# Effect of Mobile Phase Additives on Resolution of Some Nucleic Compounds in High Performance Liquid Chromatography

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**Abstract** Here we investigate the chromatographic behavior, with reversed-phase high performance liquid chromatography (RP-HPLC), of nucleic compounds (nucleobases, nucleosides, and nucleotides) on a $C_{18}$ column in several different mobile phase additives, including 1-butyl-3-methylimidazolium tetrafuloroborate ([BMIm][$BF_4$]), 1-ethyl-3-methylimidazolium methylsulfate ([EMIm][MS]) ionic liquids, ammonium formate, and potassium phosphate. The effect of the alkyl group length, the imidazolium ring, and the ionic liquid's counterions on retention and resolution of the samples were tested. The results show the potential application of a used buffer system, ion pairing system, and ionic liquid as mobile phase additives in liquid chromatography resolution of nucleic compounds. © KSBB

*Keywords: ionic liquid, buffer, ion pairing, RP-HPLC, nucleic compounds*

## INTRODUCTION

High-performance liquid chromatography (HPLC) has become the most applied chromatography technique for research and routine analysis purposes. Several factors have contributed to this growth. One is the possibility of finding conditions for optimal resolution of a sample in an easy and reliable way. The best way to obtain the best resolution conditions is, undoubtedly, interpretive optimization, which substitutes successfully for the more intuitive–although less efficient–trial-and-error approach. Different mechanisms of analyte behavior in HPLC columns are considered from the point of view of analyte distribution between the adsorbent and the bulk liquid phase. The selected mobile phase is an important parameter of the separation process.

Over the past several years there has been a growing interest in ionic liquids for their potential in different chemical processes (*e.g.* catalysis [1-4], electrochemistry [5-7], separation [8-10], *etc.*) because ionic liquids possess a variety of desirable properties. Ionic liquids are organic salts with melting points under 100°C, often lower than room temperature. Recently ionic liquids have been employed more and more as a substitute for traditional organic solvents in chemical reactions. A recent ground swell of interest in ionic liquids is now bringing this science out of academia and closer to commercialization. The natural make up of ionic liquids and the lack of volatile organic compounds can also save users considerable time and money in regulations and handling challenges. The organic mobile phase was changed by adding ionic liquids, phosphate buffer chlorides to a solution and pH adjusted. In the sodium phosphate solution, the hydroxyl (OH) is generated to form small pyramids, and phosphatidate ($PO_4^{3}$) or its compounds might help the formation of big pyramids. It is possible that sodium phosphate acts like a surface-active agent to decrease the active energy of the etching reaction, and then makes etching more effective, leading to the formation of big pyramids. Sample resolution was achieved through use of $C_{18}$ reversed-phase HPLC coupled to an ion-pairing reagent, which was added to the mobile phase. The method was previously applied with success to the separation of biological mixtures [11-18]. However, a lot of work remains to be done to clear the de-

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**Table 1.** Retention factor of nucleic compounds in binary mobile phase

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Case 1[a] | 0.167 | 0.163 | 0.673 | 0.231 | 1.534 | 1.931 | 1.933 | 2.395 | 2.382 |
| Case 2[b] | 0.502 | 0.575 | 0.663 | 1.118 | 1.661 | 1.933 | 2.041 | 0.933 | 2.462 |
| Case 3[c] | 0.492 | 0.544 | 0.732 | 0.799 | 1.510 | 1.909 | 1.984 | 2.227 | 2.301 |
| Case 4[d] | 1.170 | 1.305 | 0.730 | 2.167 | 1.476 | 1.794 | 1.928 | 2.167 | 2.167 |
| Case 5[e] | 0.499 | 0.635 | 0.492 | 1.146 | 1.510 | 2.020 | 1.608 | 0.749 | 1.881 |

1, Cytidine 5′ monophosphate disodiumsalt; 2, uridine 5′ monophosphate disodiumsalt; 3, cytosine; 4, thymidine 5′ monophosphate disodiumsalt; 5, 2-deoxyuridine; 6, thymine; 7, purine; 8, adenine; 9, 2-deoxyguanosine.

