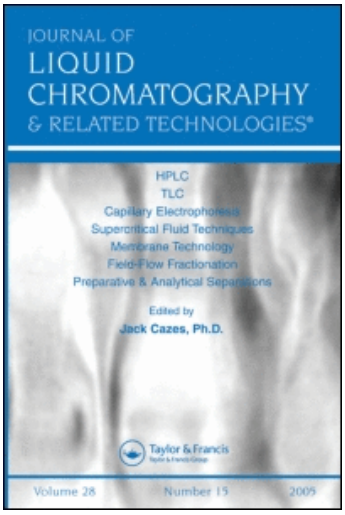

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Reversed-Phase Ion-Pair Liquid Chromatography of a Pharmaceutical Compound and Its Photolytically Transformed Isomer

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REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHY OF A PHARMACEUTICAL COMPOUND AND ITS PHOTOLYTICALLY TRANSFORMED ISOMER

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ABSTRACT

Reversed-phase HPLC is usually the method of choice for compounds that dissolve in water/organic mixtures, even for samples that contain ionizable compounds. However, reversed-phase ion-pair chromatography (RP-IPC) is often a useful alternative for such samples, particularly if the components are highly basic or acidic. With this technique an aqueous/organic mobile phase is used along with a buffer and an ion-pairing reagent to provide more retention and higher selectivity than are afforded by the column and organic solvents alone. The retention effects of ammonium acetate, sodium acetate, potassium phosphate, and triethylamine (TEA) on a pharmaceutical compound (SK&F 108566) and its stereoisomer (SB 206328) were studied. Evidence indicated that SK&F 108566 and SB 206328 exhibited ion-pair formation with simple buffer systems and the ion-pair occurred in the mobile phase prior to the complex binding rapidly to the stationary phase.

As a result, buffer systems such as sodium acetate, ammonium acetate, and potassium phosphate could be used as ion-pairing reagents. Thus, a simple HPLC system which not only provided better buffer capability but also improved selectivity through ion-pair interactions could be utilized for ionic compounds separation. One important result to emerge from this study is the potential for isocratic, multidimensional HPLC separations when reversed-phase columns are coupled and used with the appropriate buffer solutions.

INTRODUCTION

Eprosartan (SK&F 108566) is an angiotesin II antagonist that is being investigated for treatment of hypertension. It was observed that sunlight produced a rapid transformation of eprosartan into its diastereoisomer (SB 206328) within three hours (Figures. 1-2). Since no physical properties data for SB 206328 were available, it was assumed that SK&F 108566 and SB 206328 had similar solubilities and pKa values in aqueous solution. A HPLC method capable of separating these two isomers was needed to support photolysis kinetic and environmental toxicity studies as part of new drug application submission requirements.

During the method development, eprosartan and its isomer exhibited buffer concentration dependent behaviors.[1] Due to the two ionized interaction sites (dot boxes in Figure. 2), retention for eprosartan and its isomer might be better described by the ion-pair model rather than the partition (or adsorption) process generally used in reversed-phase HPLC (RP-HPLC).

Recently, Nguyen and Siegler[2] developed capillary electrophoresis (CE) separations for SK&F 108566 and its Z-isomer (SB 206328). In their method, separation as a function of pH is determined as part of resolution optimization. As will be shown in this study, the concentration of the buffer solution can also be used as part of separation optimization strategy for eprosartan and its isomer. As such, better selectivity can be obtained not only by the partition (or adsorption) process, but by the ion-pair interactions as well.

A Zorbax RX-C8 narrow bore column (150 X 2.1 mm i.d.) was chosen as the analytical separation column because it has been proved to be highly efficient and very stable with continuously analyzed samples containing bleach and hydrogen peroxide.[3] Eprosartan is a weak acid with three pKa values (4.11, 5.68, and 6.89) that also exhibits low solubility at acidic conditions (pH < 6). Eprosartan in solution at pH between 4.2 and 5.7 are predominately

