

Effects of Ionic Liquid as Additive and the pH of the Mobile Phase on the Retention Factors of Amino Benzoic Acids in RP-HPLC

J. Zheng, Y. Polyakova, and K.H. Row*

Center for Advanced Bioseparation Technology, Department of Chemical Engineering, Inha University, Incheon 402-751, South Korea

Abstract

As an organic salt, ionic liquids are widely used as new solvent media. In this paper, three positional isomers, such as *o*-amino benzoic acid, *m*-amino benzoic acid, and *p*-amino benzoic acid are separated with four different ionic liquids as additives to the mobile phase using reversed-phase (RP) high-performance liquid chromatography (HPLC). Amino benzoic acids are biologically active substances; the *p*-isomer is present in a group of water-soluble vitamins and is widely known as a sunscreen agent. The ionic liquids used are 1-butyl-3-methylimidazolium tetrafluoroborate, 1-ethyl-3-methylimidazolium tetrafluoroborate, 1-ethyl-3-methylimidazolium methylsulfate, and 1-octyl-3-methylimidazolium methylsulfate. The effects of the length of the alkyl group on the imidazolium ring and its counterion, the concentrations of the ionic liquid, and the effect of the pH of the mobile phase on the retention factor of the amino benzoic acid isomers are studied. Separation with the ionic liquid in the eluent was better than the separation without the ionic liquid. The pH mainly affected the retention and elution order of the solutes in RP-HPLC.

Introduction

Unlike traditional salts, ionic liquids are liquids composed of relatively large organic cations and inorganic or organic anions (1). Ionic liquids have some unique properties, such as negligible vapor pressure, good thermal stability, tunable viscosity, strong polarity, and miscibility with water and organic solvents, as well as good extractability for various organic compounds and metal ions (2). Ionic liquids have been applied to analytical chemistry (2), catalysis (3,4) and biocatalysis (5), synthesis (6), and separation science (7–16). When ionic liquids are diluted or immobilized on a stationary support, they may not possess all the properties of the ordinary ionic liquids, and in some cases, they may keep several kinds of intermolecular interactions. For this

reason, ionic liquids can be useful for chromatographic separations (1,15,17).

Recently, numerous amino and benzene compounds have been developed as medical and biological agents. In particular, amino compounds have resulted in many effective drugs currently in clinical and preventive use, and newer compounds with an expanded spectrum of activity are presently in continuous development. Amino benzoic acids are biologically active substances, and the *p*-isomer is included in a group of water-soluble vitamins (18,19). These vitamins play important roles in numerous biological processes. *p*-Amino benzoic acid is an essential nutrient for some bacteria and is sometimes called Vitamin B_x. However, *p*-amino benzoic acid is not essential for humans, and its activity differs from other B vitamins. This acid is taken successfully in vitamin supplements. Although humans lack the ability to synthesize folic acid from *p*-amino benzoic acid, it is sometimes marketed as an essential nutrient under the premise that it can stimulate intestinal bacteria. Also, some sulfa drugs are chemically similar to *p*-amino benzoic acid, and their antibacterial activity is due to their ability to interfere with the molecular utilization of bacteria. In addition, its most widely known use is as a sunscreen. Taking it orally will not protect from the sun: the sunscreen function is purely a matter of *p*-amino benzoic acid acting as a dye that absorbs UV light.

An interdisciplinary approach to their chemistry is currently directed towards medicinal chemistry, supramolecular chemistry, and biomedical applications. Data on the chromatographic behaviors of biological activity compounds, which is one of the most important fields in modern chemistry, gives useful information for biochemistry and combinatorial and medicinal chemistry. In addition, although modern liquid chromatography is a powerful separation method, the separation of isomers remains difficult. Furthermore, from the point of view of the adsorption theory of substances by surfaces, studying the chromatographic behavior of isomers has practical applications and theoretical interest.

One example of the separation of positional isomers of substituted benzoic acids with amine and β -cyclodextrin bonded-phase

* Author to whom correspondence should be addressed: email rowkho@inha.ac.kr.

columns in normal-phase high-performance liquid chromatography (HPLC) were achieved by Chang et al. (20). The impacts of experimental parameters on the resolution of amino benzoic acid isomers in capillary zone electrophoresis were investigated by Nielen (21). Wan et al. investigated the effect of ionic liquid on the retention and resolution of isomeric amino benzoic acids. They showed that separation can be obtained on the special porous graphitic column (Hypercarb) with phosphate buffer–acetonitrile as the mobile phase (22). The interest in ionic liquids for their potential application in separation science is increasing because ionic liquids present a variety of desirable properties. In the past decades, the dialkylimidazolium based ionic liquids have generated enormous attention.

The purpose of the present study is to investigate the potential application of different ionic liquids as additives for the separation of amino benzoic acids. Four types of ionic liquids were used as mobile phase modifiers in reversed-phase (RP)-HPLC to isolate the three positional isomers. The retention factors of these isomers were determined with a mobile phase containing four ionic liquids in water–methanol. The types of investigated ionic liquids are shown in Table I. The name, structure, and Log P of the sorbats are listed in Table II. The effects of the concentration and chemical nature of the ionic liquids on the chromatographic retention and separation of amino benzoic acid isomers were investigated; the influence of the pH of the mobile phase on the retention of amino benzoic acid isomers in RP-HPLC was also investigated.

