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# Retention Factors and Resolutions of Amino Benzoic Acid Isomers with Some Ionic Liquids

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**Abstract** Ionic liquids in the form of organic salts are being widely used as new solvent media. In this paper three positional isomers, *o*-amino benzoic acid, *m*-amino benzoic acid, and *p*-amino benzoic acids were separated with four different ionic liquids as mobile phase additives using high performance liquid chromatography (HPLC). The following ionic liquids were used: 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIm][$BF_4$]), 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIm][$BF_4$]), 1-ethyl-3-methylimidazolium methylsulfate ([EMIm][MS]), and 1-octyl-3-methylimidazolium methylsulfate ([OMIm][MS]). The effects of the alkyl group length on the imidazolium ring and its counterion, and the concentrations of the ionic liquids on the retention factors and resolutions of amino benzoic acid isomers were tested. The results of the separations with ionic liquids as the eluents were better than those without ionic liquids. Excellent separations of the three isomers were achieved using 2.0~8.0 mM/L [OMIm][MS] and 1.0~8.0 mM/L [EMIm][MS] as the eluent modifiers.

*Keywords*: ionic liquid, amino benzoic acid, retention factor, resolution, RP-HPLC

## INTRODUCTION

As novel solvents, ionic liquids (ILs) are now widely recognized as important components of green chemistry. Unlike traditional solvents, ionic liquids are composed of ions. Their unique properties such as non-volatility, non-flammability, and excellent chemical and thermal stability have made them an environmentally attractive alternative to conventional organic solvents [1]. Ionic liquids have some unique properties such as negligible vapor pressures, good thermal stabilities, tunable viscosities, strong polarities, and they are miscible with water and organic solvents. In addition, they have good extractabilities with various organic compounds and metal ions [2]. Ionic liquids have been applied to catalysis [3,4], biocatalysis [1], synthesis [5], analytical chemistry [2], *etc.* this suggests that they might also provide exclusive and interesting possibilities for separation science [6-16]. However, one should note that when ionic liquids are diluted or immobilized on a stationary support, they may not possess all the properties of ordinary ionic liquids. In some cases they may maintain several kinds of intermolecular interactions, which can be useful for chromatographic separations [14,15,17]

Recently, numerous amine and benzene compounds have been developed as medical and biological agents. During the late 20th century they were introduced as orally active compounds in medicine, and the number of such compound drugs and vitamins is likely to expand in the near future. In particular, the amino class of compounds has supplied many effective drugs that are currently in clinical and preventive use, and newer compounds with an expanded spectrum of activity are continuously in development. An interdisciplinary research approach to their chemistry is currently directed towards medicinal and supramolecular chemistry, as well as towards advanced organic materials science. Amino benzoic acid is a biologically activity substance and its *p*-isomer is found in a group of water-soluble vitamins [18,19]. It is a well-known fact that these vitamins play many important roles in numerous biological processes.

Data gathering related to the chromatographic behaviors of biologically active compounds is one of the most important fields in modern chemistry; giving useful information for the physical, combinatorial, and medicinal chemistries. In addition, modern liquid chromatography is a powerful separation method, although the separation of isomers remains difficult. Furthermore, from the theory of adsorption point of view of substances adhering to surfaces, studying the chromatographic behavior of isomers has practical applications and theoretical interests.

In this study, four different types of ionic liquids were used as mobile phase modifiers in HPLC to separate three amino benzoic acid isomers. The retention factors and resolutions of these isomers were determined from a mobile phase containing the ionic liquids. We investigated the characteristic effects of the individual ionic liquid concentrations on the chromatographic retention and

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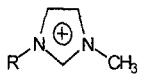
**Table 1.** Names and structures of the ionic liquids

| No. | Ionic liquid | Cation  | Anion | Formula |
|---|---|---|---|---|
| 1 | [BMIm][$BF_4$] | 1-Butyl-3-methylimidazolium | Tetrafluoroborate | $C_8H_{15}BF_4N_2$ |
| 2 | [EMIm][$BF_4$] | 1-Ethyl-3-methylimidazolium | Tetrafluoroborate | $C_6H_{11}BF_4N_2$ |
| 3 | [EMIm][MS] | 1-Ethyl-3-methylimidazolium | Methylsulfate | $C_7H_{14}N_2O_4S$ |
| 4 | [OMIm][MS] | 1-Octyl-3-methylimidazolium | Methylsulfate | $C_{13}H_{26}N_2O_4S$ |

separation of the amino benzoic acid isomers.