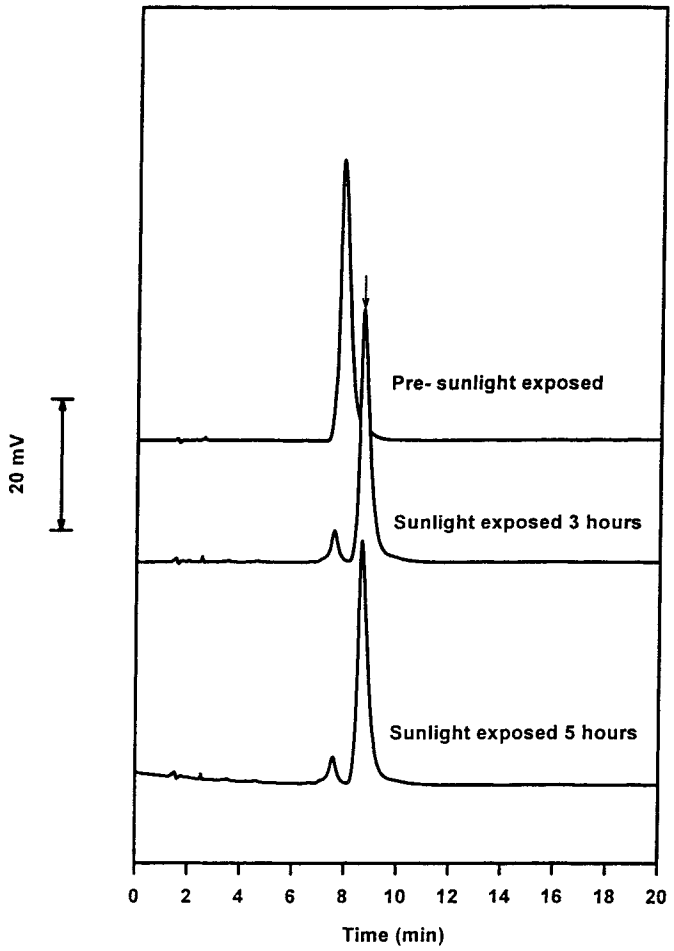

Figure 1. Chromatograms of SK&F 108566 at 0, 3 and 5 hours sunlight exposed. Arrow indicates photolysis degradant (SB 206328). Column: Zorbax C8, 150 x 4.6 mm i.d., 3.5 μm d_p. Mobile phase: 13 % (v/v) acetonitrile with 20 mM ammonium acetate, pH 7.5. Injection volume: 15 μL. Detection : UV 260 nm. Flow rate: 0.9 mL/min.

dipolar ions (or zwitterions) rather than unionized molecules (Figure 3). In the dipolar form of eprosartan, the nitrogen in the imidazole group is protonated and the carboxyl group attached to the benzene ring is dissociated. At pH > 7, the nitrogen is not ionized, both the carboxyl groups are dissociated, with a total of two negative charges. As will be demonstrated later, these two negative

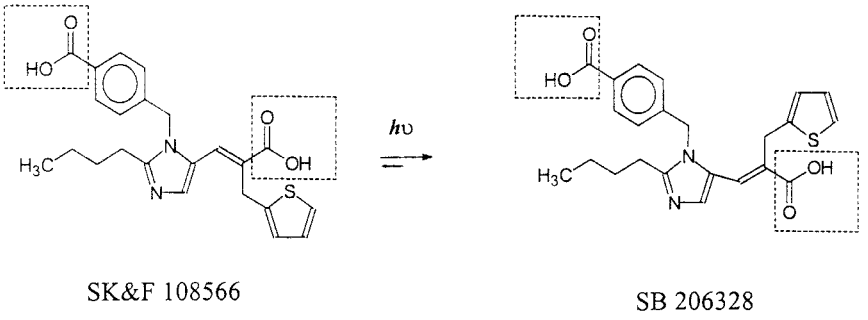

Figure 2. Eprosartan (SK&F 108566) and its phototransformation product (SB 206328). Dot boxes are the two ionized interaction sites (carboxyl groups).

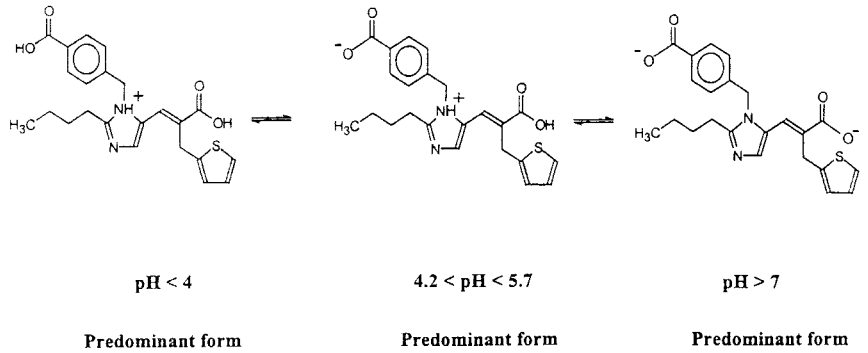

Figure 3. Three dominant forms for eprosartan at pH < 4, 4.2 < pH < 5.7 and pH > 7. See text for details.