Table I. Name and Structure of the Ionic Liquids



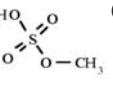
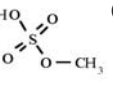
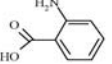
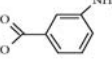
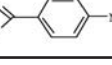
No.	Abbreviation (ionic liquid)	Cation	Formula
1	[BMIm][BF ₃]	1-Butyl-3-methylimidazolium	 C ₈ H ₁₅ BF ₄ N ₂
2	[EMIm][BF ₄]	1-Ethyl-3-methylimidazolium	 C ₆ H ₁₁ BF ₄ N ₂
3	[EMIm][MS]	1-Ethyl-3-methylimidazolium	 C ₇ H ₁₄ N ₂ O ₄ S
4	[OMIm][MS]	1-Methyl-3-	

Table II. Name, Structure, and Log P of the Amino Benzoic Acid

No.	Compound	Structure	Log P
1	<i>o</i> -Amino benzoic acid (2-ABA)		0.79
2	<i>m</i> -Amino benzoic acid (3-ABA)		0.18
3	<i>p</i> -Amino benzoic acid (4-ABA)		0.78

Experimental

Reagents

Four ionic liquids (99.99%), such as 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIm][BF₄]), 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIm][BF₄]), 1-ethyl-3-methylimidazolium methylsulfate ([EMIm][MS]), and 1-octyl-3-methylimidazolium methylsulfate ([OMIm][MS]) were purchased from C-tri Co. (Namyang, Korea) and are shown in Table I. *o*- And *m*-amino benzoic acids were purchased from Sigma-Aldrich Co. (St. Louis, MO), *p*-amino benzoic acid was from Fluka (St. Louis, MO), and all reagents were of analytical grade. Some of their properties are listed in Table II. Potassium nitrate (KNO₃) was purchased from Kanto Chemical Co. (Kanagawa, Japan) to measure the dead volume. HPLC-grade methanol (CH₃OH) was purchased from Duksan Pure Chemical Co. (Ansan, Korea), buffer solution standards of pH 4.0, 7.0, and 10.0 were from DC Chemical Co. (Japan), and distilled water was filtered with a vacuum pump (Division of Millipore, Waters) and filter (HA-0.45, Division of Millipore, Waters) before use.

Apparatus

The instrument used in this study consisted of a 600 HPLC pump (Waters, Milford, MA), 486 detector (M 7200 Absorbance Detector, Young-In Scientific Co., Korea), and Reodyne injection valve with 20- μ L sample loop. Chromate software (Ver. 3.0 Interface Eng., Seoul, Korea) connected to a PC was used as the data acquisition system. The experiments were carried out using a commercially available C₁₈ (alkyl-) bonded phase column (4.6 \times 150-mm i.d., particle size 5 μ m). The pH meter (HANNA Co. Seoul, Korea) was calibrated with buffer solution standards pH 4.0, 7.0, and 10.0.

Chromatographic conditions

Stock solutions (1 mg/mL) of the standards were prepared by dissolving the individual amino benzoic acid standards in pure water. Mixtures of amino benzoic acids were prepared as aliquots of the individual solutions of *o*-, *m*-, and *p*-isomers in the ratio of 5:5:1. Mobile phases were 5.0%, 10.0%, 15.0%, 20.0%, 25.0%, 30.0%, 50.0%, and 75.0% volume of methanol in water (pure reversed-phase systems). Various systems (0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, and 64.0 mM/L eluent) of ionic liquids were used. Using hydrochloric acid (HCl) the pure reversed-phase system with 25% volume of methanol and eluents containing 8 mM/L of ionic liquids with 25% volume of methanol was adjusted to pH 3.0, 4.0, 5.0, 6.0, and 7.0. The flow rate was fixed at 1.0 mL/min and used in the isocratic mode. A constant injection volume (5 μ L) was used throughout for the individual solutions. The injection volume of the mixture was 15 μ L. Detection was at a wavelength of 254 nm. The solutions were stored at 4°C, and the working standards were prepared again every 2 days to avoid the potential errors from decomposition of the targets. Retention factor (*k*) values were calculated using the formula:

$$k = (t_R - t_0) / t_0 \quad \text{Eq. 1}$$

where t_R is the retention time of the analyte, and t_0 is the retention time of the non-retained peak (taken as the first deviation of

the baseline following the injection of 5 μL KNO_3). Three of replicated injections were made to determine the retention time, and the average values were used to calculate the retention factors. The calibration equation of the pH meter was as follows:

$$y = x - 0.66 \quad \text{Eq. 2}$$

where x is the value of the pH meter, and y is the real value of the standard solution.

Evaluation of the results of the chromatographic experiments was carried out by mathematical statistical techniques. The relative error of a single measurement did not exceed 5%. All experimental procedures were performed at an ambient temperature.