## MATERIALS AND METHODS

### Reagents

The four ionic liquids (99.99%) used in this study are presented in Table 1. These liquids were purchased from C-tri Co. (Namyang, Korea) and include the following: 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIm][$BF_4$]), 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIm][$BF_4$]), 1-ethyl-3-methylimidazolium methylsulfate ([EMIm][MS]), and 1-octyl-3-methylimidazolium tetrafluoroborate ([OMIm][MS]). The *o*- and *m*-amino benzoic acids were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and *p*-amino benzoic acid from Fluka (St. Louis, MO, USA). All reagents were analytical grade and some of the properties are listed in Table 2. Potassium nitrate ($KNO_3$) was purchased from Kanto Chemical Co. (Japan) to measure the dead volume. HPLC grade methanol ($CH_3OH$) was purchased from Duksan Pure Chemical Co. (Ansan, Korea), and distilled water was filtered with a vacuum pump (Waters Division of Millipore, USA) and filter (HA-0.45, Waters Division of Millipore) before use.

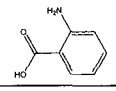
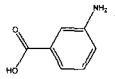
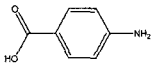
### Apparatus

An instrument with a 600 HPLC pump (Waters, USA), a 486 detector (M 7200 Absorbance Detector, Young-In Scientific Co., Korea), and a Reodyne injection valve with a 20 μL sample loop was used in this study. Chromate software (Version 3.0 Interface Eng., Korea) connected to a PC was used as a data acquisition system. The experiments were performed with the commercially available (Optimapak, Korea) $C_{18}$ (alkyl-) bonded phase column (4.6 × 150 mm i.d. and particle size 5 μm).

### Chromatographic Conditions

The individual amino benzoic acids were dissolved in water at a concentration of 1 mg/mL. A mixture of the amino benzoic acids was prepared from aliquots of the individual *o*-, *m*-, and *p*-isomer solutions in the ratio of 5:5:1. The mobile phases were 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 50.0, and 75.0 vol. % of methanol in water

**Table 2.** Names, structures, and lipophilicities (Log P) of the amino benzoic acids

| Compound[a] | Formula & Molecular weight | Structure | Log P |
|---|---|---|---|
| *o*-Amino benzoic acid (2-ABA) | $C_7H_7NO_2$ 137.14 |  | 0.79 |
| *m*-Amino benzoic acid (3-ABA) | |  | 0.18 |
| *p*-Amino benzoic acid (4-ABA) | |  | 0.78 |

[a]ABA: amino benzoic acid.

(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) of ILs in eluent (25.0 vol. % of methanol in water) were used. The flow rate was fixed at 1.0 mL/min and carried out on isocratic mode. A constant injection volume (5 μL) was used throughout for the individual solutions. The injection volume of the mixture was 15 μL. The detection wavelength used was 254 nm. The solutions were stored at 4°C and the working standards were re-prepared every two days to avoid potential errors from the decomposition of the targets. The retention factor $k$ was calculated for the isomers using the following formula:

$$k = (t_R - t_0)/t_0 \quad (1)$$

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 replicated injections were completed to determine the retention times, and the average values were used to calculate the retention factors. The resolution ($R_s$) was calculated using the following equation:

$$R_s = \frac{t_{R2} - t_{R1}}{(w_2 + w_1)/2} = \frac{2\Delta t}{w_2 + w_1} \quad (2)$$

where $t_{R1}$ and $t_{R2}$ were the retention times of the first and second peaks ($t_{R1} < t_{R2}$), respectively, and $w_1$ and $w_2$ were

the peak widths of the first and second peaks, respectively.

The retention factors reported in this study were the averages of at least three determinations. An evaluation of the experimental chromatographic results was carried out by mathematical and 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 acetonitrile-eluent has been widely used in HPLC. The parameters that affect the sorption of a substance onto the stationary phase, and hence the retention of the solutes, can depend on the nature of the stationary phase, the lipophilicity of the substance, the concentration of the solutes in the mobile phase, the ionic strength of the mobile phase, the nature and concentration of any competing modifier added to the mobile phase, and the nature and concentration of any competing modifier added to the eluent.

