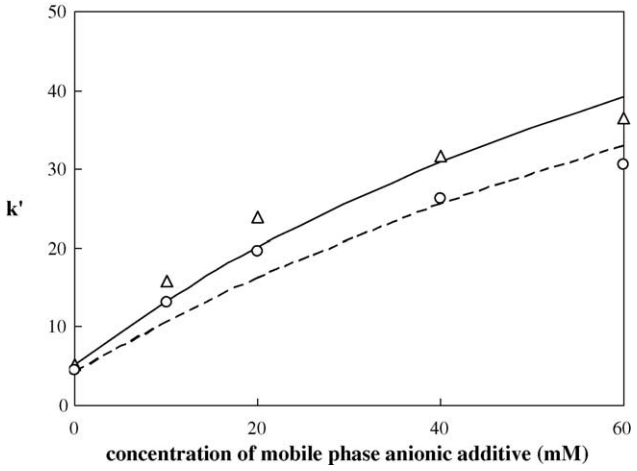

Fig. 11. Comparison of the experimental data with the predicted data based on RPLC fitting results according to the ion pair formation in the mobile phase mechanism at pH 2.8. Fitting results according to Eq. (1) based on k' results from Fig. 9 and K_{ip} from Table 1. Plot legends: lines (predicted data), dotted nortriptyline, solid amitriptyline; symbols (experimental data), (◯) nortriptyline, (△) amitriptyline.

According to Fig. 12C, the sorption isotherm of hexafluorophosphate should be saturated when its concentration is greater than 20 mM. Obviously, sorption of anionic additive alone cannot explain the trend seen in Fig. 9. Although more detailed study especially the determination of the sorption isotherms of the additive anions is needed, we believe that *ion pair formation in the mobile phase and anionic additive sorption induced dynamic ion-exchange are both important processes at pH 2.8.*

The simultaneous contributions from ion pair formation in the mobile phase and anionic additive sorption induced dynamic ion-exchange also explain the poor fitting based on pure ion pair formation model for hexafluorophosphate at pH 2.8 (see Fig. 11). In this case, retention of the cationic drugs follows Eq. (5), not (1).

Our studies clearly demonstrate that the ionic strength simultaneously affects the silanophilic interactions and perturbs the sorption of anionic additive. It is thus of critical importance that one controls the mobile phase ionic strength to get a more precise picture of how each mobile phase condition affects the retention of analytes.

