Effect of anionic additive type on ion pair formation constants of basic pharmaceuticals

Jun Dai [a], Shaun D. Mendonsa [a], Michael T. Bowser [a], Charles A. Lucy [b], Peter W. Carr [a],*

[a] *Department of Chemistry, University of Minnesota, 207 Pleasant Street SE, Minneapolis, MN 55455-0431, USA*
[b] *Department of Chemistry, Gunning/Lemieux Chemistry Centre, University of Alberta, Edmonton, Alta., Canada T6G 2G2*

Abstract

Due to their beneficial effect on selectivity, peak shape, and sample loading, the use of mobile phase anionic additives, such as formate ($HCOO^-$), chloride (Cl^-), and trifluoroacetate (CF_3COO^-), is increasing in both reversed-phase chromatography (RPLC) and liquid chromatography-mass spectrometry (LC/MS). Similarly, perchlorate is a common "ion pair" agent in reversed-phase separation of peptides. Although many studies have suggested that anions effect in chromatography is due to the formation of ion pairs in the mobile phase between the anions and cationic analytes, there has been no independent verification that ion pairs are, in fact, responsible for these observations. In order to understand the mechanisms by which anionic additives influence retention in chromatography and ionization efficiency in electrospray mass spectrometry, we studied the formation of ion pairs between a number of prototypical basic drugs and various additives by measuring the effect of anionic additives on the electrophoretic mobility of the probe drugs under solvent conditions commonly used in chromatography. For the first time, ion pair formation between basic drugs and anionic additives under conditions commonly used in reversed-phase liquid chromatography has been confirmed independently with all anions (i.e. hexafluorophosphate, perchlorate, trifluoroacetate, and chloride) used in this study. We measured ion pair formation constants (K_{ip}) for different anionic additives using capillary electrophoresis (CE) and obtained quantitative estimates for the extent of ion pairing in buffered acetonitrile–water. The data clearly indicate that different anionic additives ion pair with cationic drugs to quite different extents. The ion pair formation constants show a clear trend with the order being: $PF_6^- > ClO_4^- > CF_3COO^- > Cl^-$. However, the extent of ion pairing is not large. At a typical RPLC mobile phase additive concentration of 20 mM, the percentages of the analytes that are present as ion pairs are about 15%, 6%, and 3% for hexafluorophosphate, perchlorate, and trifluoroacetate, respectively. The fraction of the analytes present as a chloride pair is even smaller.
© 2005 Elsevier B.V. All rights reserved.

Keywords: RPLC; LC/MS; Basic pharmaceuticals; Anionic additive; Ion pair formation constant

1. Introduction

1.1. Effect of anionic additive on separation of basic compounds in RPLC and LC/MS

Due to the presence of ionizable silanol groups on silica based stationary phases, mobile phase additives are often used to reduce peak tailing and achieve better resolution in the separation of basic compounds [1,2]. The use of anionic additives, such as formate, chloride, trifluoroacetate, and perchlorate are well known for improving the separation of basic drugs, peptides, and proteins [3–12].

Roberts et al. [10] have demonstrated that anionic additives can significantly affect the retention of basic drugs in RPLC. The retention of bases in the presence of anions follows the order: $H_2PO_4^- < HCOO^- < CH_3SO_3^- < Cl^- < NO_3^- < CF_3COO^- < BF_4^- < ClO_4^- < PF_6^-$ and is consistent with the "Hofmeister Effect" (i.e. the anion's ability to cause "salting-in" and "salting-out") [13,14].

* Corresponding author.
   E-mail address: carr@chem.umn.edu (P.W. Carr).

LoBrutto and coworkers [7–9] studied the effect of both pH and the concentration of different anionic additives on the retention of small basic drugs. The effects of "chaotropic" anionic additives, such as perchlorate and trifluoroacetate, were attributed to the desolvation of the cationic analytes with water and a consequent enhancement in their hydrophobicity [8]. A somewhat vaguely defined "ion association" model was proposed to rationalize their experimental data [7,9].

In addition to their effect on retention and selectivity, other anionic additive effects in RPLC have been reported. McCalley [15] has shown that the nature of the buffer can affect both the peak shape and plate count. Gritti and Guiochon [16–20] recently demonstrated effects of concentration and type of buffer on the adsorption isotherms and overload band profiles of cationic analytes. Ion pair formation or ion-associated complexation between the cationic analytes and the anionic additives in the mobile phase was invoked to explain the experimental observations.

The effect of anionic additives on electrospray ionization efficiency in mass spectrometry is well known [21]. Mirza and Chalt [22] studied the anion effect on electrospray ionization of peptides and proteins. The decrease in the net average charge of peptide and protein ions in the presence of the various anions was shown to follow the order $CCl_3COO^- > CF_3COO^- > CH_3COO^- \approx Cl^-$. Ion pair formation in solution and ion suppression in the gas phase were proposed as the mechanisms behind the observations. The results conclusively demonstrated that the appropriate selection of eluent anion is very important for the optimization of electrospray mass spectrometry.

Similar studies were performed by Huber and Premstaller [23] on the effect of different anionic additives on protein analysis by LC/MS. They showed that formic acid gives better signal detectability than trifluoroacetate due to the suppression of gas phase ion formation by trifluoroacetate.

As discussed above, the much postulated "ion pairing" effects are very important and have attracted considerable attention in the separation of both pharmaceutically and biologically interesting analytes. Even though it is well recognized that the anionic additives have a significant effect on the retention and peak shape of analytes, especially cationic analytes, the retention mechanism of analytes in the presence of mobile phase additives, such as those mentioned above is not at all clear. Several mechanisms, including both ion pair formation in the mobile phase and dynamic ion exchange in the stationary phase, have been proposed to explain anion effects on retention [3,5,6,24,25]. Although certain types of interactions between the analytes and anionic additives (e.g. ion pair formation, "ion association", or solvent desolvation caused by anionic additives) have been suggested as possible mechanisms for the effect of small, relatively hydrophilic additives on the separation of basic drugs, to our knowledge there has been no direct confirmation of ion pair formation. No independent experiments have been done to interpret the chromatographic data. There is a great need for independent experimentation

to confirm our understanding of the mechanism by which anionic additives act to alter the retention of bases in RPLC.

1.2. Measurement of ion pair formation constants by CE

In CE, the electrophoretic mobility (μ) of a charged species can be monitored by means of the following equation:

$$\mu = \frac{L_d L_t}{V}\left(\frac{1}{t_m} - \frac{1}{t_{eof}}\right) \tag{1}$$

where t_m and t_{eof} are the migration times of the analyte and the electroosmotic flow (EOF) marker, L_t the total length of the capillary, L_d the detection length, and V the total voltage applied across the capillary.