Usually in reversed-phase HPLC, the retention time will increase as the lipophilicity of the substance is increased, and as the percentage of the modifier in the mobile phase is decreased. The character and concentration of any competing modifier that is added to the mobile phase will determine the retention times and the elution orders for the solute ions.

In this paper, we studied adjustments that were made to ionic liquids as modifiers. Each analytic test was usually performed in a reversed-phase system. As it is presented below, some phases and chromatographic modes separated the different benzoic acid isomers, however, isolating these isomers on an ordinary $C_{18}$ (alkyl-) bonded reversed-phase column with isocratic mode conditions is an intractable problem. The amino benzoic acid isomers have similar values for the octanol/water partition coefficient Log P (lipophilicity), which is one of the primary reasons they were difficult to isolate with a pure reversed-phase system (Table 2). The calculation for lipophilicity was performed using the Chem Office software [19].

The separation of positional benzoic acid isomers with amine and β-cyclodextrin bonded-phase columns in normal-phase HPLC has previously been achieved [20]. Nielen investigated the impact of experimental parameters on the resolutions of amino benzoic acid isomers in capillary zone electrophoresis [21]. Wan *et al.* examined the effects on the retentions and resolutions of isomeric amino benzoic acids showing that separation can be obtained on a Hypercarb special porous graphitic column with a phosphate buffer-acetonitrile mobile phase [22].

In our research, the positional isomers of amino benzoic acids were used as model compounds to study the effects of different ionic liquid eluents. The retention factors and resolutions of the isomers were determined with a mobile phase comprised of ionic liquids in a water-methanol solvent. Table 1 shows the specific ionic liquids that were investigated. The names, structures, molecular weights, and Log P values of the sorbats are listed in Table 2.

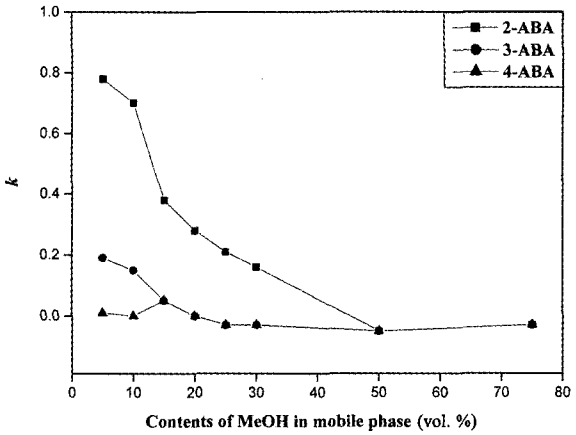



**Fig. 1.** Retention factors of amino benzoic acids with various methanol concentrations (MeOH) in the mobile phase.

### The Effects of Methanol on the Retention Behaviors of Amino Benzoic Acid Isomers

To examine the chromatographic behaviors of the isomeric acids with a pure reversed-phase system, binary mobile phases of eight different methanol concentrations (5, 10, 15, 20, 25, 30, 50, and 75 vol. %) in water were used. Samples could not be tested with methanol concentrations ranging higher than 75 vol. % due to the elution of analytes with very short retention times. The retention of acids was decreased as the methanol concentration increased in the mobile phase (Fig. 1). The elution order that was observed for the amino benzoic acid isomers was the *p*-isomer eluting before the *m*- and *p*-isomers with an eluent that contained 5~15 vol. % of methanol. All three amino benzoic acid isomers were separated using concentrations lower than 15 vol. % methanol, but the *p*-isomer could be eluted over a wide range. The co-elution of the *p*- and *m*-isomers started at a 15 vol. % of methanol, but beginning at the 50 vol. % concentration of methanol these isomers did not separated completely. The *m*- and *p*-isomers showed slight separabilities with the pure water-methanol eluents.

In all cases the retention factors for the isomeric amino benzoic acids were very low and did not exceed 0.8. The results of these experiments indicate that in a pure reversed-phase system, isomeric separation was not satisfactorily achieved using only methanol as the modifier in the mobile phase.

This completed study allowed us to select the mobile phase composition for further experiments using the ionic liquids. It is well known that the use of a highly aqueous solution (as the HPLC mobile phase) may collapse the $C_{18}$ (alkyl-) bonded phase and result in a retention time shift. At the same time, we found that a relatively high content of methanol in the eluent caused the convergence of retention times for the analytes. For these reasons an eluent of 25% methanol was chosen.