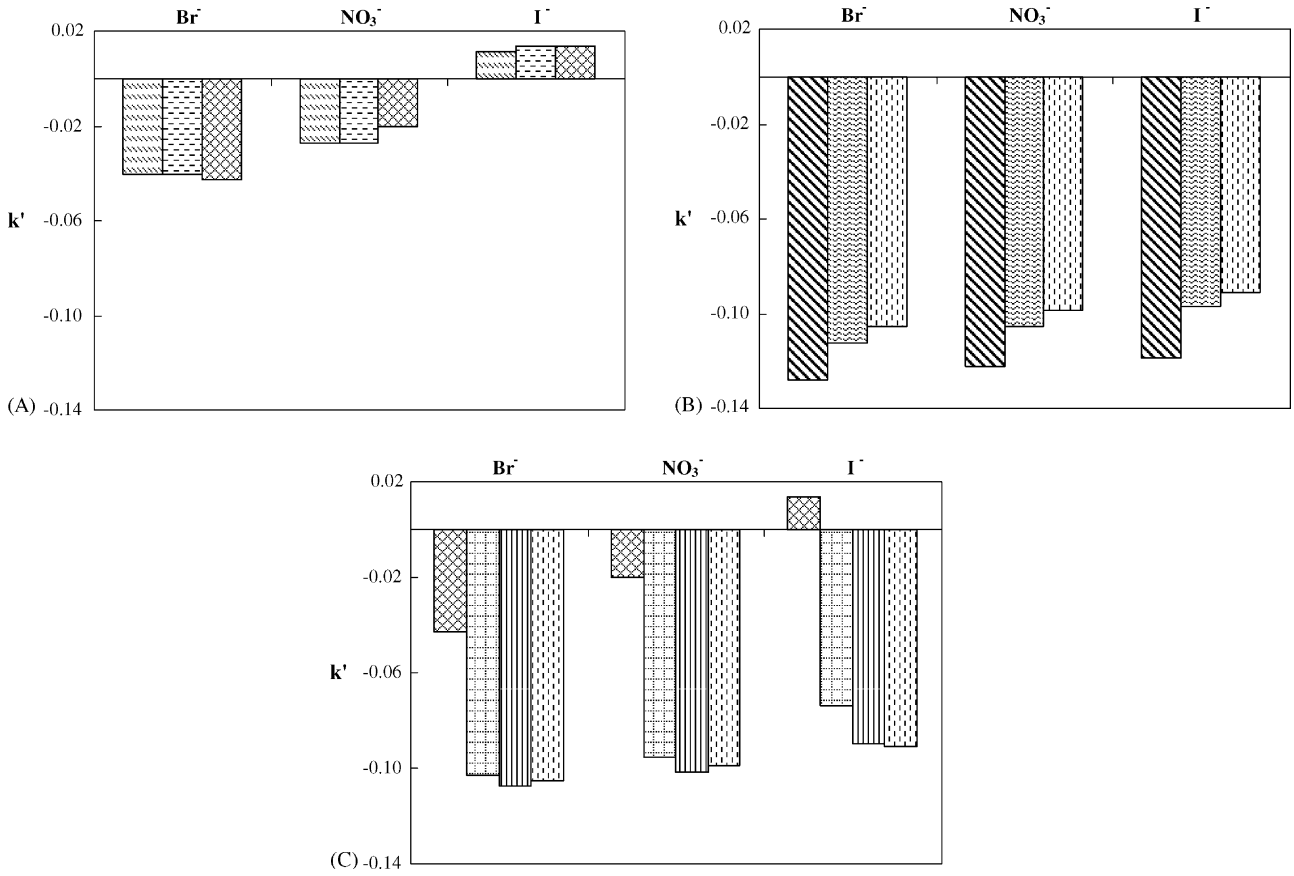

Fig. 12. Effects of anionic additive type and concentration on the exclusion of probe anions. Conditions: 35/65 acetonitrile/buffer, 0.05% formic acid at pH 2.8 and 0–60 mM NaX. (A) Different mobile phase ionic strength for chloride. From left to right, 20 mM Cl^-, 40 mM Cl^-, and 60 mM Cl^-. (B) Different mobile phase ionic strength for hexafluorophosphate. From left to right, 20 mM PF_6^-, 40 mM PF_6^-, 60 mM PF_6^-. (C) Constant ionic strength of 60 mM maintained by sodium chloride (excluding the ionic strength from 0.05% formic acid). From left to right, 0 mM PF_6^-, 20 mM PF_6^-, 40 mM PF_6^-, and 60 mM PF_6^-.

4.4.2. Studies at pH 4.8

Fig. 13 shows the analogous anion exclusion studies at pH 4.8. Even though exclusion decreases as the additive is varied from hexafluorophosphate to chloride, the differences seen with the different additives are rather small compared to the difference in behavior seen above for hexafluorophosphate and chloride at pH 2.8. We believe that at this pH (i.e., pH 4.8) the exclusion of the probe anions is more likely due to ionization of surface silanols rather than to sorption of anionic additives. This result may be peculiar to the Zorbax phase which is a more easily ionized phase as shown in its cation exchange activity measured by Synder's "*C*" parameter [42].

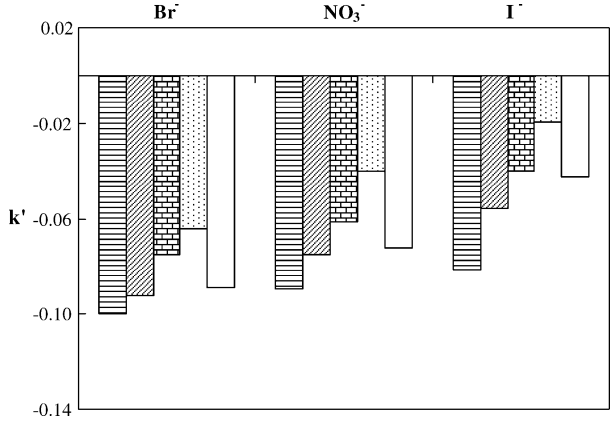

Fig. 13. Effect of the anionic additive type on the exclusion of probe anions. Conditions: 35/65 acetonitrile/buffer, 20 mM sodium acetate buffer at pH 4.8 (buffer only) and 60 mM NaX. Plot legends: from left to right, PF_6^-; ClO_4^-; CF_3COO^-; Cl^-; buffer only.