When a univalent anion present in the running buffer forms a complex with a univalent cation, the mobility of the cation decreases upon increasing the anion concentration. The type of additive and its concentration on mobility has been discussed and applied to the measurement of formation constants [26–30].

In the case of the formation of a neutral ion pair with 1:1 stoichiometry between the analyte and the additive, the ion pair formed between the analyte and the ion pairing agent has no charge

$$A^+ + X^- \overset{K_{ip}}{\rightleftharpoons} (A^+ : X^-) \tag{2}$$

$$\alpha_{free} = \frac{[A^+]}{[A^+] + [A^+ : X^-]} = \frac{1}{1 + K_{ip}[X^-]} \tag{3}$$

$$\alpha_{ion\,pair} = 1 - \alpha_{free} = \frac{K_{ip}[X^-]}{1 + K_{ip}[X^-]} \tag{4}$$

where K_{ip} is the ion pair formation constant between the analyte A^+ and anionic additive X^- in the running buffer, α_{free} the fraction of free base which has not been ion paired, and $\alpha_{ion\,pair}$ the fraction of the ion paired base.

Since the ion pairs are neutral, they migrate with the EOF. The net electrophoretic mobility only comes from the fraction of the base that has not formed ion pairs.

$$\mu = \mu^0 \alpha_{free} = \mu^0 \frac{1}{1 + K_{ip}[X^-]} \tag{5}$$

$$\frac{\mu^0}{\mu} = 1 + K_{ip}[X^-] \tag{6}$$

where the superscript 0 refers to the mobility in the absence of the ion pairing additive, and μ the electrophoretic mobility in the presence of additive at a concentration of $[X^-]$.

A plot of the relative mobility (μ^0/μ) as a function of concentration of additive $[X^-]$ based on Eq. (6) is linear, and thus, we are able to obtain the ion pair formation constant from the slope. Obviously, mobility decreases as the concentration of the ion pair agent is increased. Unfortunately, data analysis is greatly complicated by the fact that even in the absence of any direct chemical effect (i.e. ion pair formation),

the mobility of an ion is perturbed by a general non-chemical electrostatic interaction.

According to Onsager's treatment, which is related to the Debye–Hückel model of ionic interaction, the mobility of a charged species should decrease linearly as a function of the square root of the ionic strength [31,32]:

$$\mu \approx \mu_0 - \left[\frac{1.4 \times 10^6 \, |z_+ z_-|}{(\varepsilon T)^{3/2}} \frac{2g}{1 + \sqrt{g}} \mu_0 \right. \\ \left. + \frac{41.25}{\eta (\varepsilon T)^{1/2} F} \right] \sqrt{I} \tag{7}$$

where the subscript 0 denotes the analyte's mobility at infinite dilution, ε medium's dielectric constant, T the system temperature, z_+ the magnitude of the charge on the cation, z_- the charge of the anion, η the running buffer's viscosity, g an electrolyte parameter, F Faraday's constant, and I the ionic strength.

For uni-univalent electrolytes, g in Eq. (7) is equal to 0.5. The slope of mobility versus the square root of ionic strength is called the Onsager limiting slope. In the Debye–Hückel–Onsager model, i.e. Eq. (7), the ion is considered to be a point charge of zero-size. However, studies have shown that this simplification is in error when ionic strengths exceed a few millimolar [31,33]. Nonlinearities were observed for multiply charged ions and at higher ionic strengths. That is, the Debye–Hückel–Onsager model is valid only for very dilute solutions. The finite size of real ions cannot be ignored.

It has been demonstrated that the mobility as a function of the ionic strength can be estimated from Pitts' equation, which accounts for the finite size of the ion [31,33,34]. For uni-univalent electrolytes, Pitts' equation can be written as:

$$\mu \approx \mu_0 - \left[\frac{1.4 \times 10^6}{(\varepsilon T)^{3/2}} \frac{2g}{1 + \sqrt{g}} \mu_0 + \frac{41.25}{\eta (\varepsilon T)^{1/2} F} \right] \\ \times \frac{\sqrt{I}}{1 + Ba\sqrt{I}} \tag{8}$$

$$B = \left(\frac{8 \pi N_A e^2}{1000 \varepsilon k_B T} \right)^{1/2} \tag{9}$$

where a is the ion size parameter, N_A the Avogadro's number, k_B the Boltzmann constant, and e the electron charge.

Roy and Lucy [35,36] studied the effect of ionic strength on ionic mobility at different percentages of organic modifier in both acetonitrile–water and methanol–water systems. According to their studies, the effect of ionic strength on mobility increases as the amount of organic modifier in the buffer is increased. The effect is also solute dependent.

In addition to its direct effect on mobility, ionic strength also influences the ion pair formation constant. The ion pair formation constant at a given ionic strength can be expressed as [37]:

$$K_{ip} = K_{ip}^0 \frac{\gamma_{A^+} \gamma_{X^-}}{\gamma_{AX}} \tag{10}$$

where K_{ip}^0 is the ion pair formation constant at zero ionic strength, and γ_{AX}, γ_{A^+}, γ_{X^-} the activity coefficients of the ion pair, analyte, and additive, respectively.

According to the Debye–Hückel equation, the activity coefficient in reasonably dilute solutions can be written as [33]:

$$-\log \gamma_i = \frac{A z_i^2 \sqrt{I}}{1 + Ba\sqrt{I}} \tag{11}$$

$$A = \left\{ \frac{2 \pi N_A}{1000 (\varepsilon k_B T)^3} \right\}^{1/2} \left(\frac{e^3}{2.303} \right) \tag{12}$$

where B is defined by Eq. (9), a as in Eq. (8), and z_i the magnitude of charge on the ion of interest.

For 1:1 stoichiometric ion pair formation with a uni-univalent analyte and additive, z_{AX} equals zero and $z_{A^+}^2$ ($z_{X^-}^2$) equals 1. If we assume the same ion size parameter for all ions (i.e. a in Eq. (11) is the same for all species), Eq. (10) can be rewritten as:

$$\log K_{ip} = \log K_{ip}^0 - \frac{2A\sqrt{I}}{1 + Ba\sqrt{I}} \tag{13}$$

According to the above discussion, it is clear that maintaining a constant ionic strength during any study of the effect of ion pairing on mobility is essential.