Results and Discussion

Chemically modified silica with aqueous–methanol eluents has been widely used in RP-HPLC. The parameters that affect the sorption of the substances onto the stationary phase and, hence, the retention of solutes include many effects, such as the nature of the stationary phase, the lipophilicity of the substance, the concentration of the solute in the mobile phase, the ionic strength of the mobile phase, and the nature and concentration of any competing modifier added to the eluent. The strength of a mobile phase is defined by its polarity, and the ability to dissolve more polar compounds. Solvent selectivity, on the other hand, is the ability to dissolve compounds that have the same polarity. Each solvent will show a favorable kind of interaction with sample solutes having the same polarity. The optimization of solvent strength and/or selectivity is a major goal in chromatographic method development. The complexity of mobile phases used to achieve this goal is different

Usually in RP-HPLC, retention time will increase with the increasing lipophilicity of the substance and with decreasing percentage of the organic modifier in the mobile phase. The character and concentration of any competing modifier added to the mobile phase will determine the retention times and the elution order of the solutes.

In our present paper, the use of ionic liquids as modifiers was studied in reversed-phase chromatographic system. As shown later, although some phases and chromatographic modes separated benzoic acids, the isolation of these isomers on the ordinary C_{18} (alkyl-) bonded phase column in a reversed-phase system with isocratic conditions is an intractable problem. Amino benzoic acid isomers have close values of the octanol–water partition coefficient $\text{Log } P$ (lipophilicity), and this is one of the basic reasons for the difficulty of their isolation with a pure reversed-phase system (Table II). Lipophilicity was calculated using the Chem Office software (19).

In this study, the binary mobile phase of methanol in water (pure reversed-phase system) was used. Generally, organic modifiers (methanol or acetonitrile) added to the eluent will have an influence on the analysis time and sometimes even on the resolution of the analytes. The introduction of an organic modifier will also bring changes in the hydrophobicity of the mobile phases and analytes, and variations in the mobilities of the ana-

lytes and their elution order may occur. To investigate the chromatographic behavior of isomeric acids with a pure reversed-phase system, binary mobile phases of eight different methanol content (5–75% volume) in water were tested. These pilot experiments showed that from a wide range of methanol concentration, if the volume is greater than 50%, samples could not be tested because of the elution of the analytes with too short retention times. Retention of the investigated acids decreased as the methanol concentration of the mobile phase increased. Using 5–15% volume methanol, the *o*-isomer is eluting before the *m*- and *p*-isomers. The two amino benzoic acid isomers could be separated with lower than 15% volume methanol, but the *o*-isomer eluted as very broad peak. The coelution of *p*- and *m*-isomers begins from 15% volume of methanol, and starting with 50% volume of methanol, these isomers can't be separated at all. The *m*- and *p*-isomers show slight separability with pure water–methanol eluents. For these reasons, the eluent with 25% methanol was chosen and used in the future experiments.

It should be noted that in all cases with the water–methanol eluent, the retention factors of the isomeric amino benzoic acids were very low and did not exceed 0.8. These experiments indicated that in a pure reversed-phase system no satisfactory separation can be achieved using only methanol as the modifier of the mobile phase.

pH of the mobile phase containing different types and concentrations of ionic liquids

To investigate the influence of the pH of the mobile phase containing different types and concentrations of ionic liquids, 0.5–64.0 mM/L of ionic liquids were added to the pure mobile phase as additives. The pH of the original pure mobile phase (25% volume of methanol in water) was 6.8, and after addition of different concentrations of ionic liquids, the pH changed clearly as shown Figure 1. Ionic liquids may be either protonic or aprotic in nature. Protonic ionic liquids are formed by proton transfer between molecules that are acids and bases, respectively. Ionic liquids cannot be adequately characterized on the basis of polarity or any single parameter. They are complex entities compared with the relatively simple solvents used in most chromatographic systems.

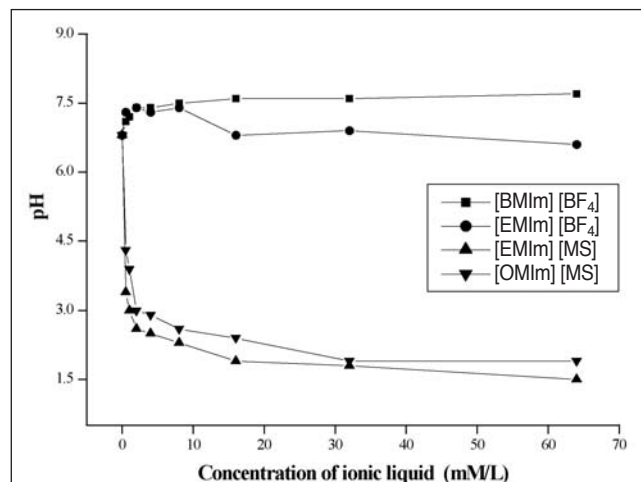


Figure 1. pH of the mobile phase (25% volume methanol in water) containing different amounts of ionic liquids.

graphic processes. They are capable of a wider range of intermolecular interactions than most other traditional mobile phase modifiers. These include dispersion, dipolar, inductive, hydrogen bonding, hydrophobic, and ionic interactions. A multi-parameter scale, that takes into account the many different possible solvent properties that can be used to properly characterize ionic liquids as well as other modifiers.

The pH of the mobile phase containing [BMIm][BF₄] and [EMIm][BF₄] was slightly increased; on the other hand, the pH of the mobile phases containing [EMIm][MS] and [OMIm][MS] were decreased by increasing the ionic liquid concentration. This phenomenon can probably be determined by the structure of the [BF₄] and [MS] anions of the ionic liquids. The structure of the methylsulfate anion ([MS]), includes a hydroxyl group. Thus, the methylsulfate anion can easily donate the hydrogen cations, but tetrafluoroborate ([BF₄]) is a weak basic anion.