The effects of anionic additive type on exclusion of probe anions at pH 4.8 and 2.8 are compared in Fig. 14. The effect is clearly greater at pH 2.8 than at pH 4.8.

According to the above discussion, the various anions have different ability in both ion pairing in the mobile phase and sorption into the stationary phase. We believe that the trend in ion pairing ability from chloride to hexafluorophosphate (see Table 1) is due to the considerable difference in their propensity to transfer from water to a nonaqueous solvent [43]. This trend in ion pairing is also consistent with a number of related as well as some seemingly unrelated processes. First, it is in agreement with the observed order of effect of anionic additive type on the retention of cations in RPLC (see Figs. 4 and 7). Second, it is the inverse of the so called "Hofmeister effect" or "salting-out" series which relates the ability of a salt to

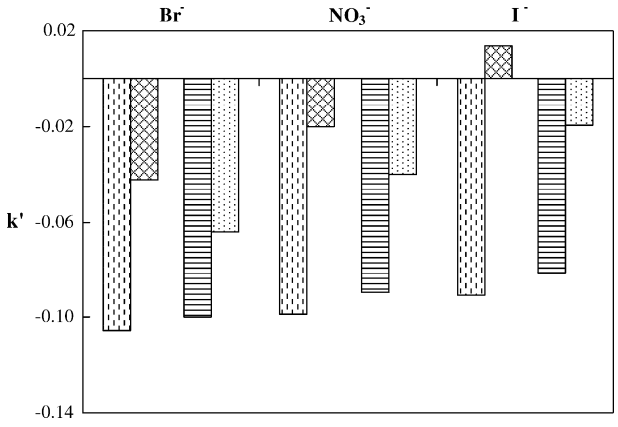

Fig. 14. Comparison of effect of anionic additive type on the exclusion of probe anions at pH 2.8 and 4.8. Conditions: 35/65 acetonitrile/buffer, 0.05% formic acid at pH 2.8 or 20 mM sodium acetate buffer at pH 4.8 and 60 mM NaX. From left to right, 60 mM PF_6^- at pH 2.8, 60 mM Cl^- at pH 2.8, 60 mM PF_6^- at pH 4.8, and 60 mM Cl^- at pH 4.8.

reduce the solubility of proteins and non-polar species in water [12,17]. Third, it agrees with the order of retention of these anions ($ClO_4^- > BF_4^- > CF_3COO^- > Cl^- > H_2PO_4^-$) on anion exchange materials [44]. Fourth, it is consistent with the sequence of anionic interference effects in liquid membrane anion selective electrodes ($ClO_4^- > BF_4^- > NO_3^- > Cl^- > HCOO^- > H_2PO_4^-$) [45]. Last, it agrees with the order of the anions in promoting *ion pair extraction* of basic compounds out of water into nonpolar solvents ($ClO_4^- > Br^- > NO_3^- > Cl^-$) [46–49]. We point out these parallel trends because it is all too easy to detect the common trend in the effect of anions in chromatography and ion pair formation and jump to the conclusion that ion pair formation is responsible for the chromatography when the root is really a more fundamental property shared by the potentially unrelated phenomena. *In this case it is the strength of hydration of the anion by water that is the governing phenomena which controls both the anions ability to ion pair* (*as seen in the trend of* K_{ip}) *and its ability to sorb into a less polar environment* (*as seen in the trend of* k'_{max}). As we discussed in the introduction, we believe that the relatively hydrophobic anions, such as hexafluorophosphate and perchlorate, form ion pair more easily and also tend to sorb more readily into the stationary phase because such anions desolvate more easily than do anions like chloride and phosphate which are so strongly hydrated.

4.5. Effect of anionic additive type on the separation of neutral compounds

The effect of anionic additive concentration on the retention of acetophenone is shown in Fig. 15. Although the change in the retention factor is very small compared to that of cations, it is clear that various anion additives affect the retention of neutral compounds quite differently. Except for chloride, increasing the concentration of all other additives tends to slightly diminish the retention of acetophenone, that is, all but chloride cause an increase in water solubility; they "salt-in" the neutral analyte whereas chloride causes a minor amount of "salting-out". The results on benzene are the same as for acetophenone. Similar decreases in retention for neutral compounds were observed before [7,8].

