Chinese Journal of Chemistry, **2004**, *22*, 549—552 **Article**

Ionic Liquids as Mobile Phase Additives for Separation of Nucleotides in High-Performance Liquid Chromatography

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Ionic liquids are a type of salts that are liquid at low temperature (＜100 ℃). Because of their some special properties, they have been widely used as new "green solvents" for many chemical reactions and liquid-liquid extraction in the past several years. In this paper, a new method for the separation of nucleotides is developed and the essential feature of the method is that 1-alkyl-3-methylimidazolium salts are used as mobile phase additives, resulting in a baseline separation of nucleotides without need of gradient elution and need of organic solvent addition as currently used in RP-HPLC. This study shows the potential application of ionic liquids as mobile phase additives in reversed-phase liquid chromatography.

Keywords ionic liquid, 1-alkyl-3-methylimidazolium, nucleotide, high-performance liquid chromatography

Introduction

Most organic salts are solid at ambient temperature, but ionic liquids (IL) or room temperature ionic liquids (RTIL) are a type of salts that are liquid at low temperature (＜100 ℃). Because RTIL are good solvents for both inorganic and organic materials, they are non-volatile, nonflammable, thermally stable, and recyclable solvents, and they have some particular properties in chemical reactions *etc.*, currently they are being investigated widely as "green chemistry" solvents.[1-5] Common ionic liquids, which are combined with Cl^-, $Cl^-/AlCl_3$, BF_4^- (TFB), PF_6^- (HFP), and NO_3^- anions, are composed of 1,3-dialkylimidazolium and 1-alkylpyridinium cations. In recent years the interest of RTIL in separation science is also growing rapidly (*e.g.* supercritical-fluid extraction,[6] gas chromatography,[7] and electrophoresis[8-11]). Poole *et al.*[12] firstly reported the application of ethylammonium and propylammonium nitrate in liquid chromatography. Our group firstly reported the application of 1-alkyl-3-methylimidazolium-based ionic liquids as additives in liquid chromatography to separate some basic compounds,[13,14] showing that ionic liquids can inhibit the adsorption of basic compounds on a reversed-phase column.

Nucleotides, the break-down products of DNA and RNA, play an important role in cell metabolism,[15-24] and nucleotide mutations are linked to cancer and genetic diseases.[15-19,25] Since some nucleoside analogs are effective drugs in the treatment of AIDS, cancer, and other diseases, their triphosphate metabolites must frequently be monitored.[15,20-24] Analysis of nucleotides in body tissues and cells is widely used in biochemical, medical, and pharmacological studies. In addition, for quality control in food industry concentrations of nucleotides also need to be determined.[26] Thus, a critical need for the analysis of nucleotides emerges. Nucleotides can be quantified by high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Although HPLC has some advantages, organic modifiers (methanol, acetonitrile and tetrahydrofuran) and gradient elution are often required to separate nucleotides.[27-29]

The purpose of this study is to explore the potential application of RTIL as mobile phase additives in liquid chromatography, because many organic additives have low solubility in water, which limits their use in reversed-phase liquid chromatography. Taking advantage of the special properties of RTIL, it is hopeful to extend the number of mobile phase additives in HPLC. In this study, seven ionic liquids were evaluated as additives to separate five nucleotides. These nucleotides are baseline separated by using 1-alkyl-3-methylimidazolium-based ionic liquids as additives on a common reversed-phase C_{18} column without gradient elution and any other modifiers. The addition of RTIL provides better resolution and peak symmetry. The possible separation mechanism seems to involve the interactions between nucleotides and the RTIL. To the best of our knowledge, this study is the first report on the separation of nucleotides using ionic liquids as additives in HPLC.

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Experimental

Chemicals

Nucleotides including 5'-monophosphate adenosine (AMP), 5'-monophosphate cytidine (CMP), 5'-monophosphate uridine (UMP), 5'-monophosphate guanosine (GMP), and 5'-monophosphate inosine (IMP) were purchased from Sigma Chemical Corp. (U.S.A.). 1-Methylimidazole, alkylchloride, sodium tetrafluoroborate and pyridine were purchased from Fluka Chemical Corp. (U.S.A.). All other chemicals were purchased from the Shanghai or Tianjing Chemical Reagent Corp. (China) and are of analytical-reagent grade. Deuterium oxide was purchased from the Beijing Chemical Reagent Corp. (China), which is NMR reagent. De-ionized water was used.