The organic solvent-water mixtures used as mobile phase in many RP-HPLC separations are in fact, at least ternary mixtures with organic solvent, water, and organic-solvent-water aggregates. Solutes dissolved in these mixtures are solvated differently by these three mixture components and may lead to chromatographic behaviors in organic solvent-rich mixtures different from those expected in water-rich mixtures. In particular, as the pH scale of the medium changes with the mobile phase composition, the pH value of a HPLC buffer solution and the dissociation constants of many ionizable solutes will change to a different degree.[4] In this study, high buffer concentrations were used (>200 mM) and maintaining the mobile phase pH at 7.5 was very

critical for full ionization of eprosartan and its isomer. Thus, the organic modifier concentration should be kept as low as possible but not too low as to alter the column stationary phase characteristics.[5] In such, pH in the mobile phase was not deviated too far from aqueous solution and ionizable compounds should behave normally in the mobile phase. Organic modifier (acetonitrile) was fixed at 10% and the mobile phase pH was adjusted to 7.5. By preselecting the separation column, mobile phase pH and organic modifier content, the ion-pair effects on eprosartan and its diastereoisomer can be investigated.

MATERIALS AND METHODS

Materials

Ammonium acetate, sodium acetate, and potassium phosphate pH 7.5 stock solutions (350 mM each) were prepared with HPLC grade water and adjusted to pH 7.5 with ammonia hydroxide, potassium hydroxide, or 50% (w/w) sodium hydroxide and then diluted to volume with HPLC grade water. Solutions were diluted to 300, 250, 200, 100, 50, 25, 10, and 1 mM with water and adjusted with ammonia hydroxide, sodium hydroxide, acetic acid, or phosphate acid to pH 7.5, if necessary. Triethylamine (TEA) pH 7.5 stock solution (200 mM) was prepared by adding 28 mL to 970 mL HPLC water followed by pH adjustment to 7.5 through addition of trifluoroacetic acid (TFA). The final volume was brought to 1000 mL with HPLC water. Solutions were diluted to 100, 50, 25, 10, 5, and 1 mM with HPLC grade water and adjusted with TFA to pH 7.5 if necessary. These solutions were mixed and filtered through a separate 0.4-0.5 mm polycarbonate, PVDF, or equivalent membrane filter. A 100 μg/mL eprosartan (SK&F 108566) stock solution (SmithKline Beecham Pharmaceutical, King of Prussia, USA) was prepared with 10 mM pH 7.5 potassium phosphate buffer. A 0.1% (v/v) toluene solution was prepared by taking 10 μL toluene and the final volume was brought to 10 mL with methanol. A 100 μg/mL benzoic acid solution was prepared with HPLC water. All the chemicals except eprosartan were purchased from J. T. Baker (Phillipsburg, NJ, USA).

Methods

All measurements were obtained from a Hewlett-Packard 1090 HPLC system with a diode array detector (Hewlett Packard, Palo Alto, CA, USA). The wavelength was set at 260 nm. A Nelson 900 Series interface and the PE

Access*Chrom VAX chromatographic software (Perkin-Elmer Nelson System, Cupertino, CA, USA) were used for data collection and analysis. The sampling rate was 1 pt/s and the samples were injected at least three times. Chromatographic separations were carried out on a 150 x 2.1 mm i.d. Zorbax C8 column (MacMod Analytical Inc., Chadds Ford, PA, USA) at room temperature. The injection volume was 10 μL. Various concentrations of ammonia acetate, sodium acetate, potassium phosphate, and triethylamine pH 7.5 solution were used as mobile phase A and HPLC-grade acetonitrile as mobile phase B. The elution profile was 10 % B isocratic. The run time was adjusted according to various buffer systems. Repeated injections of 100 mg/L eprosartan was used to measure the reproducibility of retention times. The eprosartan test solution aliquots were transferred to quartz test tubes (16 x 150 mm) which were exposed to ambient sunlight at various hours, and stored at 4°C.