Effect of ionic liquid concentration on the retention of amino benzoic acid isomers

As reported by other authors (1,17), ionic liquid cations in the eluent will adsorb onto the C₁₈ silica based surface, causing

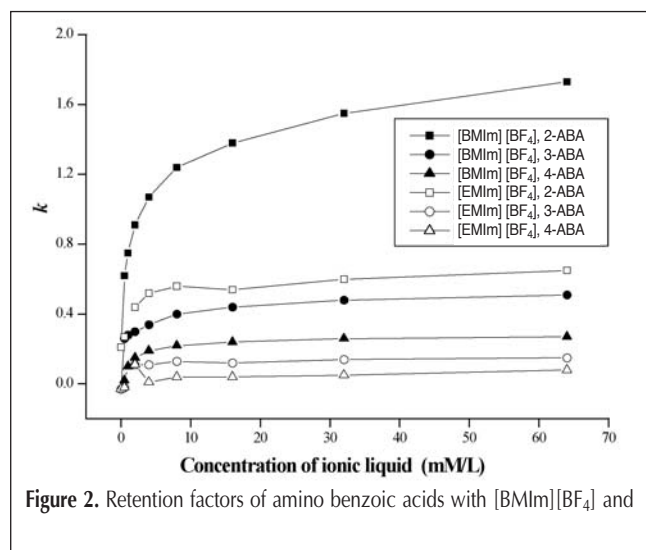


Figure 2. Retention factors of amino benzoic acids with [BMIm][BF₄] and

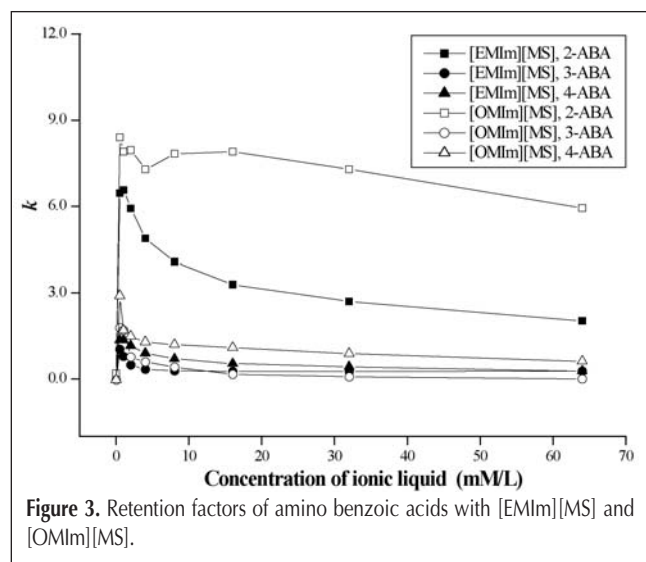


Figure 3. Retention factors of amino benzoic acids with [EMIm][MS] and [OMIm][MS].

changes in their properties. Imidazolium cations can interact with silanol groups and compete with the polar group of the analytes for the silanol groups on the alkylsilica surface in a column. Therefore, it can effectively shield residual silanols and improve peak shape, while also decreasing the retention time of the analytes. Another application of ionic liquid in chromatography is suppression of the deleterious effects of free silanols by using imidazolium tetrafluoroborate ionic liquid (24). Addition of imidazolium tetrafluoroborate ionic liquid to the mobile phases at concentrations of 0.5–1.5% volume as silanol-blocking additives was markedly more efficient than the addition of the standard mobile phase additives, such as triethylamine and dimethyloctylamine.

Used ionic liquids containing imidazolium cation or an inorganic anion absorb UV light, making detection problematic. Alkyl-imidazolium cations have a strong absorbance from 200–280 nm. Thus, [BMIm][BF₄] in water at the concentrations 5×10^{-5} mol/L has an absorbance maximum at approximately 215 nm (15). A 10% (v/v) solution of 1-butyl-3-methyl-imidazolium chloride in acetonitrile has an absorbance of 0.5–2.5 AU in the UV region using a 1 cm cuvette (25). Accordingly, a signal strength depression at UV detection was observed. The influence of the ionic liquid on the detection of the analytes was also evaluated by measuring the peak heights of the individual standards in different concentrations of ionic liquids. The experiments showed that the decreasing trend of the peak heights with increasing ionic liquid concentration in the range of 0.5–64.0 mM/L is negligible. However, this reduction was negligible at 254 nm and did not affect on the results of experiments.

In the next step, the influence of the ionic liquids concentration in the range of 0.5–64.0 mM/L was tested. It is important to note here that after each experiment with a certain concentration of the ionic liquid and before the next experiment with the subsequent concentration of the ionic liquid, the column was flushed for at least 3 h to remove the ionic liquid used at the previous concentration and to fully equilibrate the column.