2. Experimental

2.1. Capillary electrophoresis

All CE experiments were performed with a P/ACE MDQ capillary electrophoresis system (Beckman Coulter Inc., Fullerton, CA) equipped with a UV-absorbance detector. The data acquisition and processing were controlled by 32 Karat software from Beckman. Fused-silica capillaries with an outside diameter of 363 μm and an internal diameter of 52 μm were obtained from Polymicro Technologies (Phoenix, AZ). UV-absorbance detection was set at 254 nm. The capillary was thermostated to 25 °C, and the absence of significant Joule heating was confirmed experimentally.

2.2. Reagents

All chemicals were reagent grade or better. Cationic drugs were purchased from Sigma (Sigma Corp., St. Louis, MO). HPLC grade acetonitrile was from Burdick & Jackson (Muskegon, MI). HPLC water was obtained from a Barnsted Nanopure deionizing system (Dubuque, IA) 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 Corp., Kenosha, WI) before use. Other chemicals

used in this study were purchased from Aldrich (Aldrich Chemical Co., Milwaukee, WI).

2.3. Procedure

The buffers at pH 4.8 were prepared from acetic acid and sodium acetate. The buffers at pH 2.3 were prepared from 5 mM hydrochloric acid. Running buffers with different anionic additives were prepared by adding the sodium salts of each anion to the buffer. Sodium chloride was used to adjust the ionic strength for some of the experiments. The concentrations of buffers and sodium salts were reported with respect to the volume of the aqueous-organic mixture. The samples were prepared at about 0.05 M in 35/65 acetonitrile–water and then diluted to about 10^{-4} M with the corresponding running buffers. Acetophenone was used as the neutral marker.

The total length of the capillary for experiments at pH 4.8 is 32 cm, with a length to the detector of 10 cm. The capillary for experiments at pH 2.3 had a total length of 60 cm and a length to detector of 50 cm. Each end of the capillary was burned approximately 0.5 cm to remove the polyimide coating to avoid detachment of the polymer which happens upon exposure to buffers containing organics [36,38]. Freshly made capillaries were conditioned using the following rinses (15 psi): 0.10 M sodium hydroxide (30 min), followed by water (30 min), and then the running buffer (30 min). The capillary was conditioned in the buffer for 10 min at a voltage of 10 kV before the first run of the day. Between each run, the following rinses were performed (20 psi): 0.1 M sodium hydroxide (2 min), followed by water (2 min), and then by running buffer (2 min). An open beaker of the same composition as the running buffer was put inside the instrument compartment to saturate the atmosphere to suppress evaporation of the running buffer [36].

Separations at pH 4.8 were performed at a voltage of 10 kV with the short end of the capillary (10 cm) as the injection side. Samples were injected by pressure at 0.2 psi for 4 s. During the separation, a pressure of 100 psi was applied to both ends of the capillary to avoid bubble formation [39].

Determination of the electrophoretic mobility at pH 2.3 was performed according to the method of Williams and Vigh [40] to overcome the slow EOF problem. First, a mixture of analytes and acetophenone was injected into the capillary at 0.6 psi for 4 s. Second, the sample band was pushed along the capillary by applying 1.0 psi for 3.5 min. Subsequently, a voltage of 10 kV was applied for 2.0 to 4.0 min, depending on the mobility of the analyte. After the voltage separation, acetophenone was injected again at 0.6 psi for 4 s. Finally, the three sample bands were pushed past the detector by a pressure of 1.0 psi for 9.0 min. The mobility was calculated using the following equation:

$$\mu = \frac{(t_{N1} - t_A)L_d L_t}{V(t_{N2} + 0.5t_{in} - t_d)(t_{migr} - 0.5t_{rampup} - 0.5t_{rampdown})} \quad (14)$$

where L_t and L_d are the total and detection length of the capillary; V the voltage applied across the capillary during the voltage separation step; t_A, t_{N1}, and t_{N2} the migration times of the analyte, the first neutral marker, and the second neutral marker; t_{migr} the time during which V is applied; t_{in} the injection time; t_d the experimentally determined delay time (5.3 s); t_{rampup} and $t_{rampdown}$ (both 0.17 min) the time required for changing voltage from 0 to V.

3. Results and discussion

3.1. Effect of ionic strength on the mobility of a charged species

As discussed, increases in ionic strength and ion pair formation strength both lead to a decrease in mobility. The effects of ionic strength on the mobility of basic analytes (see Fig. 1 for structures) are shown in Fig. 2. A theoretical line based on Pitts' equation is also included. The coefficients and solvent parameters in Eq. (8) used for acetonitrile–water (35/65, v/v) are [36,41]: viscosity 0.92 centipoises, dielectric constant 63.0, Ba 2.65, and μ_0 in theoretical line is calculated using μ at 20 mM sodium acetate buffer without any additive. In dilute solutions, to a good approximation, we can assume that η is constant at different ionic strengths.

Li et al. [31] and Lucy and Roy [36] have optimized the value of Ba in Eq. (8) and successfully used 2.4 in aqueous solution for both anions and amines. The values of both B and a are solvent dependent. A value of 2.65 is used here for Ba taking into consideration of the amount of acetonitrile used. We assumed that a was the same as in Li's work.

As seen from Fig. 2, the rate of decrease in mobility as a function of ionic strength differs considerably from hexafluorophosphate to chloride. The anions
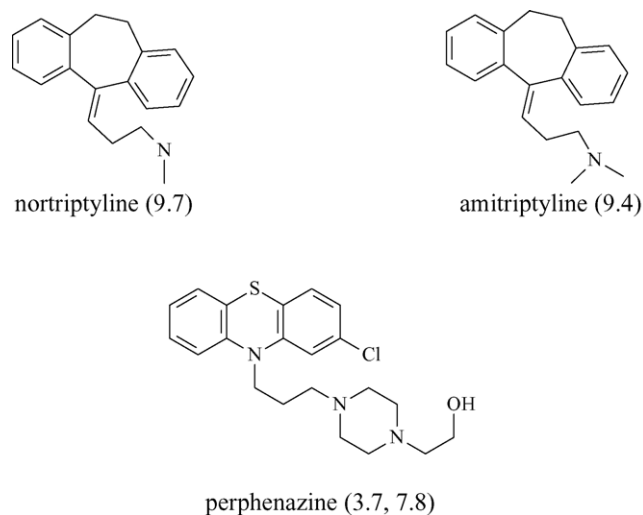

nortriptyline (9.7) amitriptyline (9.4)