Two explanations can be provided for this phenomenon. The first is that the sorbed anionic additives reduces the hydrophobicity of the stationary phase [8]. However, this theory cannot explain the effect of chloride since it is in the opposite direction. Also, as we discussed, there is barely any sorption of these anionic additives to the stationary phase at pH 4.8. The second interpretation, which we believe is correct, comes from changes in the mobile phase [10,17,48,50]. That is, anionic additives such as perchlorate and hexafluorophosphate tend to break the hydrogen bonding structure of the mobile phase (i.e., they are chaotropic) and decrease the retention of neutral compounds; while anions such as chloride are structure-making which tend to increase the hydrogen

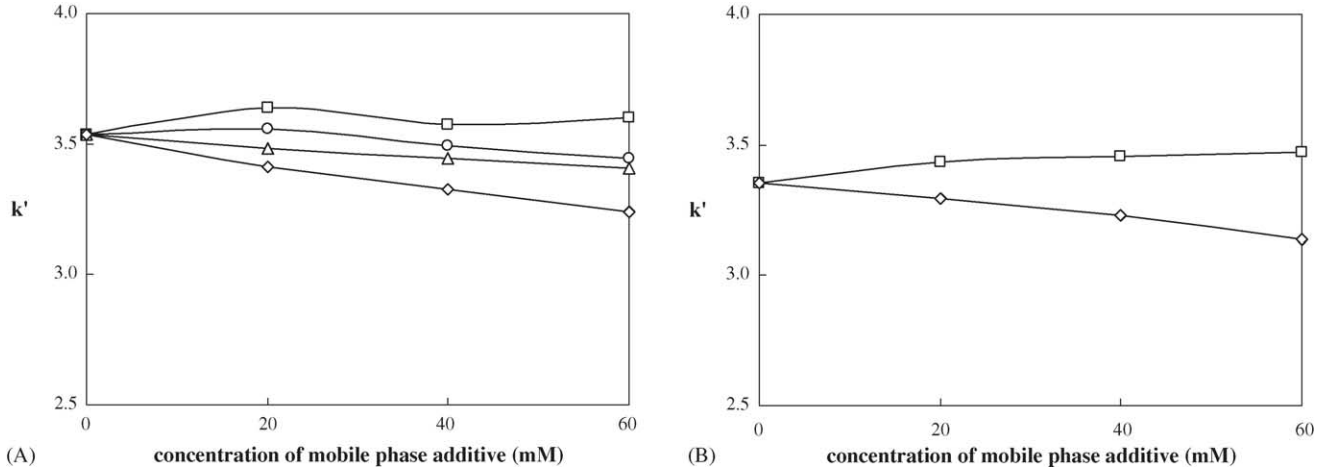

Fig. 15. Effects of anionic additive type and concentration on the retention of acetophenone. Plot legends: (◊) PF_6, (△) ClO_4^-, (◯) CF_3COO^-, (□) Cl^-. (A) pH 4.8, conditions same as Fig. 5; (B) pH 2.8, conditions same as Fig. 8.

bonding structure of mobile phase (i.e., chloride exhibits the "salting-out" effect) and increase the retention of neutral analyte. As seen from Fig. 15, the so called "salting-out" or "chaotropic" effect is a rather small for these small nonpolar solutes.

5. Conclusions

We have noted the following trends:

(1) The nature of the anionic additive has a very significant effect on the retention of cationic drugs at both pH 4.8 and 2.8. The retention of basic drugs in the presence of anionic additives follows the order: $Cl^- < CF_3COO^- < ClO_4^- < PF_6$.
(2) Ion pair formation constants as measured by CE and the retention factor of bases as measured by RPLC show the same trend: $Cl^- < CF_3COO^- < ClO_4^- < PF_6^-$.
(3) Excellent fitting of RPLC retention data based on K_{ip} values measured by CE is obtained at pH 4.8, but not at pH 2.8.
(4) The exclusion of anionic probes at pH 4.8 scarcely varies with the type of additive. At pH 2.8, the type of anionic additive has a considerable effect on the exclusion of anionic probes.
(5) The concentration of added salts affects the retention of basic analytes through its impact on silanophilic (Coulombic) interactions and by chemically specific anionic additive effects.
(6) Quite different behavior of the basic drugs as a function of the concentration of sodium chloride was observed at pH 4.8 and 2.8 due to the change in the degree of ionization of the surface silanols for the Zorbax phase used in this study.
(7) Anionic additives have only very small effects on retention of neutral analytes. However, sodium chloride increases the retention of neutrals and all other sodium salts tested (CF_3COO^-, ClO_4^-, and PF_6^-) decreased retention.