The constancy of efficiency and peak tailing factor showed that the use of ionic liquids is not harmful to the column. In order to

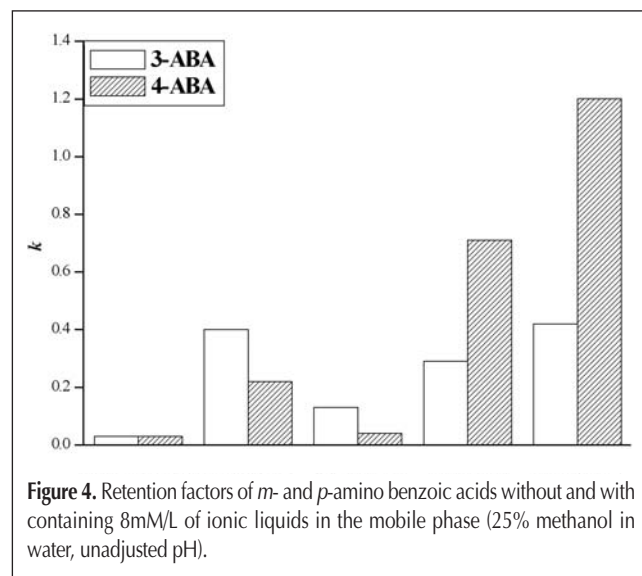


Figure 4. Retention factors of *m*- and *p*-amino benzoic acids without and with 8 mM/L of ionic liquids in the mobile phase (25% methanol in water, unadjusted pH).

test the possible effects of the ionic liquid on the C₁₈ (alkyl)-bonded phase, the column was evaluated before and after exposure to the ionic liquid, using an aqueous methanol media as the mobile phase, with the solutes and benzene as a testing marker.

The dependencies of the retention factors (*k*) on the [BF₄] anion-containing ionic liquid content of the eluent are shown in Figure 2. It is obvious that identical trends were obtained for all analytes. The increase of the [BF₄] anion concentrations of the ionic liquids in the eluents causes an increase of the retention of all sorbats. In this case, the elution order was changed and the *p*-isomer eluted before the *m*- and *o*-isomers. Figure 3 demonstrates the effect of the [MS] anion content of the ionic liquids in the mobile phase on the retention factors of amino benzoic acid isomers. The solute retention generally increases with increasing the concentration of ionic liquids to 0.5mM/L in the eluent, but the retention of the solute decreases with the further increase of the concentration of ionic liquids. Equal dependences were obtained for all analytes. Here, the elution order of *m*-, *p*-, and *o*- was observed for amino benzoic acid isomers.

Ionic liquid concentration remarkably affects the retention and separation of the analytes. Figures 2 and 3 show that they do not clearly change when adding more than 8mM/L of the ionic liquid. It is interesting to note that without ionic liquids, the peaks of the *m*- and *p*-isomers were almost completely overlapped, whereas after the addition of ionic liquids to the eluent these isomers were partially or completely resolved. Figure 4 shows the retention of *m*- and *p*-isomers without ionic liquid and with the mobile phase containing 8mM/L of four different ionic liquids. If ionic liquids were added to the eluent, only partial separation of the *m*- and *p*-isomers was achieved; however, the elution order of the isomers can also be controlled. The previously described data shows that the nature of the ionic liquid distinctly affects the chromatographic behavior of the solutes. However, using the unadjusted mobile phase containing ionic liquids, better results were obtained in the separation of amino benzoic acid isomers than without ionic liquids.

To investigate the effect of the counterion, [EMIm], different counterions ([BF₄] and [MS]) were compared. In comparison to [EMIm][BF₄] (Figure 2), [EMIm][MS] (Figure 3) provides a longer retention time for all analytes, but it also provides better resolution for the *o*-/*m*- and *m*-/*p*-isomers. It should be noted that the anion also has an influence on the elution order. Thus, ionic liquids composed of the inorganic [BF₄] ion with different organic counterions (i.e., [BMIm] and [EMIm]) are compared. The elution order *p* > *m* > *o* was observed with tetrafluoroborate anions, and this sequence was same with a pure water–methanol reversed-phase system.

In the case of methylsulfate anions, the sequence was *m* > *p* > *o*. It has been proposed that the elution order of the amino benzoic acid isomers was determined by the number of contact points available to the solute–adsorbent interactions. The different separations, resulting from two ionic liquids with different counterions as the eluents, may be due to the association with solutes at the water–methanol media, and it seems that [EMIm] with [MS] is superior to that

with [BF₄] in the separation of amino benzoic acid isomers. However, further investigations on the mechanism of such interaction are needed before clearer explanations of the phenomena could be provided.

Optimization and ruggedness of the testing are a part of method development, for instance, application of HPLC in routine analyses. Usually, an optimal chromatographic system is defined in terms of more than one criterion, for example optimum separation and analysis time. Criteria have to be evaluated to achieve these goals with the resolution, selectivity, and the maximum retention factor.

In the chromatographic experiment, the optimum separation condition is determined by factors such as base line resolution, reduction of concentration of the additive, and minimum time of analysis. Complete separation of *m*- and *p*-isomers is achieved at the concentration of [EMIm][MS] 1.0mM/L and [OMIm][MS] 2.0mM/L. With a further increase of the concentration of [EMIm][MS], the resolution of *m*-/*p*- and *p*-/*o*-isomers slowly decreases. Increase of the concentration of [OMIm][MS] results in an analogous tendency. Excellent separation of the isomers is achieved with an eluent of 2.0–8.0mM/L [OMIm][MS] and 1.0–8.0mM/L [EMIm][MS].