perphenazine (3.7, 7.8)
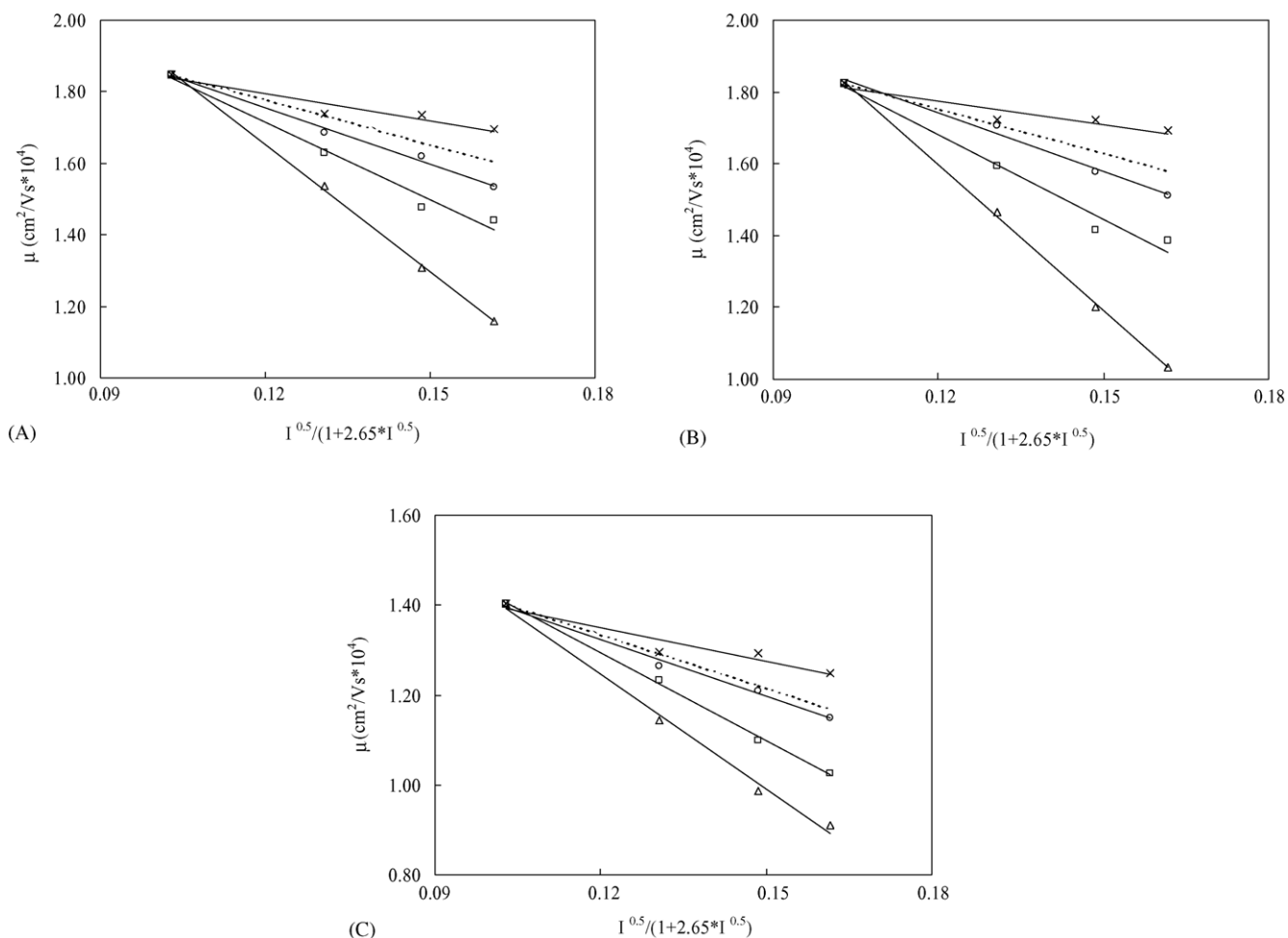
Fig. 1. Structures and pK_as of the basic drugs.

Fig. 2. Effect of ionic strength and type of additive on mobility based on the Pitts' equation. Conditions: 35/65 acetonitrile/buffer, 20 mM sodium acetate buffer at pH 4.8, and 0 to 60 mM NaX ($X^- = PF_6^-$, ClO_4^-, CF_3COO^-, Cl^-). Plot legends: ($\triangle$) PF_6^-; ($\square$) ClO_4^-; ($\bigcirc$) CF_3COO^-; ($\times$) Cl^-; dotted line, theoretical prediction (based on Pitts' equation with $\eta = 0.92$ cP, $\varepsilon = 63.0$, $Ba = 2.65$, and where μ_0 is calculated from μ at 20 mM sodium acetate buffer without any additive. We assume that perphenazine is singly charged). (A) nortriptyline; (B) amitriptyline; (C) perphenazine.

tested show a clear trend for their effect on mobility: $PF_6^- > ClO_4^- > CF_3COO^- > Cl^-$. The greater rate of decrease in mobility for hexafluorophosphate, perchlorate and trifluoroacetate cannot be explained by the general dependence of mobility on ionic strength as described in Eq. (8) since the ionic strength is the same for all four salts. This indicates that ion pairing must be taking place with some of the salts.

Previously, Roy and Lucy [34–36] had assumed that the Pitts' plots would be nonlinear if ion pairing were present. However, even in the case of hexafluorophosphate, where ion pairing is strongest, linear behavior is still observed (Fig. 2). Thus, linearity alone cannot be used as a criterion for the absence of ion pairing. Rather, differences in the slope in Fig. 2 from that predicted by the Pitts' equation are better indicators.

However, we do observe that the decrease in mobility when sodium chloride was used is very small; surprisingly it is even weaker than that predicted by the Pitts' equation. Since we worked at pH 4.8, it is possible that basic drugs

weakly adsorb on the capillary wall [42–44], and thus, as salt is added the competition between the cation of the salt and the basic drug decreases the fraction of drug adsorbed at any instant, thereby, increasing its mobility. It is also possible that we have underestimated the ion size parameter (a) to be used for basic drugs. However, only absurdly large values of a, about 15 Å, make the theoretical slope less than the slope in chloride media. Our best guess is that a wall effect is the chief issue. Based on Fig. 2, we cannot decide whether there is ion pairing between the drugs and chloride. Nevertheless, Fig. 2 demonstrates that even if there is ion pairing for chloride ion, it is very small.

According to our chromatographic studies, chloride does show a very weak ion pairing ability with the cationic drugs used in current study [45].