RESULTS AND DISCUSSION

Reversed-Phase Ion-Pair Chromatography (RP-IPC)

Ion-pair chromatography is a derivative technique that has quickly gained wide acceptance as a versatile and efficient method for the separation of ionized and ionizable molecules by high-performance liquid chromatography. Normally we would expect such ionic solutes to show little or no retention on lipophilic stationary phases when typical reversed-phase eluents are used. However, retention and subsequent separation of these solutes on non-polar stationary phases can be achieved by the addition to the eluent of a lipophilic reagent with the opposite charge to that of the solute ion. The effect on the solute is to modify its polarity, usually by complete neutralization of the charge by suitable choice of a counterion of opposite charge. In this fashion, the retention of solutes having the opposite charge to the ion-pairing reagent is increased when the concentration of the reagent in the eluent is increased.

The retention mechanism by which separation occurs in reversed-phase ion-pair chromatography (RP-IPC) is not without controversy. At least three models (ion-pair, dynamic ion-exchange and ion-interaction model) have been proposed to explain different aspects of the separation process. Each model represents an extreme situation or boundary condition and actual separations probably involve a mixed model mechanism or occur by a mechanism yet to be defined. It is not the purpose of this paper to discuss the details of the retention mechanisms as they have been previously described.[6-11]

However, one model[12-15] that pictured retention as a result of ion-pair formation in the mobile phase (*m*) followed by the partition (or adsorption) of this ion-pair on the stationary phase (*s*) will be used throughout this study.

In this study the mobile phase was maintained at pH 7.5. Therefore, SK&F 108566 and SB 206328 were in the form of the divalent anionic specie R-$(COO^-)^2$ (represented hereafter as R^{-2}). C^+ represented the counterion, such as TEA. Under these conditions, the following equilibria apply.[16]

$$\left(R^{-2}\right)_m + 2(C+)_m \overset{k1}{\leftrightarrow} (RC_2)_m \tag{1}$$

$$(RC_2)_m \overset{k2}{\leftrightarrow} (RC_2)_s \tag{2}$$

$$\left(R^{-2}\right)_m \overset{k3}{\leftrightarrow} \left(R^{-2}\right)_s \tag{3}$$

$$D = \frac{\left[r^{-2}\right]_s + [RC_2]_s}{\left[R^{-2}\right]_m + [RC_2]_m} \tag{4}$$

$$k' = D\frac{V_s}{V_m} = \frac{V_s}{V_m} * \frac{K_3 + K_1K_2[C+]^2_m}{1 + K_1[C+]^2_m} \tag{5}$$

Where D, k', *m* and *s* are distribution ratio, capacity factor, mobile phase and stationary phase, respectively. Equation 5 describes a rectangular hyperbolic relationship between the square of counterion concentration in the mobile phase $[C^+]_m$ and the retention of the solute species (k'). If ion-pair formation does not occur in the mobile phase, this model also predicts the linear behavior of capacity factor (k') on $[C^+]_m$. In fact, the side reaction of ion-pair formation in the mobile phase accounts for the nonlinearity (leveling off), as will be demonstrated in next section.

The organic modifier plays a significant role in determining chromatographic retention and selectivity. The retention of charged solutes undergo ion-pair interactions that are more sensitive to organic solvent composition changes than those of nonionic substances. It has been shown[16]