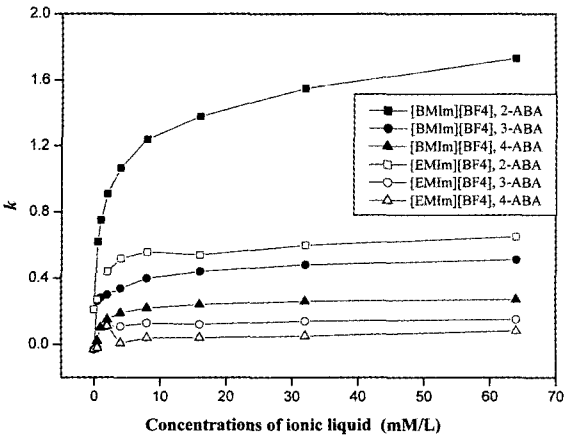



**Fig. 2.** Retention factors of amino benzoic acids with [BMIm][BF4] and [EMIm][BF4] (mobile phase 25% vol. methanol in water).

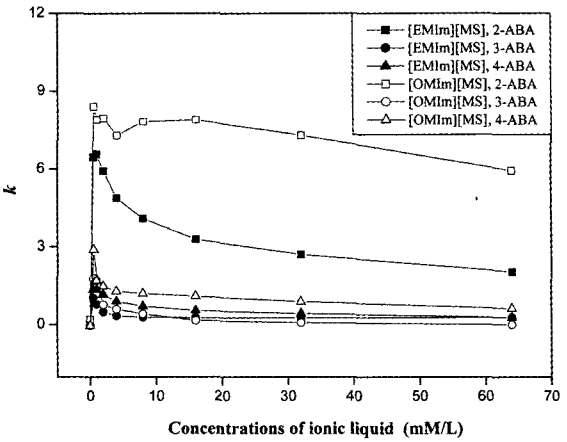



**Fig. 3.** Retention factors of amino benzoic acids with [EMIm][MS] and [OMIm][MS] (mobile phase 25% vol. methanol in water).

## The Effects of the Ionic Liquid Concentrations on the Retentions and Resolutions of Amino Benzoic Acid Isomers

In this next step we tested the influence of ionic liquid concentrations that ranged from 0.5~64.0 mM/L. The ionic liquids that were used were chosen because they are water-soluble (hydrophilic) and combine the most common cations and anions. It is important to note that after each experiment the column was flushed for at least 3 h to remove the previously used ionic liquid concentration and/or to fully equilibrate the column.

The constancy in the efficiency and peak tail factors showed that the use of the ionic liquids was not harmful to the column. To test the possible effects of the ionic liquids on the $C_{18}$ (alkyl-) bonded phase, the column was evaluated before and after exposure to each ionic liquid using a methanol-water solution as the mobile phase with the solutes.

The retention factors ($k$) based on the eluents containing the [$BF_4$] anion-containing ionic liquids are shown in Fig. 2. It is clear that identical trends were obtained for all analytes. Increases in the [$BF_4$] anion concentration of the eluents caused increased retentions of all the sorbats. In this case the elution order was changed, and the *p*-isomer eluted before the *m*- and *o*-isomers. Fig. 3 demonstrates the effects of the mobile phase ionic liquids [MS] anion content on the retention factors of the amino benzoic acid isomers. The retentions of the solutes generally increased with 0.5 mM/L increases in the ionic liquid concentration, but there was a threshold concentration above, which the solute retentions decreased with further increases in the ionic liquid concentration. The retention results obtained for the analytes showed they were all equally dependent on the ionic concentrations of the eluents. Here, the observed elution orders were *m*-, *p*-, and *o*- for the amino benzoic acid isomers.

The concentrations of the ionic liquids remarkably affected the retentions and separations of the analytes (Fig. 4). It is interesting to note that when the eluent did not contain ionic liquids, the peaks of the *m*- and *p*-isomers were almost completely overlapped, whereas after the addition of the ionic liquids these isomers were partially (b~d) or completely (e) resolved.