To definitively eliminate the wall effect and help explain the RPLC results in chloride media [45], we repeated the chloride ion CE experiment at pH 2.3. At this pH, the wall effect is minimized. 5 mM hydrochloric acid was used as the low pH buffer [46].
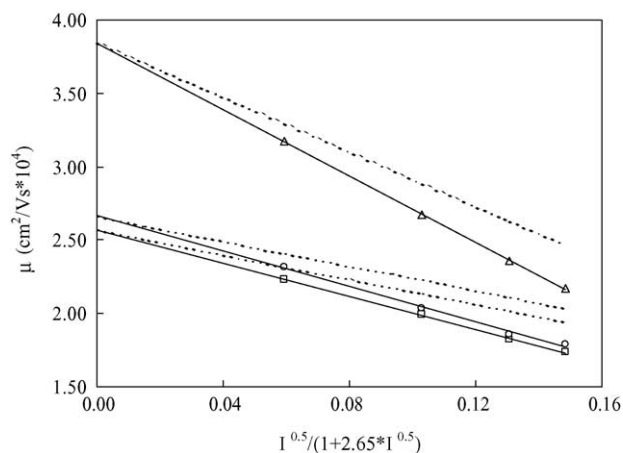
Fig. 3. Effect of ionic strength on mobility based on the Pitts' equation. Conditions: 35/65 acetonitrile/buffer, 5.0 mM hydrochloric acid at pH 2.3, and 0 to 55 mM NaCl. Plot legends: (○), nortriptyline; (□) amitriptyline; (△) perphenazine; dotted lines, theoretical predictions (based on Pitts' equation with $\eta = 0.92$ cP, $\varepsilon = 63.0$, $Ba = 2.65$, and where μ_0 is obtained from the intercept of the experimental lines. We assume that perphenazine is doubly charged).

Fig. 3 shows the effect of concentration of chloride ion on the mobility of basic drugs. Theoretical lines based on Pitts' equation are included. As seen in Fig. 3, the decrease of mobility as a function of the salt concentration is greater than the predictions by Pitts' equation for all three drugs, confirming the formation of ion pairs between the analytes and chloride ions.

Table 1 compares the mobility of basic drugs at pH 2.3 and 4.8. As the salt concentration increases, the effect of pH on mobility decreases, which is a result of the blocking of silanol groups by the higher salt concentration [47]. We want to point out that perphenazine, a diamine with pK_as of 3.7 and 7.8, has significantly higher mobility at pH 2.3 compared to the value at pH 4.8. We believe that this is a result of the change in its charge state from one to two. As can be seen from Table 1, at an ionic strength of 60 mM, the difference in the

Table 1
Effect of ionic strength and running buffer pH on mobility

Analyte/pH/mobility[a]		Ionic strength		
		20 mM	40 mM	60 mM
Nortriptyline	2.3[b]	2.034	1.858	1.793
	4.8[c]	1.849	1.740	1.734
	Ratio[d]	1.10	1.07	1.03
Amitriptyline	2.3	1.994	1.822	1.737
	4.8	1.824	1.722	1.721
	Ratio	1.09	1.06	1.01
Perphenazine	2.3	2.676	2.362	2.171
	4.8	1.403	1.295	1.294
	Ratio	1.91	1.82	1.68

[a] Unit of mobility is cm²/Vs × 10⁴.
[b] CE running conditions same as Fig. 3.
[c] CE running conditions same as chloride in Fig. 2.
[d] Ratio of mobility at pH 2.3 vs. the value at pH 4.8.

mobility at these two pHs is less than 5%, which indicates the effectiveness of blocking "wall effect" by addition of salts.

Based on Eq. (10), the ion pair formation constant must also be affected by the ionic strength. Under our conditions of 35/65 acetonitrile–water mixture, the change in ion pair formation constant as the ionic strength is varied from 20 mM to 80 mM is about 15%. Although this is not a big effect, it can be important.

To suppress any inadvertent effect of ionic strength on mobility, we used sodium chloride to maintain the ionic strength constant. As seen in Fig. 2, chloride does not have a strong ion pairing effect. Also, since hydrochloric acid is a strong acid, chloride does not alter the pH of the solvent. A total ionic strength of 80 mM was used to limit possible wall adsorption.

3.2. Effect of type of anionic additives on ion pair formation constants

Since CE only involves interactions in the fluid phase, we believe that the ion pair formation constant obtained from CE studies should give accurate estimates of the extent of ion pair formation in the mobile phase in RPLC and LC/MS. Fig. 4 and Table 2 show the curve fitting according to Eq. (6). For comparison purposes, results with and without ionic strength adjustment are presented. As seen in Table 2, chloride is a very weak ion pair agent. There were only very slight changes in the mobility of each analyte as we varied the concentration of sodium chloride.

In view of how weak ion pairing effects are, mobility measurements at higher additive concentrations are needed to obtain really accurate estimates of ion pair formation constants. However, since our purpose is to study the ion pair effect in RPLC, in which a typical mobile phase additive concentration would be around 20 mM, we kept the additives' concentration at fairly low levels. The use of a low salt concentration also limits Joule heating problems in the CE measurements, which became evident when we did use higher salt concentrations.

Even though we worked within a relatively small range of additive concentrations, it is clear to us that the different anionic additives used here show quite different abilities to form ion pairs with these cationic drugs. The trend in K_{ip} is obviously in the order: $PF_6^- > ClO_4^- > CF_3COO^- > Cl^-$.

The trend in K_{ip} is consistent with a number of anion related phenomena: the effect of anion type on the retention of cations in RPLC, the so called "Hofmeister Series" or salting-out coefficient, the extractability of anions in promoting ion pair extraction of cationic drugs out of water into nonpolar solvents, the order of retention of these anions in anion exchange chromatography, the sequence of anion interference effects in liquid membrane anion selective electrodes, and most fundamentally the hydration energetics of the anions.