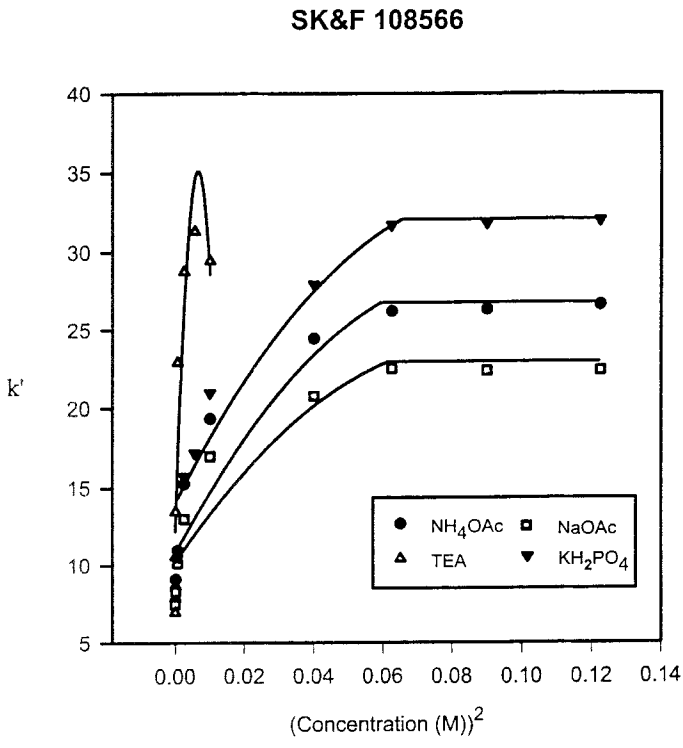

Figure 4. Capacity factor of eprosartan (SK&F 108566) versus various buffer systems and triethylamine (TEA) on a reversed-phase column. Column: Zorbax C8, 150 x 2.1 mm i.d., 5 μm particle size. Mobile phase: 10 % (v/v) acetonitrile with various buffers, pH 7.5. Injection volume: 10 μL. Detection : UV 260 nm. Flow rate: 0.3 mL/min.

that a small variation in acetonitrile concentration in the mobile phase can lead to a significant error in k' in the ion-pair mode. As discussed previously, at higher organic modifier concentration the pH of the mobile phase is not well defined and the ionic compounds will behave unpredictably. In order for ion-pair mode separation to be meaningful, the organic modifier content should be as little as possible. Bosch *et al.*[4] has demonstrated that the content of organic solvent in RP-HPLC has a strong influence on the pKa values of solutes and, therefore, on the retention of ionizable solutes in RP-HPLC. The situation is more severe in gradient elution, where organic content in the mobile phase usually ranges from 0 to 100%. Thus, for RP-IPC to be a useful separation technique for ionic compounds, the concentration of organic modifier should

not exceed 15% (v/v) to ensure the pH value in the mobile phase is not deviated too much from the non-organic aqueous solution. Recently, Kirkland has provided practical guidelines for rapidly and effectively developing rugged RP-HPLC methods specifically for ionizable compounds.[17] However, the effects of high organic solvent contents on pKa and retentions of the ionic compounds are still not addressed when in fact many pharmaceutical compounds are ionizable.

Retention Effects on SK&F 108566 and SB 206328

Triethylamine (TEA) has been used as a ion-pairing reagent for anionic compounds.[18-20] Thus it was no surprise that TEA exhibited ion-pair effects on eprosartan and its isomer, which were anionic at pH 7.5 (Figure 4). However, cations from buffer systems such as sodium (Na^+), ammonium (NH_4^+) and potassium (K^+) also showed a similar ion-pair effects but at a lesser extent than those observed in TEA (Figures 4 and 5). While Na^+, NH_4^+ and K^+ exhibited leveling off trends in capacity factor (k'), a maximum in k' was observed when TEA was used as a counterion (Figures 4 and 5). As mentioned earlier, the nonlinear behaviors of k' on $[C^+]_m$ in Figures 4 and 5 indicated that the ion-pair was formed in the mobile phase. The parabolic behavior of TEA might in part be a competing equilibrium phenomenon. Knox and Laird[21] have suggested that aggregates of counterions (TEA in this case) may enhance the solubilization of solute ions in the mobile phase and thus accelerate the movement of such ions through the column. Kraak *et al.*[22] have provided further evidence for this behavior. It is possible that for TEA not only is ion-pair formation observed in the mobile phase but dynamic ion-exchange as well. In the dynamic ion-exchange model, the ion-pairing reagent is adsorbed onto the stationary phase surface where it behaves as a dynamically coated ion-exchanger.

Separation and retention are due to ionic interactions between the ionized solute molecules and the counterions adsorptively bound to the stationary phase. It is also possible that at high concentrations of counterion both mechanisms occur simultaneously, whereas at low concentrations of counterion only one mechanism is operative. The dual retention process has been observed by Hsu *et al.*[5] in an aminopropyl normal-phase column. If both mechanisms contribute significantly to retention, then a decrease in retention may be expected as counterion concentration increases. The fact that all the counterions (with the exception of TEA) do not exhibit parabolic behaviors indicated that only one retention mechanism occurs in this study. TEA also has been used to eliminate the secondary retention. More information is needed to determine the actual retention mechanism.