[a]Mobile phase: water/methanol = 85/15 (vol. %).

[b]Mobile phase: water/methanol = 85/15 (vol. %), 0.025 M $NaH_2PO_4$, pH 3.

[c]Mobile phase: water/methanol = 85/15 (vol. %), 0.2 M ammonium formate.

[d]Mobile phase: water/methanol = 85/15 (vol. %), 13 M [BMIm][$BF_4$].

[e]Mobile phase: water/methanol = 85/15 (vol. %), 10 M [OMIm][MS].

tailed mechanism.

In this study, we report the influence of the different mobile phase modifiers on the retention and separation of nucleic compounds.

## MATERIALS AND METHODS

### Apparatus

An analytical HPLC system was used with an M930D solvent delivery module equipped with a M930D solvent delivery pump (Young-In Co., Korea), a UV M720 absorbance detector (Young-In Scientific Co., Korea). Experiments were performed with a commercially available Optimapak $C_{18}$ (alkyl-) bonded phase column (4.6 × 250 mm i.d. and 5 μm particles) from Rs-Tech Co. (Daejeon, Korea).

### Reagents

Nucleic compound standards (cytidine 5′ monophosphate disodiumsalt, uridine 5′ monophosphate disodiumsalt, cytosine, thymidine 5′ monophosphate disodiumsalt, 2-de-oxyuridine, thymine, purine, adenine, and 2-deoxyguanosine) were purchased from Fluka (St. Louis, MO, USA). 1-Butyl-3-methylimidazolium tetrafluoroborate ([BMIm][$BF_4$]) and 1-ethyl-3-methylimidazolium methylsulfate ([EMIm][MS]), were purchased from C-tri Co. (Namyang, Korea). Ammonium formate was obtained from Kanto Chemical Co. (Japan) and HPLC-grade methanol, sodium phosphate, and phosphoric acid were purchased from Duksan Pure Chemical Co. (Ansan, Korea). Distilled water was filtered with a vacuum pump (Division of Millipore, Waters, USA) and a filter (HA-0.45, Division of Millipore) prior to use.

### Chromatographic Conditions

Each analyte was dissolved in water to make a final concentration of 1,000 μg/mL. The eluent was the mixture of methanol-water (15:85, v/v). The modified mobile phases were prepared by dissolving known amounts of ionic liquid ([BMIm][$BF_4$] or [OMIm][MS]) in the eluent. The eluent buffer was prepared from stock solutions of 0.025 M sodium phosphate monobasic and the pH 3 was adjusted by addition of phosphoric acid. The ion pairing system was prepared using 0.2 M ammonium formate. The injection volume of the mixture was 5 μL. Analyses was performed throughout at ambient temperature (297 K). Flow rate of 0.5 mL/min flowed in isocratic mode and the elution profiles were monitored at λ of 270 nm.

## RESULTS AND DISCUSSION

Previous studies in our laboratory [19] demonstrated resolution of nucleic compounds while employing the same ionic liquids. In this study, we extend this research and compare the influence of different mobile phase modifiers (ionic liquids, buffer, and ion ion-pairing) on the retention and separation of nucleic compounds. In this paper, the nucleic compounds tested were divided into five groups: 1) with pure organic group, 2) with sodium phosphate monobasic and phosphoric acid group, 3) with ammonium formate group, 4) with [BMIm][$BF_4$], ionic liquid group, and 5) with [OMIm][MS] ionic liquid group. Retention factors of each analyte were calculated with the equation $k = (t - t_0)/t_0$ and listed in Table 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 mL $KNO_3$).