As discussed in the introduction and demonstrated by our recent studies [45], the anions show significantly different effects on retention of basic drugs in RPLC. The retention of basic drugs in the presence of anionic additives
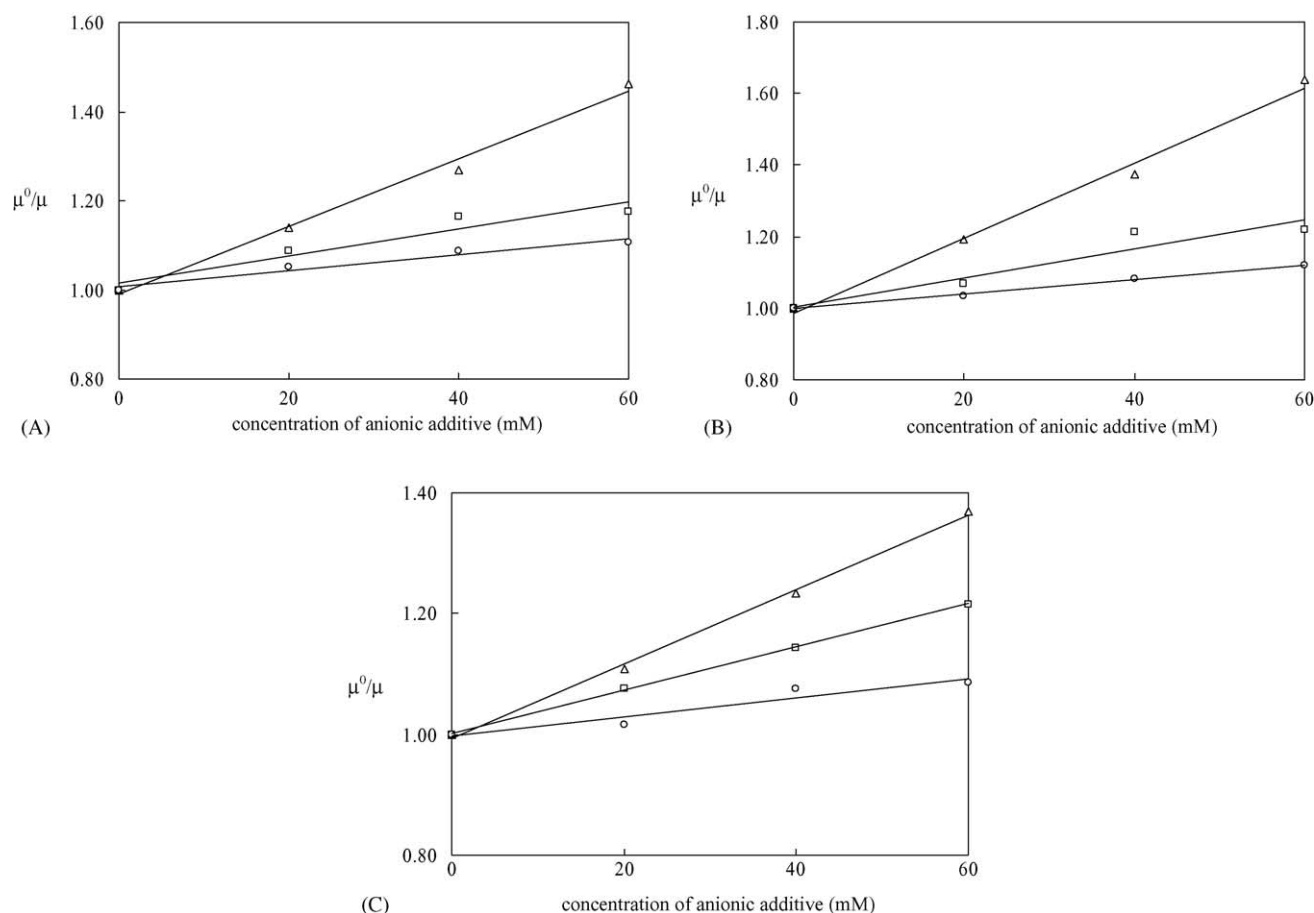
Fig. 4. Effect of anionic additives on mobility at a constant ionic strength of 80 mM maintained by sodium chloride (including the ionic strength from acetate buffer). Conditions: 35/65 acetonitrile/buffer, 20 mM sodium acetate buffer at pH 4.8 and 0 to 60 mM NaX (X = PF_6^-, ClO_4^-, CF_3COO^-, Cl^-). Plot legends, same as Fig. 2. (A) nortriptyline; (B) amitriptyline; (C) perphenazine.

follows the same order as ion pair formation constant, i.e. $PF_6^- > ClO_4^- > CF_3COO^- > Cl^-$ [45].

The type of anion in a salt has a considerable differential effect on the solubility of proteins and nonelectrolyte in aqueous solutions; which is often referred as the "Hofmeister" or "Lyotropic" Series. The series follows the order: $H_2PO_4^- > SO_4^- > CH_3COO^- > Cl^- > Br^- > NO_3^- > ClO_4^-$ [13,14,48]. The anions at the beginning of the series cause the greatest "salting out" effect. That is, they show the greatest reduction in the solubility of proteins and non-electrolytes [49]. Such anions are called kosmotropes. On the other hand, certain anions cause "salting in" that is an increase in solubility, and are termed chaotropes [50,51]. The salting in/out or chaotropism/kosmotropism phenomena are associated with the interactions between the anions and the aqueous media (i.e. structure breaking and structure making effects). The chaotropic anions tend to break the "water structure" and make aqueous solvents less "polar" [51,52].

Anions can affect the extraction of amines from water to organic solvent by promoting ion pairs with the amines [48,53–57]. The extraction efficacy follows the sequence: $ClO_4^- > Br^- > NO_3^- > Cl^-$. Higuchi and coworkers [53,54]

have demonstrated that anions show very different extents of extraction of basic pharmaceuticals and this is due to differences in their ion pair extraction equilibrium constants.

Anions have different retentions on anion exchange materials following a similar sequence: $ClO_4^- > BF_4^- > CF_3COO^- > Cl^- > H_2PO_4^-$ [58].

According to the compilation by Umezawa and Umezawa [59], the sequence of anionic interference effects in liquid membrane anion selective electrodes frequently shows the order: $ClO_4^- > BF_4^- > NO_3^- > Cl^- > HCOO^- > H_2PO_4^-$.

The above anion effect phenomena are both related and seemingly unrelated. The relative positions of anions in each sequence are consistent. We point out these parallel trends because it is all too easy to detect the commonality in the trend in the effect of anions in liquid 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 a seemingly unrelated phenomenon. A more detailed interpretation of anion effects in chromatography is presented in our recent work [45].

According to the compilation by Marcus [60,61], the transfer free energies of various anions from water to non-aqueous solvents differ very greatly. Small anions (e.g. Cl^-, F^-) tend

Table 2
Effect of type of anionic additives on ion pair formation constants[a]

	Nortriptyline						
	1[b]	2[c]	1	2	1	2	2
	PF_6^-	PF_6^-	ClO_4^-	ClO_4^-	CF_3COO^-	CF_3COO^-	Cl^-
$R^{2\,d}$	0.9915	0.9992	0.9220	0.9404	0.9604	0.9779	0.7994
S.D.[e]	0.022	0.009	0.028	0.038	0.011	0.016	0.021
Intercept [f]	0.990	1.003	1.016	1.023	1.008	1.011	1.015
S.D.[g]	0.019	0.008	0.023	0.032	0.009	0.013	0.018
K_{ip} $(\times M^{-1})$[h]	7.58	9.98	3.01	4.81	1.76	3.31	1.35
S.D.[i]	0.50	0.20	0.62	0.86	0.25	0.35	0.48