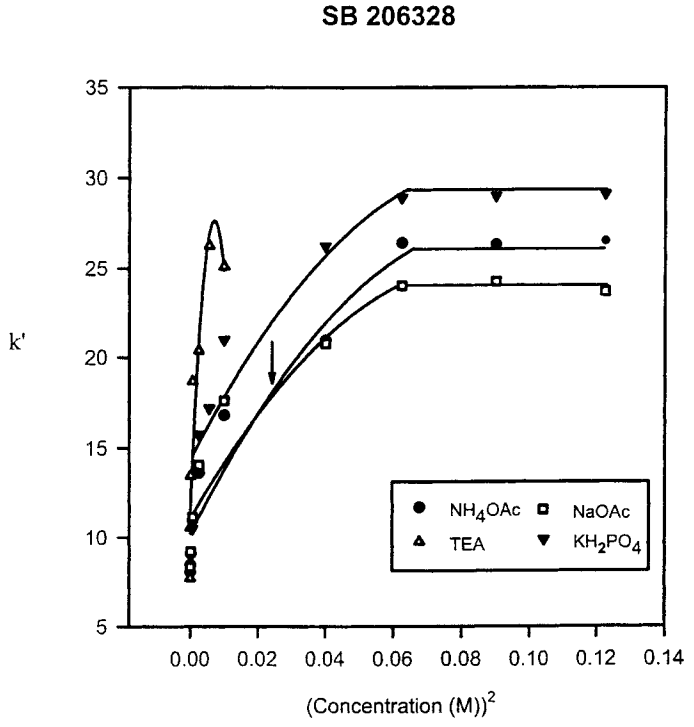

Figure 5. Capacity factor of SB 206328 versus various buffer systems and triethylamine (TEA) on a reversed-phase column. Arrow indicated the cross retention of SB 206328 in sodium acetate and ammonium acetate. Column: Zorbax C8, 150 x 2.1 mm i.d., 5 μm particle size. Mobile phase: 10 % (v/v) acetonitrile with various buffers, pH 7.5. Injection volume: 10 μL. Detection : UV 260 nm. Flow rate: 0.3 mL/min.

Since no peak distortion was observed, the silanol secondary retention effects from the Zorbax RX-C8 column were ignored. It is suggested here that ion-pair effects observed due to the buffer systems may be originated from simple acid-base interaction between buffer cations and solutes (i.e. SK&F 108566 and SB 206328). In any given liquid phase oppositely charged ions will have some tendency to attract one another, depending upon the dielectric constant of the medium, the solvation of the individual ions, and the types of interaction that cause ion association. Oppositely charged ions can be sufficiently bound to one another such that the resulting entity acts as a partially or fully neutralized species. For the affinity of acid-base interaction, Pearson[23] has suggested a simple rule for predicting the stability of complexes

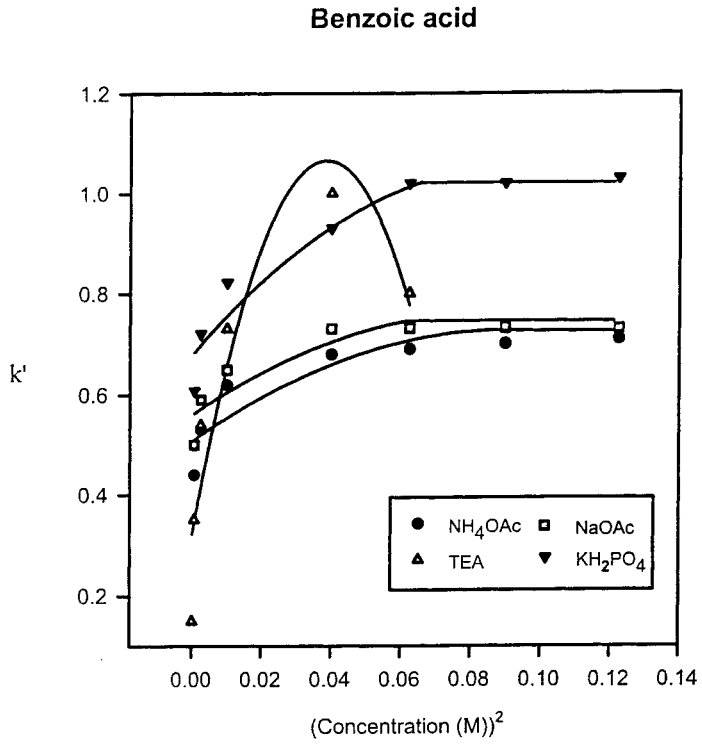

Figure 6. Capacity factor of benzoic acid versus various buffer systems and triethylamine (TEA) on a reversed-phase column. Column: Zorbax C8, 150 x 2.1 mm i.d., 5 μm particle size. Mobile phase: 10 % (v/v) acetonitrile with various buffers, pH 7.5. Injection volume: 10 μL. Detection : UV 260 nm. Flow rate: 0.3 mL/min.