Effect of mobile phase pH on the retention factors

For ionic or ionizable solutes, changing the pH or adding an ion-pairing reagent to the mobile phase may result in better separations. The effect of pH (on the degree of protonation and deprotonation) and solutes types (e.g., monoprotic and diprotic acids and bases, as well as zwitterionic compounds) was the cause for the chromatographic retention behavior observed for ionic or ionizable solutes.

The influence of pH on the retention of ionizable solutes was significantly different than the influence of the modifier content. Variations in the mobile phase pH can result in nonlinear retention behavior and therefore the developed models have to be more complex. To provide a satisfactory explanation for the elution order under the existing theories (26–28), the effects of pH in RP-HPLC was investigated. The joint effects of the various factors determine the migration time of the analyte. To investigate the variation of the elution order and elution time with pH change, mobile phases with five different pH values were tested.

Table III compares the retention factors and resolutions of *m*- and *p*-amino benzoic acids on mobile phases with and without

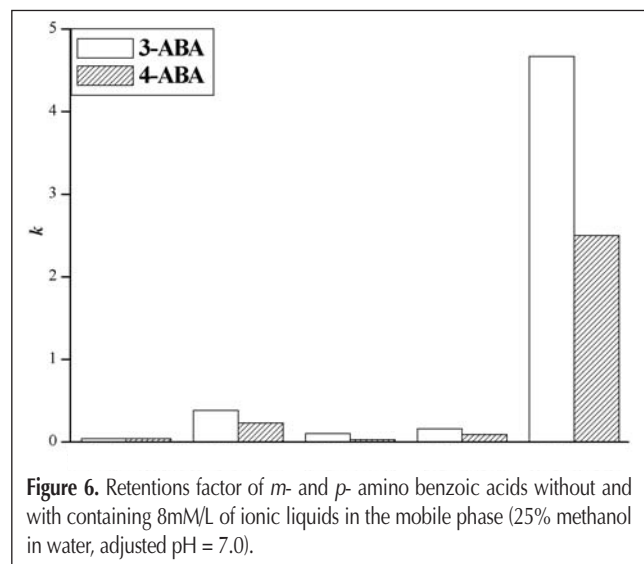
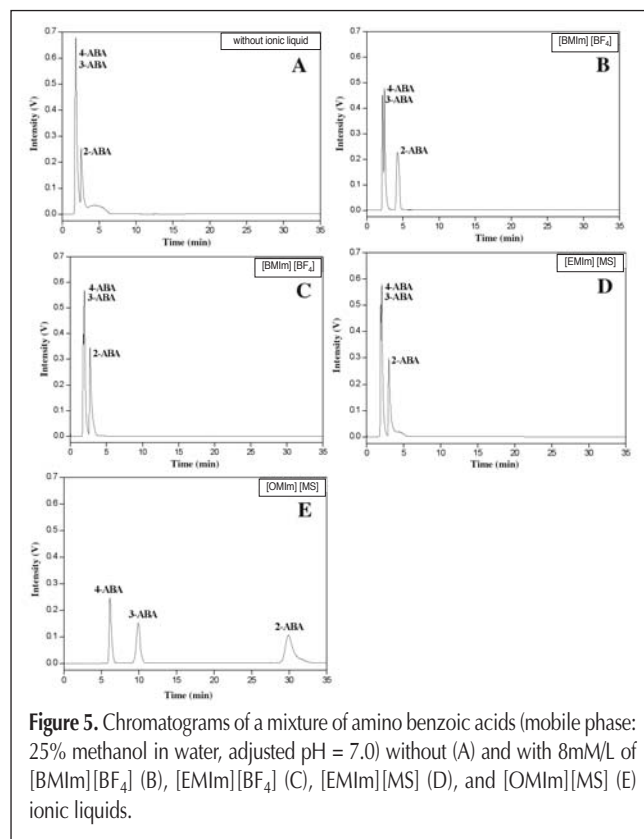
Table III. Retention Factor (*k*) and Resolution (*R_s*) of *m*- and *p*-Amino Benzoic Acids With and Without 8mM/L of Ionic Liquid in Mobile Phase

Mobile phase (water–MeOH, 75:25 vol. %)	unadjusted pH		pH = 3.0			pH = 7.0			<i>R_s</i>
	<i>k</i>		<i>R_s</i>	<i>k</i>		<i>R_s</i>	<i>k</i>		
	3-ABA	4-ABA		3-ABA	4-ABA		3-ABA	4-ABA	
without ionic liquid	0.03	0.03	0.00	0.68	1.24	2.06	0.04	0.04	0.00
[BMIm][BF ₄]	0.40	0.22	1.07	0.68	1.30	2.26	0.38	0.23	0.89
[EMIm][BF ₄]	0.13	0.04	0.44	0.71	1.30	2.03	0.10	0.03	0.31
[EMIm][MS]	0.71	4.08	2.37	0.71	1.27	2.48	0.16	0.09	0.42
[OMIm][MS]	1.20	7.83	2.55	0.65	1.28	1.71	4.67	2.50	4.19

8mM/L of ionic liquids, and the pH of the mobile phase was unadjusted and adjusted to 3.0 and 7.0 by HCl. Table III clearly shows the effect of pH.