### HPLC Separation of a Standard Mixture

Factors that influence retention and chromatographic efficiency of nucleic compounds include the ionic liquid, the pH, the buffer, and the ion pairing agent content, need to be investigated in order to optimize sample separation. The first step of the present work was performed with a pure organic mobile phase system. The binary mobile phase of methanol in water (15% methanol) was used. Generally, organic modifiers (methanol or acetonitrile) added into the eluent will influence analysis time and sometimes even resolution of analytes. The organic modifier also brings changes of hydrophobicity to the mobile phase and analytes, and variations in mobilities of the analytes and their elution order may occur. Fig. 1 shows a representative chromatogram of a standard

**Table 2.** Resolution of nucleic compounds in binary mobile phase

| | $R_{12}$ | $R_{23}$ | $R_{34}$ | $R_{45}$ | $R_{56}$ | $R_{67}$ | $R_{78}$ | $R_{89}$ |
|---|---|---|---|---|---|---|---|---|
| Case 1 | 0.034 | 7.285 | 6.206 | 9.943 | 2.582 | 0.012 | 2.525 | 0.070 |
| Case 2 | 0.898 | 1.145 | 5.546 | 4.899 | 2.488 | 0.887 | 9.323 | 14.049 |
| Case 3 | 0.340 | 1.554 | 0.442 | 4.764 | 3.011 | 0.440 | 1.532 | 0.462 |
| Case 4 | 1.019 | 3.984 | 8.188 | 3.405 | 2.239 | 1.040 | 1.253 | 0.000 |
| Case 5 | 0.855 | 0.898 | 4.654 | 3.251 | 4.142 | 2.610 | 6.810 | 9.657 |

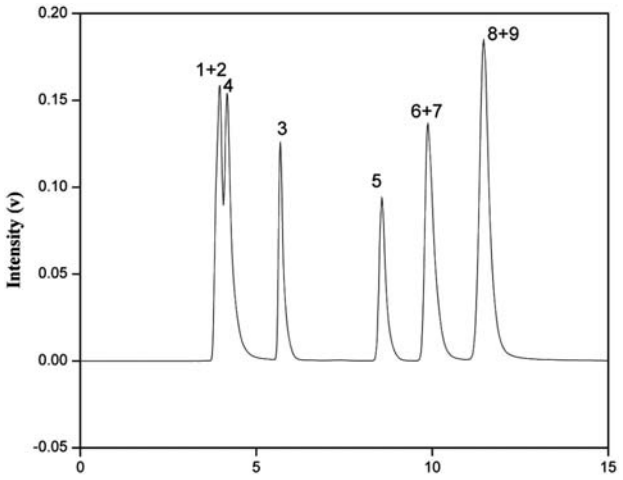


**Fig. 1.** Chromatogram of nucleic compounds by HPLC (Mobile phase: water/methanol = 85/15. 1, Cytidine 5′ monophosphate disodiumsalt; 2, uridine 5′ monophosphate disodiumsalt; 3, cytosine; 4, thymidine 5′ monophosphate disodiumsalt; 5, 2-deoxyuridine; 6, thymine; 7, purine; 8, adenine; 9, 2-deoxyguanosine).