formed between acids and bases: hard acids prefer to bind to hard bases and soft acids prefer to bind to soft bases. It should be noted that this statement is not a explanation or a theory but a simple rule of thumb which enables the user to predict qualitatively the relative stability of acid-base adducts. According to hard-soft acid-base (HSAB) model,[23] NH^+_4, Na^+ and K^+ are classified as hard acids and carboxyl group ($CH3COO^-$) is classified as a hard base. Thus, the interactions between buffer cations (NH^+_4, Na^+ and K^+) and the carboxyl group are likely to be strong. The fact that SK&F 108566 and SB 206328 both have two carboxyl groups should make the hard acid-base interactions even stronger.

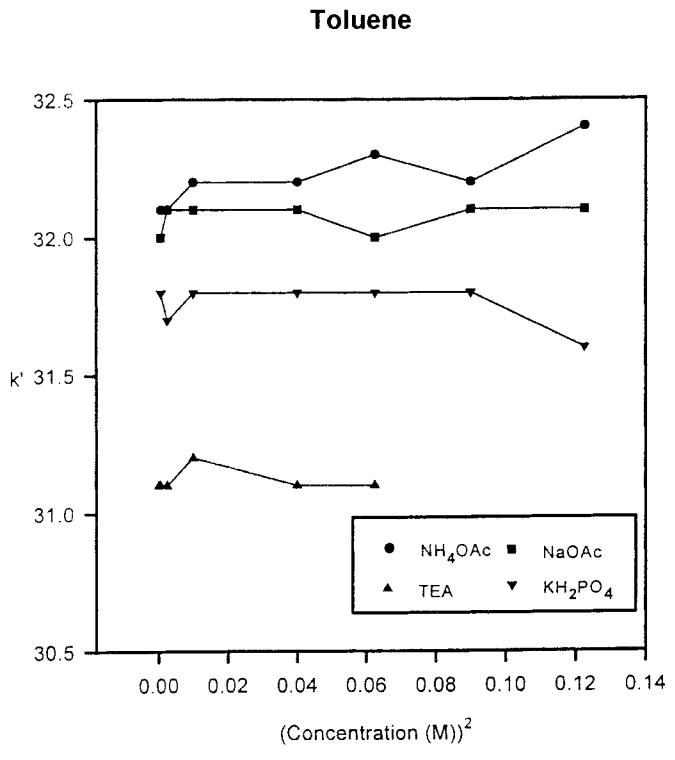

Figure 7. Capacity factor of toluene versus various buffer systems and triethylamine (TEA) on a reversed-phase column. Column: Zorbax C8, 150 x 2.1 mm i.d., 5 μm particle size. Mobile phase: 10 % (v/v) acetonitrile with various buffers, pH 7.5. Injection volume: 10 μL. Detection : UV 260 nm. Flow rate: 0.3 mL/min.

Benzoic acid and toluene were used to test the HSAB hypothesis. Because of the carboxyl group, benzoic acid is a hard acid.[23] Toluene with its benzene functional group is a soft acid.[23] The ion-pair interactions similar to those observed in Figures 4 and 5 were also observed for benzoic acid (Figure 6). However, the effect is at least one order of magnitude smaller than those observed in Figure 4 and 5. Benzoic acid has one carboxyl group, thus the ion-pair interaction with the counterions is predicted to be weaker than those compounds with multiple carboxyl groups such as SK&F 108566 and SB 206328. Again, TEA had the largest influence on the retention than those cations from three buffer systems (Figures 4-6). Toluene, a compound with a soft acid (benzene ring) showed little or no effect at all (Figure 7).