The effects of [$BF_4$] and [MS] ionic liquid concentrations on the resolutions ($R_s$) of the isomers are shown in Figs. 5 and 6, respectively. As a rule in chromatographic experiments, optimal separation conditions are determined by factors such as a resolution value above 1.5 and reductions in the additive concentration and the time required for analysis. Complete separation of the *m*- and *p*-isomers was achieved at concentrations of 1.0 mM/L [EMIm][MS] and 2.0 mM/L [OMIm][MS]. However, with a further increase in the [EMIm][MS] concentration the resolution of the *m*-/*p*- and *p*-/*o*-isomers slowly decreased. Increases in the concentration of [OMIm][MS] provoked an analogous tendency. Ultimately, excellent separations of the isomers were achieved with eluents in the ranges of 2.0~8.0 mM/L [OMIm][MS] and 1.0~8.0 mM/L [EMIm][MS].

With the ionic liquid adjustments of the eluent, the separation efficiencies and symmetries of the four peaks were greatly improved indicating that the ionic liquids had the ability to shield residual silanols.

Although ionic liquids are rather "young" modifiers, their great potential for use in HPLC has already been demonstrated. Most authors explain the influencing separation mechanisms of the ionic liquids according to the interactions of the cations with the surface sorbent. Previous studies have shown that ionic liquids form layers (pseudo-stationary phase) on the surface of the modified silica gel [16,23]. Thus with low ionic liquid concentrations, the cations could interact and compete with the silanol groups (specific electrostatic interactions) on the alkyl silica base surface, and with the polar groups of the analytes. At the same time, the nonpolar alkyl groups of the stationary phase could interact with the different alkyl groups of the heterocyclic ring or the quaternary cations (unspecific type of interactions and hydrophobic interactions). Therefore, this phenomenon can efficiently shield

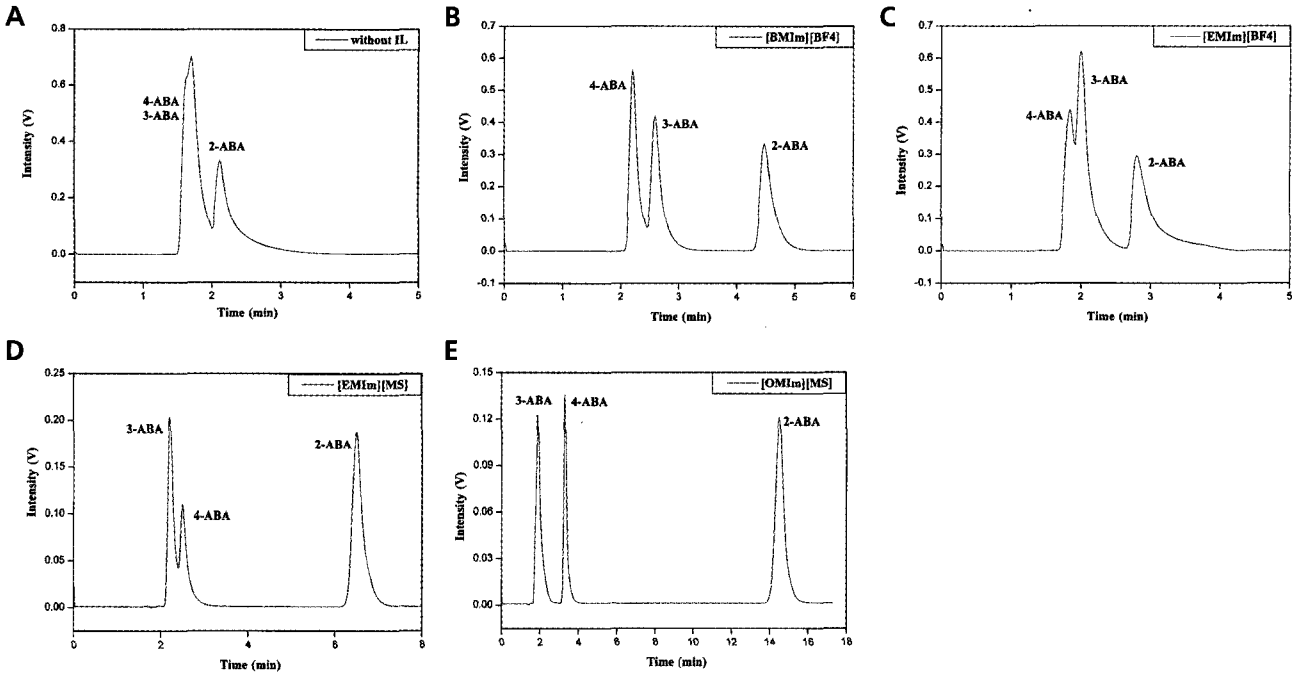



**Fig. 4.** Chromatograms of an amino benzoic acid isomer mixture on mobile phases (25% vol. methanol in water) with and without 32 mM/L of ionic liquid; (A) no ionic liquids, (B) [BMIm][BF4], (C) [EMIm][BF4], (D) [EMIm][MS], and (E) [OMIm][MS].