	Amitriptyline						
	1	2	1	2	1	2	2
	PF_6^-	PF_6^-	ClO_4^-	ClO_4^-	CF_3COO^-	CF_3COO^-	Cl^-
R^2	0.9924	0.9997	0.9144	0.9368	0.9966	0.9900	0.7747
S.D.	0.029	0.007	0.039	0.045	0.0037	0.011	0.020
Intercept	0.987	0.997	1.004	1.023	0.998	1.002	1.013
S.D.	0.024	0.006	0.032	0.037	0.003	0.009	0.016
K_{ip} $(\times M^{-1})$	10.46	12.83	4.01	5.46	2.01	3.53	1.14
S.D.	0.65	0.17	0.87	1.00	0.08	0.25	0.44

	Perphenazine						
	1	2	1	2	1	2	2
	PF_6^-	PF_6^-	ClO_4^-	ClO_4^-	CF_3COO^-	CF_3COO^-	Cl^-
R^2	0.9976	0.9816	0.9995	0.9916	0.9147	0.9661	0.8265
S.D.	0.010	0.039	0.002	0.018	0.015	0.021	0.026
Intercept	0.993	1.025	1.002	1.009	0.997	1.016	1.016
S.D.	0.008	0.033	0.002	0.015	0.013	0.018	0.022
K_{ip} $(\times M^{-1})$	6.16	9.07	3.57	6.20	1.57	3.54	1.82
S.D.	0.22	0.88	0.06	0.40	0.34	0.47	0.59

[a] Mobility results fitted according to Eq. (6).
[b] CE running conditions same as Fig. 4.
[c] CE running conditions same as Fig. 2.
[d] Square of correlation coefficient of the fitting.
[e] Standard errors of the fitting.
[f] Intercept of the fitting.
[g] Standard errors of the intercept.
[h] Slope (i.e. ion pair formation constant) of the fitting.
[i] Standard errors of the slope.

to be strongly solvated by protic solvents like water and alcohols, while large anions (e.g. ClO_4^-, I^-) are relatively weakly solvated. Thus, the energies required to transfer anions from water to non-aqueous solvents are considerably smaller for anions like perchlorate than those for anions like chloride. This is consistent with the trend of ion pair formation constants observed here. We believe that ion pairs with anions like hexafluorophosphate form more easily because such anions desolvate more readily than do anions like chloride, which are so strongly hydrated.

3.3. Effect of concentration of anionic additive on the fraction of ion paired base

Table 3 shows the extent of ion pairing at different concentrations. An example of the effects of anionic additive concentration on the percentage of the analyte (amitriptyline) that is present as ion pairs is given in Fig. 5.

As seen in Table 3, from 0 mM to 60 mM, the fraction of the analyte that is present as an ion pair differs from trifluoroacetate to hexafluorophosphate. Nevertheless, the extent of ion pairing is not large. At a typical RPLC mobile phase additive concentration of 20 mM, the percentage of the analyte that is present as ion pairs are about 15%, 6%, and 3% for hexafluorophosphate, perchlorate, and trifluoroacetate, respectively. The fraction of the bases present as a chloride pair is even smaller. As expected, we note that the strength of ion pairing does not differ radically among the cations. Diamond has pointed out that the order of ion pair extraction among amines follows the trend: primary > secondary > tertiary [57]. We did not detect large differences in the behavior of the

Table 3
Effect of concentration of anionic additives on the fraction of ion paired analytes[a]

Analyte	Anion	$\alpha_{ion\ pair}$ (%)			Concentration needed for 50% ion-pairing (mM)
		20 mM	40 mM	60 mM	
Nortriptyline	PF_6^-	13	23	31	130
	ClO_4^-	6	11	15	330
	CF_3COO^-	3	7	10	570
Amitriptyline	PF_6^-	17	29	39	100
	ClO_4^-	7	14	19	250
	CF_3COO^-	4	7	11	500
Perphenazine	PF_6^-	11	20	27	160
	ClO_4^-	7	13	18	280
	CF_3COO^-	3	6	9	640

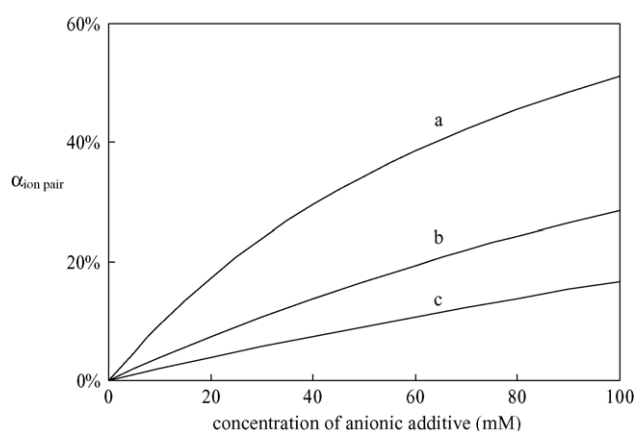[a] Values are based on Eq. (4) and K_{ip}s are from Table 2 at constant ionic strength.

Fig. 5. Effect of concentration of additive on the fraction of the ion paired analyte. $\alpha_{ion\ pair}$ is calculated by Eq. (4) and K_{ip}s are based on the fitting results of amitriptyline in Table 2 at constant ionic strength. (a) PF_6^-; (b) ClO_4^-; (c) CF_3COO^-.

drugs studied here even though different types of amines were tested.

4. Conclusion

For the first time ion pair formation between basic drugs and anionic additives under conditions commonly used in RPLC has been independently confirmed by capillary electrophoresis. Ion pair formation constants measured by CE show the same trend as their effect on retention in RPLC: $Cl^- < CF_3COO^- < ClO_4^- < PF_6^-$. The differences in the strength of ion pairing for the different bases are a good deal smaller than the differences between the anions. The CE data give a quantitative estimate of the extent to which analytes are ion paired, which is valuable in interpreting data in RPLC and LC/MS when ion pair agent are used.

We believe our studies offer very useful information for further method developments in ion pair chromatography and will be helpful for ion pair agent application in RPLC and LC/MS.