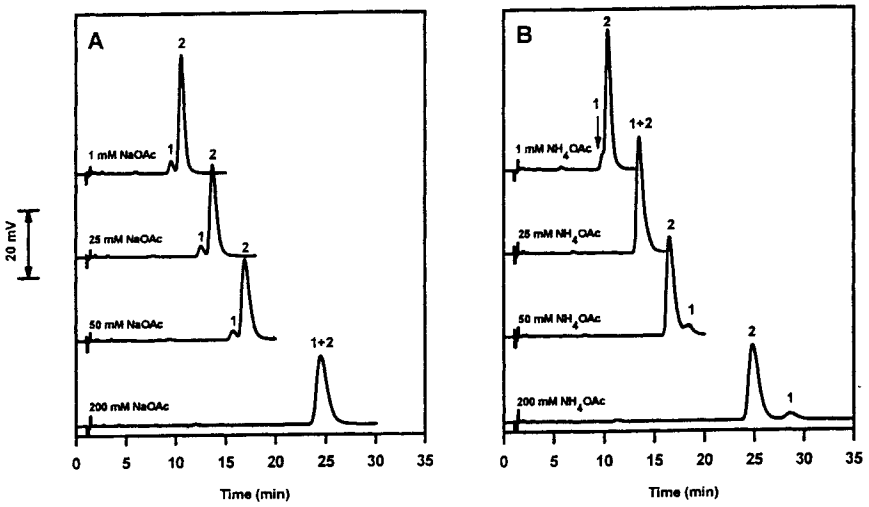

Figure 8. Chromatogram of 1. SK&F 108566 and 2. SB 206328 in (a) sodium acetate and (b) ammonium acetate. Column: Zorbax C8, 150 x 2.1 mm i.d., 5 μm particle size. Mobile phase: 10 % (v/v) acetonitrile with various buffers, pH 7.5. Injection volume: 10 μL. Detection : UV 260 nm. Flow rate: 0.3 mL/min.

The results from these test compounds were in agreement with the HSAB Model. The HSAB model also suggests the ion-pair effects between cationic pharmaceutical compounds and the anions from buffer systems. Currently, this is under investigation.

A retention reversal of SK&F 108566 and SB 206328 has been observed (Figure 8). At low sodium acetate concentrations (< 50 mM), SK&F 108566 was less retained than SB 206328. At ammonium acetate concentration greater than 50 mM, SK&F 108566 was retained longer than SB 206328. A similar retention reversal has been reported for phenol and aniline with the cyano phase.[24] The reasons for SK&F 108566 and SB 206328 retention reversal were not clear. However, it suggests that for the optimum separation of SK&F 108566 and SB 206328 with the sodium acetate, the buffer concentration should be kept below 50 mM as above 50 mM these two compounds will coelute. For optimum separation with ammonium acetate the buffer concentration should be above 50 mM. Buffer concentration therefore provides another way of controlling solvent strength and selectivity. This characteristic can be used to improve the separation in RP-HPLC, where the separation is based on partition (or adsorption) on the nonspecific alkyl bonded phase.

Manipulation of secondary solvent effects is the most effective means of altering selectivity but can also be the most unpredictable.[25,26] The Snyder solvent selectivity triangle[27] has provided a foundation for the development of systematic mobile phase optimization strategies in HPLC.[28] It is proposed here that for ionic compounds separation a similar approach such as the Snyder triangle could also be used as the separation optimization strategies.

According to HSAB model, buffer systems could be described by hard, borderline, and soft acids or bases. Buffer systems could, therefore, be placed in a triangular coordinate system according to these tendencies. As demonstrated by Smith and Cooper[24, 29] in normal phase columns, a selectivity matrix based on the ion-pair model can then be used to provide information regarding the relative retention of specially chosen probes and gives insight into the retention mechanism responsible for observed behavior.

CONCLUSIONS

A simple buffer system can be used to provide retention and selectivity in the same fashion as the ion-pairing reagent does but at a smaller degree. The optimum separation can be achieved simply by controlling the buffer concentration. Although the ion-pair effects from buffer systems may not be as effective as the ion-pairing reagents, problems associated with ion-pairing reagents such as long equilibration times, irreversible adsorption of the ion-pairing reagent, and reproducibility can be avoided. Because the separation can be controlled by a simple buffer system, the potential of reducing organic solvent waste and the associated disposal cost also make it worthwhile to investigate the ion-pair effects on ionizable pharmaceuticals from buffer systems.

The ion-pair effects just described can be used to extend the reversed-phase columns selectivity, and thus applicability. The results presented here can also lead to the development of mobile phase programming using coupled reversed-phase columns and isocratic buffer systems.

Finally, another approach for separating ionizable compounds is capillary zone electrolysis (CZE), since only non-organic aqueous buffer systems are involved in the separation, the effects of the organic solvent on the solute's pKa and retentions are eliminated. A more predictable and rugged separation method can be achieved.

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