the residual silanols and improve the peak shapes, as well as influence the chromatographic retention times of the sorbates.

The relationship between the concentrations of the ionic liquid modifiers and the thickness and stability of this layer may generally be qualified using the following factors. The addition of an ionic liquid modifier to a mobile phase leads to competition between the ionic liquid cations and the polar groups of the sorbates, affecting the polar silanols groups on an alkyl silica surface. Thus, the ionic liquid modifier also disables the alkyl groups of the stationary phase. This leads to a sharp decrease in the possibility for dispersion interactions between the sorbate and the alkyl groups of the stationary phase. If the concentrations of the ionic liquid are slightly increased, cation interactions with the silanol groups on the alkyl silica surface due to specific interactions, or due to hydrophobic and non-specific interactions, gradually strengthen. This then results in an increase in the carbon content of the stationary phase.

With a further increase in the ionic liquid modifier concentration, cations interact with the silanol groups through electrostatic interactions, producing a weak bilayer electronic structure. Cations also interact with the alkyl groups through hydrophobic and non-specific interactions. Thus, we have one explanation for better understanding of the dynamic modification of the sorbent by an ionic liquid, and the formation of the pseudo-stationary phase. Unfortunately, the influence of the ionic liquids on chromatographic retention is currently unclear. The latter fact together with the successful examples of ionic liquids being used in chromatographic separations offers a powerful stimulus for further studies in this direction.

According to the works cited, ionic liquids may suppress the free silanols on the stationary phase, resulting in better retention of the acids under investigation. In addition, the attraction between imidazolium cations and the ionized solutes may play an important role.

## The Effects of the Alkyl Groups of Imidazolium Cations and the Retentions and Resolutions of Amino Benzoic Acid Isomers

To study the effects of different alkyl groups of imidazolium cations on the separation of amino benzoic isomers, we made additions of two ionic liquids of equal concentrations consisting of different alkyl groups ([BMIm] [EMIm]) and a similar anion [$BF_4$]. It is apparent that [BMIm][$BF_4$] provided a better resolution; however, the improvement is at the expense of a longer analytical time.

With an increase in alkyl chain length on the imidazolium cations, the retentions of all compounds were increased (Fig. 2) and the resolutions were also increased (Fig. 5). As presented above, [BMIm][$BF_4$] and [EMIm] [$BF_4$] did not allow for the successful separation of *m-/p-* isomers at a full range of tested concentrations (0.5~65.0 mM/L), but *o-/m-* isomers were adequately isolated ($R_s$ > 1.5) on 2.0 mM/L.

In comparison with a pure reversed-phase system (in our case a water-methanol system), these ionic liquids significantly improved the peak shape, decreased the peak tailing, and increased the resolution; the reason may be that [BMIm][$BF_4$] and [EMIm][$BF_4$] shielded the residual silanols. It seems that [BMIm][$BF_4$] was the most effective eluent in shielding residual silanol, and perhaps

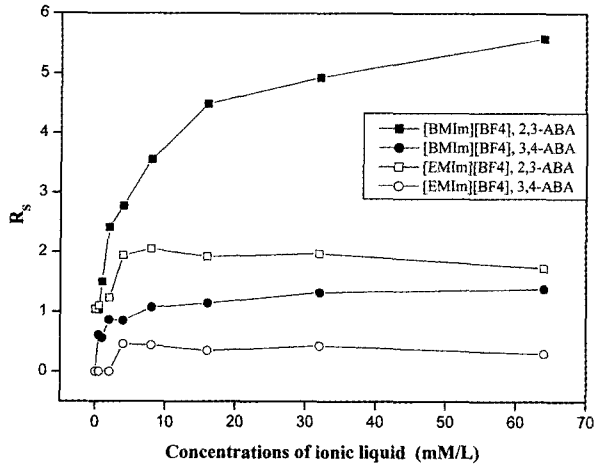



**Fig. 5.** Resolutions of amino benzoic acids with [BMIm][BF4] and [EMIm][BF4] (mobile phase 25% vol. methanol in water).