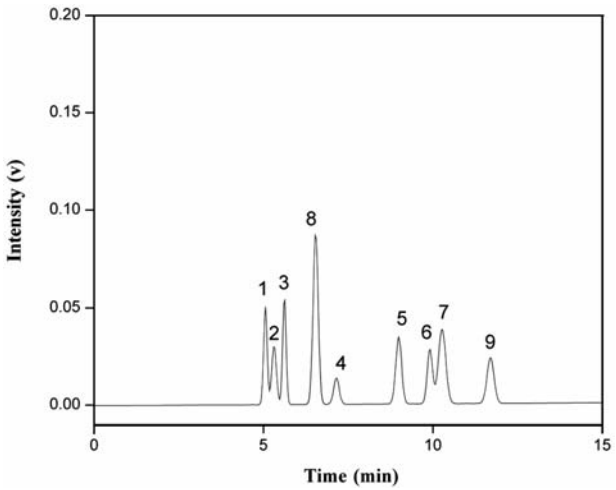


**Fig. 2.** Chromatogram of nucleic compounds by HPLC (Mobile phase: water/methanol = 85/15, 0.025 M $NaH_2PO_4$, pH 3, $H_3PO_4$. 1, Cytidine 5′ monophosphate disodiumsalt; 2, uridine 5′ monophosphate disodiumsalt; 3, cytosine; 4, thymidine 5′ monophosphate disodiumsalt; 5, 2-deoxyuridine; 6, thymine; 7, purine; 8, adenine; 9, 2-deoxyguanosine).

mixture containing cytidine 5′ monophosphate disodiumsalt (5-CMP), uridine 5′ monophosphate disodiumsalt (5-UMP), cytosine (CYT), thymidine 5′ monophosphate disodiumsalt (5-TMP), 2-deoxyuridine (2-DEU), thymine (THM), purine (PUN), adenine (ADN), and 2-deoxyguanosine (2-DEG). In this system, 5-CMP, 5-UMP, and 5-TMP, THM and PUN, ADN and 2-DEG were not fully resolved. These experiments indicate that in a pure reverse-phase system the separation was not satisfactorily achieved using only methanol as mobile phase modifier.

## Buffer System

The pH of the aqueous mobile phase can have a significant influence on separation. Because the usable eluent pH range is 3.0~8.5 for this column, typical eluents used in non-suppressed ion-chromatography, such as phosphoric acid solutions, were employed. In this work, phosphate buffer solution with UV absorbance was selected because its pH value could be easily controlled over a wide range. A decrease in pH reduces the number of negatively charged residues on a protein, thus reducing the interaction with cationic analytes [20]. While this behavior is reminiscent of the binding of charged analytes to ion-exchange materials, the pH-dependence of specific interactions within distinct binding sites often appears to be more complex. The retention behavior of each analyte was evaluated based on its retention factor ($k$) (Table 1). As can be seen in Fig. 2, when 15% methanol is added to 0.025 M $NaH_2PO_4$ buffer, pH 3 ($H_3PO_4$), it gave rise to a baseline separation of 5-CMP and 5-UMP, THM and PUN, ADN and 2-DEG with a resolution of 0.898, 0.887, and 14.049, respectively (Table 2), which is the greatest resolution value obtained in this study. In this case the elution order changed and the 5-TMP eluted before the ADN (Table 3). These results indicated the low concentrations of modifier at room temperature shorten separation time of the nucleic compounds and this condition is economical.

## Ion-pairing System

In this study, we report a highly sensitive and simple ion-pairing HPLC method with UV detection. Variation of the ionic strength by the phase ion pairing agents and addition of organic modifiers are among the most widely used means to influence retention and resolution in chromatography separa-

**Table 3.** Elution order of the of nucleic compounds in different mobile phase

| Order | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Case 1 | 1 + 2 | | 4 | 3 | 5 | 6 + 7 | | 8 + 9 | |
| Case 2 | 1 | 2 | 3 | 8 | 4 | 5 | 6 | 7 | 9 |
| Case 3 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Case 4 | 3 | 1 | 2 | 5 | 6 | 7 | | 4 + 8 + 9 | |
| Case 5 | 1 + 3 | | 2 | 8 | 4 | 5 | 7 | 9 | 6 |