In the two (protonated or deprotonated state) states, the solutes will be retained to a different degree. The solutes were either in their protonated or deprotonated form completely for pH values, different from the ionization constant. The complicating aspect of pH as an independent variable in mobile phase optimization is the multiplex character of the response (the retention factor) as a function of this pH.

At lower pH, the ionizable analytes are fully protonated. The elution order of the amino benzoic acid isomers was *m*-, *p*-, and



o- at pH 3.0, the retention factors of the three isomers were very stable, and also all substances were fully separated, even without an ionic liquid. Thus, the acidic working environment renders improved scope for optimal separation in a pure reversed-phase system. When the pH of the mobile phase was adjusted to 3.0 for the separation of *m*- and *p*-amino benzoic acids, an optimum resolution value ($1.5 \leq R_s \leq 2.0$) was obtained with the [OMIm][MS] ionic liquid.

Adjusting the pH from 3.0 to 7.0, the elution order of *p*- and *m*-isomers changed (Table III, Figures 5 and 6). Figure 5 shows the chromatograms on a mobile phase of pH = 7.0 with and without 8mM/L of ionic liquids. The retention factors of *m*- and *p*-amino benzoic acids with and without the addition of ionic liquids to the mobile phase are displayed in Figure 6. The retention factors were constant, but the resolution was unsatisfactory except for the [OMIm][MS] ionic liquid. When the pH of the mobile phase was adjusted to 7.0, and using the [OMIm][MS] ionic liquid, *m*- and *p*-amino benzoic acids were completely separated, as oppose to the others. This result may be explained by potential interactions between the [MS] anion and the solute and by the high hydrophobicity of the long alkyl chain of the ionic liquid cation with neutral condition (pH 7.0). It should be noted that when the pH of the mobile phases was adjusted to 4.0, 5.0, and 6.0 values, every isomer existed in two form: as a molecular form and as an ionic form, and both forms were detected in two peaks. The amino benzoic acid isomers include amino and hydroxyl groups; thus, they are affected very much by the pH variety of the mobile phase. Therefore, when the mobile phase is in acidic or approximately neutral conditions (i.e., pH = 3.0 or pH = 7.0), the amino benzoic acid isomers will have just one molecular form or ionic form. The retention factor of ionizable solutes varies considerably when the pH is changed. These results have shown that not only the addition of modifiers is important, but also adjusting the mobile phase pH.

The reason for the elution order was assumed to be the position of the amine group. Amino benzoic acid isomers have an interaction between the amino group and the acidic group. Therefore, its original hydrophobicity depends on the distance between them; in the other words, the original hydrophobicity of *m*- and *p*- isomers in the pure mobile phase or in neutral condition (pH = 7.0) results in the sequence *m*- < *p*-, but in pH = 3.0, their hydrophobicity will be opposite because the *p*- isomer will interact more than *m*- isomer with the hydrogen cation.

Unfortunately, the existing theories (11,13) and our presented investigations cannot provide a satisfactory explanation for the complex effects of ionic liquids on separation. At this time, additional experiments with acetonitrile and other solvent to separate compounds are in progress, and a comprehensive evaluation of the interaction mechanism of ionic liquids and their application in HPLC is developed.

Conclusion

In this paper, the effects of four ionic liquids and changes of the pH on the retention and separation of amino benzoic acid isomers was studied. Ionic liquids showed promising perfor-

mance as additives in the separation of these isomers, as discussed. The length of the alkyl group on the imidazolium ring and its counterion and the concentrations of the ionic liquids can also affect the separation. According to these results, it was assumed that the separation mechanism is complex, and when ionic liquids are used as additives in HPLC, the strong interactions between the imidazolium cation and its counterion and solutes will also play important roles. Part of the ionic liquids coat on the surface of the stationary phase, on which they suppress the free silanol groups and improve the shape of peaks and their resolution. As a result, excellent separation of these isomers was achieved using 2.0–8.0mM/L [OMIm][MS] and 1.0–8.0mM/L [EMIm][MS] as the modifier with eluents of unadjusted pH. It was assumed that the elution order depends on the pH of the mobile phase. The role of the ionic liquids is multiple, and further investigations are needed in order to qualitatively and quantitatively explain the phenomena.

Acknowledgment

The authors are grateful for the financial support of the Center for Advanced Bioseparation Technology, Inha University.