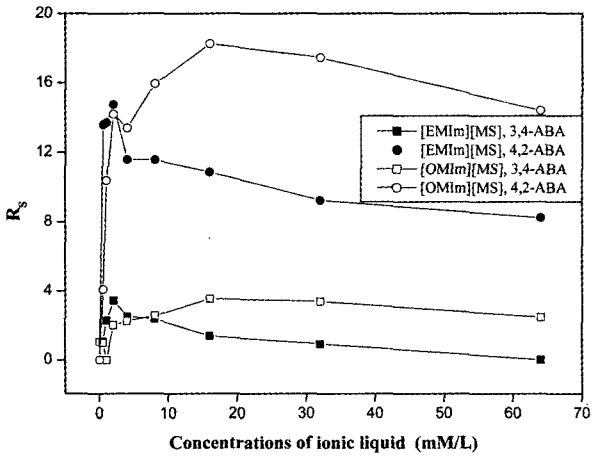



**Fig. 6.** Resolutions of amino benzoic acids with [EMIm][MS] and [OMIm][MS] (mobile phase 25% vol. methanol in water).

this is the reason why peak forms and resolutions improved with an increase in alkyl chain length.

### The Effects of Counterions on the Retention and Resolution of Amino Benzoic Acid Isomers

The data mentioned above show that the nature of the ionic liquid distinctly affects the chromatographic behavior of the solutes. To investigate the effects of counterions, [EMIm] with [$BF_4$] and [MS] counterions were compared. As Figs. 2 and 3 show, the [EMIm][$BF_4$] and [EMIm][MS] eluents provided longer retention times for all of the analytes, however, they had better resolutions ($R_s$) for the *o*-/*m*- and *m*-/*p*- isomers (Figs. 5 and 6). It should be noted that the anions also had an influence on the elution order. Thus, ionic liquids composed of organic [BMIm] with different inorganic counterions, *i.e.* [$BF_4$] and [MS] were compared. The elution order of *p* > *m* > *o* was observed with tetrafluoroborate anions and this succession was found to be the same with a pure water-methanol reversed-phase system.

In the case of methylsulphate anions the resulting succession was *m* > *p* > *o*. It has been proposed that the number of contact points available for solute adsorbent interactions determines the elution order of the amino benzoic acid isomers. The dissimilar separations that resulted from the two ionic liquids containing different counterions as the eluents, may be due to an association with solutes in the water-methanol media. It seems that an eluent of [EMIm] with [MS] is superior to that with [$BF_4$] in the separation of amino benzoic acid isomers.

Unfortunately, the existing theories [24-26] and the investigation we presented here cannot provide a satisfactory explanation for the elution orders that are seen for isomers. Neither is consistent with the predictions that the *m*-isomer should be eluted before the *o*- and *p*-isomers with [MS] anion-containing ionic liquids. At this time additional experiments are in progress to separate other compounds. Also comprehensive conversations are developing on the interaction mechanisms of ionic liquids and their applications in HPLC.

## CONCLUSION

In this paper, we discussed the effects of four ionic liquids on the retention and separation of amino benzoic acid isomers. The length of the alkyl group on the imidazolium ring and its counterion, and the concentrations of ionic liquids affected separation. According to these results we presume that complex mechanisms would be involved when ionic liquids are used as additives in HPLC. Although the strong interactions between the imidazolium cation and its counterion of ionic liquids and solutes would also play important roles. Portions of the ionic liquids can coat the surface of the stationary phase where they suppress free silanols and improve the shapes of peaks and the resolutions. As a result, excellent separations of the isomers in this study were achieved using 2.0~8.0 mM/L [OMIm][MS] and 1.0~8.0 mM/L [EMIm][MS] as the eluent modifiers.

The roles of ionic liquids are multiplex and numerous, therefore, further investigations are needed to qualitatively and quantitatively explain the phenomena. However, in this study ionic liquids showed a promising performance as additives in HPLC. More detailed discussions on the interaction mechanisms of ionic liquids and their application in HPLC are currently in progress.

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