Our observations lead to several important conclusions.

At pH 4.8, the excellent fitting of the RPLC retention data by the K_{ip} values measured by CE, the small effect of additive types on anion exclusion, and the small amount of additive anion sorption due to the presence of ionized silanol groups lead us to believe that the ion pair formation retention mechanism dominates at this pH. These results provide a solid clarification for the retention processes that are involved. Ion pair formation with chloride ion takes place, which is consistent with the results we obtained from CE experiments, but it is very weak and is scarcely stronger than a non-specific general electrostatic effect. In contrast, trifluoroacetate, perchlorate, and hexafluorophosphate form stronger ion pairs in the order given.

Due to the change in the silanophilicity (i.e., the ionization of surface silanols), the situation at pH 2.8 is rather different for the relatively hydrophobic inorganic anions (i.e., hexafluorophosphate). The electrostatic exclusion of anions by ionized silanol groups becomes much less at pH 2.8 and the type of anionic additive has a considerable effect on anion exclusion. In addition, the pure ion pair formation mechanism does not fit the data well at pH 2.8. All three observations support our contention that *at pH 2.8 the sorption of the additive anions and the concomitant dynamic ion-exchange process must be considered along with the probably somewhat more important ion pair formation in the mobile phase mechanism*.

At constant ionic strength, the fact that the exclusion of anion probes remains unchanged (when concentration of hexafluorophosphate is greater than 20 mM) and the retention of cations continues to increase as the concentration of hexafluorophosphate is increased clearly demonstrate that *ion pair formation in the mobile phase must also contribute to the retention of cationic drugs at pH 2.8*.

However, for the relatively hydrophilic inorganic anions (e.g., chloride), ion pair formation in the mobile phase remains the principal retention mechanism. The insignificance of anion exclusion (e.g., iodide is slightly retained) at pH 2.8 when chloride is used as the anionic additive indicates that the sorption of chloride on the stationary phase is virtually nil.

The so called "salting-out" or "chaotropic" effect cannot explain the above anionic additive effects. We believe that the strength of solvation of the anion by water (i.e., energy of hydration for the anion) is the governing phenomenon which controls both the anion's ability to ion pair and its ability to sorb into a less polar environment.

The amount of ion pairing in the mobile phase is not very big, which is reasonable considering the high percentage of aqueous mobile phase used. The extent of ion pairing in pure organic solvents is much greater than in water. Given the small fraction of any cation that is actually present as an ion pair, we feel that one can neglect the effect of such interactions in considering cation displacement studies. That is, a given cation is just as effective as an ionized silanol group "blocking agent" in chloride as in perchlorate media.

Ionic strength and chemically salt concentration effects play a very important role in the separation of cationic drugs in RPLC. Faulty control in a series of experiments can cause considerable confusion and mislead one as to the relative importance of the ion-exchange and ion pair formation processes. Maintaining ionic strength constant, which has not been done before in the study of the effect of small hydrophilic anionic additives on retention in RPLC, is critical to get an accurate picture of how each mobile phase condition affects the retention of analytes.

As clearly shown, at pH conditions as low as 2.8, there are still some silanophilic interactions with ionized silanol groups on certain types of even high quality (type-B) silica, specifically the SB C_{18} column we used in this study. Our model equation, which is the first to simultaneously treat both ion pairing and silanophilic interactions, clearly shows that the competitive effects from these two processes can be

very misleading. That is, the apparent lack of dependence of retention on the added salt concentration could lead one to conclude that there is neither ion pairing nor silanophilic interaction when, in fact, both are taking place in opposing directions.

As shown above, our focus is on understanding and clarifying the role of ion pairing in the retention process of basic drugs in RPLC. In a subsequent paper, we will discuss the effect of anionic additives on selectivity and peak broadening of basic drugs.

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