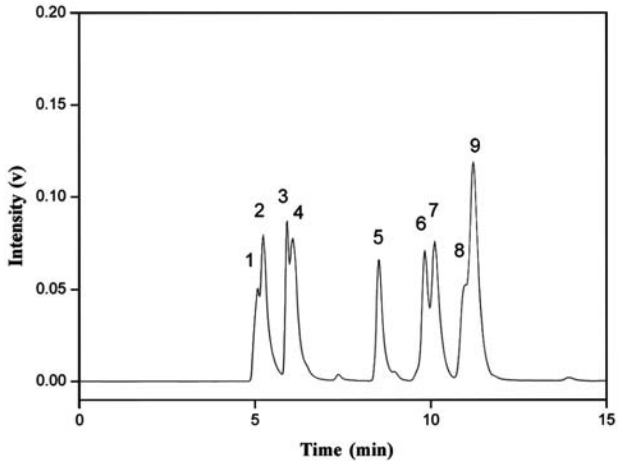


**Fig. 3.** Chromatogram of nucleic compounds by HPLC (Mobile phase: water/methanol = 85/15, 0.2 M ammonium formate. 1, Cytidine 5′ monophosphate disodiumsalt; 2, uridine 5′ monophosphate disodiumsalt; 3, cytosine; 4, thymidine 5′ monophosphate disodiumsalt; 5, 2-deoxyuridine; 6, thymine; 7, purine; 8, adenine; 9, 2-deoxyguanosine).

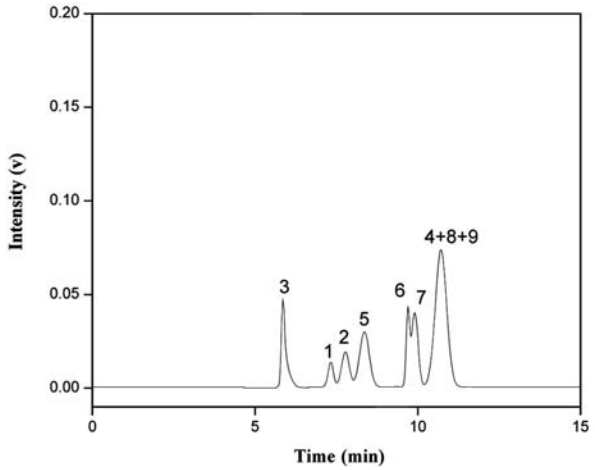


**Fig. 4.** Chromatogram of nucleic compounds by HPLC (Mobile phase: water/methanol = 85/15, 13 mM [BMIm][$BF_4$]. 1, Cytidine 5′ monophosphate disodiumsalt; 2, uridine 5' monophosphate disodiumsalt; 3, cytosine; 4, thymidine 5' monophosphate disodiumsalt; 5, 2-deoxyuridine; 6, thymine; 7, purine; 8, adenine; 9, 2-deoxyguanosine).

tion [17]. Freshly prepared standard mixtures, with known concentration and aliquots of each sample, were assayed by ion-pairing HPLC for separation of 5-CMP, 5-UMP, CYT, TMP, 2-DEU, THM, PUN, and, 2-DEG. The HPLC separation presented here was possible with the use of a $C_{18}$ reversed-phase HPLC column coupled with an ion-pairing reagent, which was added to the mobile phase. We successfully applied this method to the separation of nucleic compounds. Pure systems can not separate 5-CMP and 5-UMP, THM and PUN, ADN and 2-DEG. Addition of organic modifier 0.2 M ammonium formate to the eluent will influence resolution of these analytes. Fig. 3 demonstrated the effect of the ion pairing agent content in the mobile phase on retention of nucleic compounds. Table 2 (case 3) also shows the resolution of nucleic compounds with some improvements.

### Effect of [BMIm][$BF_4$] on Retention Behavior of Nucleic Compounds

Fig. 4 shows the chromatogram of nucleic compounds with 13 mM [BMIm][$BF_4$] used as the eluent modifier. Comparison to the pure eluent, it is obvious the addition of an ionic liquid to the mobile phase results in better separation of 5-CMP and 5-UMP, THM and PUN. First, the resolutions of four nucleic compounds were improved, especially for 5-CMP and 5-UMP. Although the separation of 5-CMP and 5-UMP was incomplete, it was superior to that using only water as the eluent. Second, after the addition of [BMIm][$BF_4$] to the eluent, separation efficiency and symmetry of the four chromatographic peaks improved greatly. As for basic compounds with polar functional groups, severe band tailing, band broadening and low plate numbers in the chromatogram often occurred because of residual silanols on $C_{18}$ column. But, when ionic liquid is used as the mobile phase additive in HPLC, it exists in the solution and also coats the $C_{18}$ column imidazolium cations can interact with free existing silanol groups and the polar group of the analytes competes for the silanol groups on the alkylsilica surface. Therefore, the ionic liquid can effectively shield the residual free silanols and improve the peak shapes.