References

- X. Xiao, Z. Liang, L. Xial, and S. Jiang. Ionic liquids as additives in high performance liquid chromatography analysis of amines and the interaction mechanism of ionic liquids. *Anal. Chim. Acta.* **519**: 207–11 (2004).
- J. Liu, J. Jonsson, and G. Jiang. Application of ionic liquids in analytical chemistry. *Trends Anal. Chem.* **24**: 20–27 (2005).
- D. Zhao, M. Wu, Y. Kou, and E. Min. Ionic liquids: applications in catalysis. *Catal. Today* **74**: 157–89 (2002).
- C.M. Gordon. New developments in catalysis using ionic liquids. *Appl. Catal. A* **222**: 101–17 (2001).
- Z. Yang and W. Pan. Ionic liquids: Green solvents for nonaqueous biocatalysis. *Enzyme Microb. Technol.* **37**: 19–28 (2005).
- J. Sun, S. Fujita, and M. Arai. Development in the green synthesis of cyclic carbonate from carbon dioxide using ionic liquids. *J. Organomet. Chem.* **15**: 3490–97 (2005).
- J. Liu, N. Li, G. Jiang, J. Liu, J.A. Jonsson, and M. Wen. Disposable ionic liquid coating for headspace solid-phase microextraction of benzene, toluene, ethylbenzene, and xylenes in paints followed by gas chromatography-flame ionization detection. *J. Chromatogr. A* **1066**: 27–32 (2005).
- J. Peng, J. Liu, G. Jiang, C. Tai, and M. Huang. Ionic liquid for high temperature headspace liquid-phase microextraction of chlorinated anilines in environmental water samples. *J. Chromatogr. A* **1072**: 3–6 (2005).
- K. Tian, S. Qi, Y. Cheng, X. Chen, and Z. Hu. Separation and determination of lignans from seeds of Schisandra species by micellar electrokinetic capillary chromatography using ionic liquid as modifier. *J. Chromatogr. A* **1072**: 181–87 (2005).
- C. He, S. Li, H. Liu, K. Li, and F. Liu. Extraction of testosterone and epitestosterone in human urine using aqueous two-phase systems of ionic liquid and salt. *J. Chromatogr. A* **1072**: 143–49 (2005).
- L. He, W. Zhang, L. Zhao, X. Liu, and S.X. Jiang. Effect of 1-alkyl-3-methylimidazolium-based ionic liquids as the eluent on the separation of ephedrine by liquid chromatography. *J. Chromatogr. A* **1007**: 39–45 (2003).
- L. He, Jr., W.Z. Zhang, B. Wen, X. Liu, and S.X. Lang. Separation of ephedrine using 1-butyl-3-methylimidazolium tetrafluoroborate ionic liquids as eluent in high-performance liquid chromatography (HPLC). *Chin. Chem. Lett.* **14**: 603–604 (2003).
- M.P. Marszał?, T. Baczek and R. Kaliszan. Reduction of silanophilic interactions in liquid chromatography with the use of ionic liquids. *Anal. Chim. Acta.* **547**: 172–78 (2005).
- M.M. Waichigo, T.L. Riechel, and N.D. Danielson. Ethylammonium acetate as mobile phase modifier for reversed phase liquid chromatography. *Chromatogr.* **61**: 17–23 (2004).
- W. Zhang, L.J. He, L. Xia, and S.X. Jiang. Ionic liquids as mobile phase additives for separation of nucleotides in HPLC. *Chin. J. Chem.* **22**: 549–52 (2004).
- C.H. Lee, J.W. Lee, and K.H. Row. Optimum solvent selectivity and gradient mode for deoxyribonucleosides in reversed-phase high-performance liquid chromatography. *J. Chromatogr. A* **1072**: 337–44 (1998).
- Y. Polyakova, Y. M. Koo, and K. H. Row. Application of ionic liquids as mobile phase modifier in HPLC. *Biotechnol. Bioprocess Eng.* **11**: 1–6 (2006).
- T. Gichner, G. Voutsinas, A. Patrinely, A. Kappas, and M.J. Plewa. Antimutagenicity of three isomers of amino benzoic acid in Salmonella typhimurium. *Mutation Research* **309**: 201–10 (1994).
- D. Strack. ChemOffice Ultra 2000 CambridgeSoft, Phytochemistry. **57**: 143–44 (2001).
- C. Allen Chang, Q. Wu, and L. Tan. Normal-phase high-performance liquid chromatographic separations of positional isomers of substituted benzoic acids with amine and β -cyclodextrin bonded-phase columns. *J. Chromatogr. A* **361**: 199–207 (1986).
- M.W.F. Nielen. Impact of experimental parameters on the resolution of positional isomers of aminobenzoic acid in capillary zone electrophoresis. *J. Chromatogr. A* **542**: 173–83 (1991).
- Q.H. Wan, P.N. Shaw, and D.A. Barrett. Chromatographic behaviour of positional isomers on porous graphitic carbon. *J. Chromatogr.* **697**: 219–27 (1995).
- R. Kaliszan, M.P. Marszał, M.J. Markuszewski, T. Baczek, and J. Pernak. Suppression of deleterious effects of free silanols in liquid chromatography by imidazolium tetrafluoroborate ionic liquids. *J. Chromatogr. A* **1030**: 263–71 (2004).
- W. Zhang, L. He, Y. Gu, X. Liu, and S. Jiang. Effect of ionic liquids as mobile phase additives on retention of catecholamines in reversed-phase high-performance liquid chromatography. *Anal. Lett.* **36**: 827–38 (2003).
- M. Koel. Physical and chemical properties of ionic liquids based on the dialkylimidazolium cation. *Proc. Estonian Acad. Sci. Chem.* **49**: 145–55 (2000).
- B.J. Bassler, R. Kaliszan, and R.A. Hartwick. Retention mechanisms on metallic stationary phases. *J. Chromatogr. A* **461**: 139–47 (1989).
- G. Gu and C.K. Lim. Separation of anionic and cationic compounds of biomedical interest by high-performance liquid chromatography on porous graphitic carbon. *J. Chromatogr. A* **515**: 183–92 (1990).
- Y.I. Yashin. Chromatographic retention parameters and molecular structure. *Pure & Appl. Chem.* **61**: 2021–26 (1989).

Manuscript received May 9, 2006;
revision received October 25, 2006.