References

[1] X. Yang, J. Dai, P.W. Carr, J. Chromatogr. A 996 (2003) 13.
[2] X. Yang, J. Dai, P.W. Carr, Anal. Chem. 75 (2003) 3153.
[3] C. Horvath, W. Melander, I. Molnar, P. Molnar, Anal. Chem. 49 (1977) 2295.
[4] E. Tomlinson, T.M. Jefferies, C.M. Riley, J. Chromatogr. 159 (1978) 315.
[5] B.A. Bidlingmeyer, S.N. Deming, W.P. Price, J.B. Sachok, M. Petrusek, J. Chromatogr. 186 (1979) 419.
[6] J.H. Knox, R.A. Hartwick, J. Chromatogr. 204 (1981) 3.
[7] R. LoBrutto, A. Jones, Y.V. Kazakevich, J. Chromatogr. A 913 (2001) 189.
[8] R. LoBrutto, A. Jones, Y.V. Kazakevich, H.M. McNair, J. Chromatogr. A 913 (2001) 173.
[9] A. Jones, R. LoBrutto, Y. Kazakevich, J. Chromatogr. A 964 (2002) 179.
[10] J.M. Roberts, A.R. Diaz, D.T. Fortin, J.M. Friedle, S.D. Piper, Anal. Chem. 74 (2002) 4927.
[11] Y. Machida, H. Nishi, K. Nakamura, J. Chromatogr. A 830 (1999) 311.
[12] X. Yang, L. Ma, P.W. Carr, J. Chromatogr. A (2005) in press.
[13] D. Eagland, in: F. Franks (Ed.), Water, Comprehensive Treatise, Wiley-Interscience, New York, 1972, p. 305.
[14] L.R. Jacob, J. Chromatogr. Libr. 61 (Protein Liquid Chromatography) (2000) 235.
[15] D.V. McCalley, J. Chromatogr. A 987 (2003) 17.
[16] F. Gritti, G. Guiochon, J. Chromatogr. A 1028 (2004) 197.
[17] F. Gritti, G. Guiochon, J. Chromatogr. A 1033 (2004) 43.
[18] F. Gritti, G. Guiochon, J. Chromatogr. A 1033 (2004) 57.
[19] F. Gritti, G. Guiochon, J. Chromatogr. A 1038 (2004) 53.
[20] F. Gritti, G. Guiochon, J. Chromatogr. A 1041 (2004) 63.
[21] A.P. Bruins, J. Chromatogr. A 794 (1998) 345.
[22] U.A. Mirza, B.T. Chalt, Anal. Chem. 66 (1994) 2898.
[23] C.G. Huber, A. Premstaller, J. Chromatogr. A 849 (1999) 161.
[24] J.-G. Chen, S.G. Weber, L.L. Glavina, F.F. Cantwell, J. Chromatogr. A 656 (1993) 549.
[25] J.J. Stranahan, S.N. Deming, Anal. Chem. 54 (1982) 2251.
[26] M.T. Bowser, D.D.Y. Chen, J. Phys. Chem. A 103 (1999) 197.

[27] M.T. Bowser, D.D.Y. Chen, Anal. Chem. 70 (1998) 3261.

[28] S. Descroix, A. Varenne, L. Geiser, S. Cherkaoui, J.-L. Veuthey, P. Gareil, Electrophoresis 24 (2003) 1577.

[29] M. Stefansson, M. Novotny, Anal. Chem. 66 (1994) 3466.

[30] I. Bjørnsdottir, S. HonoréHansen, S. Terabe, J. Chromatogr. A 745 (1996) 37.

[31] D. Li, S. Fu, C.A. Lucy, Anal. Chem. 71 (1999) 687.

[32] S.P. Porras, M.-L. Riekkola, E. Kenndler, J. Chromatogr. A 924 (2001) 31.

[33] T. Erdey-Gruz, Transport Phenomena in Aqueous Solutions, John Wiley & Sons, New York, 1974.

[34] K.I. Roy, C.A. Lucy, J. Chromatogr. A 964 (2002) 213.

[35] K.I. Roy, C.A. Lucy, Anal. Chem. 73 (2001) 3854.

[36] K.I. Roy, C.A. Lucy, Electrophoresis 23 (2002) 383.

[37] B.W. Rasmussen, M.J. Bjerrum, J. Chromatogr. A 836 (1999) 93.

[38] F. Baeuml, T. Welsch, J. Chromatogr. A 961 (2002) 35.

[39] S.M.C. Buckenmaier, D.V. McCalley, M.R. Euerby, J. Chromatogr. A 1004 (2003) 71.

[40] B.A. Williams, G. Vigh, Anal. Chem. 68 (1996) 1174.

[41] C. Schwer, E. Kenndler, Anal. Chem. 63 (1991) 1801.

[42] A. Cifuentes, P. Canalejas, A. Ortega, J.C. Dýez-Masa, J. Chromatogr. A 823 (1998) 561.

[43] B. Verzola, C. Gelfi, P.G. Righetti, J. Chromatogr. A 868 (2000) 85.

[44] M.R. Schure, A.M. Lenhoff, Anal. Chem. 65 (1993) 3024.

[45] J. Dai, P.W. Carr, J. Chromatogr. A (2005) in press.

[46] K.I. Roy, C.A. Lucy, Electrophoresis 24 (2003) 370.

[47] J.S. Green, J.W. Jorgenson, J. Chromatogr. 478 (1989) 63.

[48] D.J.W. Grant, T. Higuchi, Solubility Behavior of Organic Compounds, John Wiley & Sons, New York, 1990.

[49] R.E. Verrall, in: F. Franks (Ed.), Water, Comprehensive Treatise, Plenum, New York, 1972, p. 211.

[50] K.D. Collins, Biophys. J. 72 (1997) 65.

[51] P.M. Wiggins, Physica A 314 (2002) 485.

[52] A. Suggett, in: F. Franks (Ed.), Water, Comprehensive Treatise, Wiley-Interscience, New York, 1972, p. 519.

[53] T. Higuchi, K. Kato, J. Pharm. Sci. 55 (1966) 1080.

[54] T. Higuchi, A. Michaelis, T. Tan, A. Hurwitz, Anal. Chem. 39 (1967) 974.

[55] N.A. Gibson, D.C. Weatherburn, Anal. Chim. Acta 58 (1972) 159.

[56] K. Miyabe, S. Taguchi, I. Kasahara, K. Goto, J. Phys. Chem. B 104 (2000) 8481.

[57] R.M. Diamond, Solvent extraction chemistry, in: J. Rydberg, D. Dyrssen, J.O. Liljenzin (Eds.), Proceedings of the International Conference, Interscience, New York, 1967, p. 349.

[58] J.S. Fritz, D.T. Gjerde, Ion Chromatography, Wiley-VCH, Weinheim, 2000.

[59] K. Umezawa, Y. Umezawa, Selectivity Coefficients for Ion-Selective Electrodes, University of Tokyo Press, Tokyo, Japan, 1983.

[60] Y. Marcus, Pure Appl. Chem. 55 (1983) 977.

[61] Y. Marcus, Ion Properties, Dekker, New York, 1997.