### Effect of [EMIm][MS] on Retention Behavior of Nucleic Compounds

In this case the elution order and shape was the same as with buffer system (Table 3). Ionic liquids can play a variety

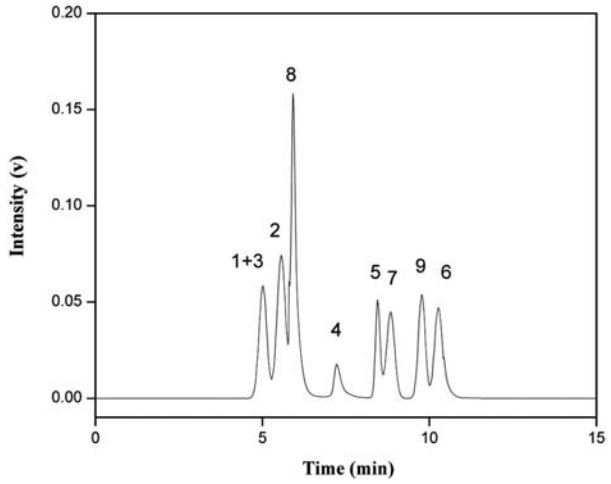


**Fig. 5.** Chromatogram of nucleic compounds by HPLC (Mobile phase: water/methanol = 85/15, 10 mM [OMIm][MS]. 1, Cytidine 5′ monophosphate disodiumsalt; 2, uridine 5′ monophosphate disodiumsalt; 3, cytosine; 4, thymidine 5′ monophosphate disodiumsalt; 5, 2-deoxyuridine; 6, thymine; 7, purine; 8, adenine; 9, 2-deoxyguanosine).

of roles, including coating residual free silanols and modification of the stationary phase. Fig. 5 shows sample elution with 10 mM of the [EMIm][MS] ionic liquid. This condition can successful separate each sample except for 5-CMP and CYT. It has been proposed that the elution order of the nucleic compounds was determined by number of contact points available to the solute adsorbent interactions. As a rule in chromatographic experiment, the optimum separation condition is determined by several factors including base line resolution, reduction of additive concentration, and analysis time.

The different separations that resulted from the two eluent modifier ionic liquids with different anions and cations may be due to the association with nucleic compounds in water-methanol solvent. It seems that [EMIm] with [MS] is superior to [BMIm] with [$BF_4$] in the separation of nucleic compounds. However, ionic liquids showed promising performance as additives in the separation of nucleic compounds. Further investigation on the mechanism of such interactions is needed before a more clear explanation of these phenomena can be provided.

## CONCLUSION

In this study, the effects of mobile phase with ionic liquids, buffer, and ion pairing systems on the separation of nucleic compounds were investigated. The nature of the modifiers can affect the separation of ionic analytes. The two ionic liquids tested here have improved effect on the retention and resolution of nucleic compounds. The length of the alkyl on the imidazolium ring and its counterion can affect the resolution, because part of the ionic liquids coated on the surface of the stationary phase could suppress the free silanols of the surface. Comparison of ionic liquids with standard mobile phase additives such as ammonium formate showed the former to have advantages as silanol suppressors in HPLC. The interactions between ionic liquids and nucleic compounds are complicated and further investigation is needed in order to quantitatively explain some of the phenomena. As a result, excellent separation of these nucleic compounds was achieved with a buffer system as the modifier of the adjusted pH of eluents. We suppose that the elution order depends on the pH of the mobile phase.

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