Preparation of ionic liquids

The ionic liquids used in this study were synthesized as described in the references.[30,8] All ionic liquids were placed in a silica-gel desiccator to prevent from the uptake of water prior to the preparation of the mobile phases.

Apparatus and chromatographic conditions

HPLC separation was performed on an Agilent 1100 system (Agilent Technologies, U.S.A.) equipped with a quaternary pump, an autosampler, a thermostatted column compartment and a diode-array multiple-wavelength UV-Vis detector (DAD). All separations were carried out on a reversed-phase Hypersil ODS-C_{18} column (150 mm×4.6 mm I.D., 5 μm particle size) purchased from the Dalian Institute of Chemical Physics, Chinese Academy of Sciences. The chromatographic conditions for the HPLC system were set as follows: UV absorbance at 260 nm with a 4 nm slit width and a reference at 360 nm with a 64 nm slit width; column temperature: 25 ℃; mobile phase: aqueous ionic liquids (EMIm-BF_4, ProMIm-BF_4, BMIm-BF_4, ProMIm-Cl, BMIm-Cl, PenMIm-Cl and N-B-P-BF_4); flow-rate: 1.0 mL/min.

The spectral data were obtained on a Specord UV-Vis scanning instrument (Zeiss Jena, Germany).

Results and discussion

In this study, 1-alkyl-3-methylimidazolium-based and *N*-alkylpyridinium-based ionic liquids combined with chloride or tetrafluoroborate (BF_4^-) anions were evaluated as mobile phase additives to separate five nucleotides in HPLC. The alkyl groups on the imidazolium cations include ethyl, propyl, butyl, and pentyl.

Choice of UV detection wavelength

Figure 1 shows the representative UV spectra of BMIm-BF_4, BMIm-Cl, and N-B-P-BF_4 in water at the same concentrations (5×10^{-5} mol•L^{-1}). All these ionic liquids exhibit an absorbance maximum at about 215 nm, while N-B-P-BF_4 has another absorbance maximum at about 258 nm. Monophosphate nucleotides display an absorbance maximum at about 260 nm, so the UV detection wavelength was chosen at 260 nm in this study as reported.[31]

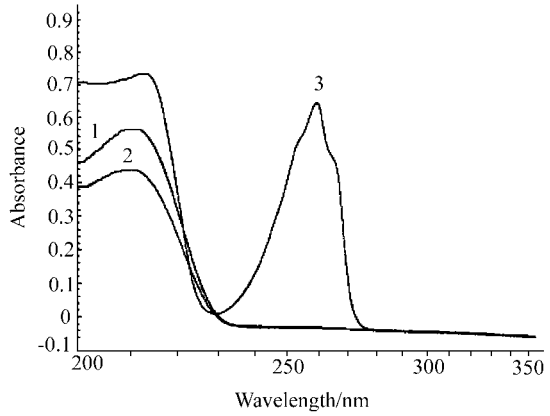

Figure 1 UV spectra of (1) BMIm-BF_4, (2) BMIm-Cl and (3) N-B-P-BF_4 in water.

Influence of BMIm-BF_4 concentration

To examine the effect of RTIL, water solutions of BMIm-BF_4, as an example, in different concentrations (*i.e.* from 0 to 35 mmol • L^{-1}, pH=6.5—7), were used as the mobile phases. Only four peaks of nucleotides (CMP, UMP, GMP and IMP) are found within 60 min using only water as the mobile phase, GMP and IMP can not be baseline separated, and the bands tail seriously on the C_{18} column (Figure 2A). After the addition of BMIm-BF_4, the retention time of nucleotides changes drastically compared with that using only water. The retention time of nucleotides, which slightly increases in the concentration range of BMIm-BF_4 from 0 to 2 mmol • L^{-1}, decreases rapidly with the increase of the concentration of BMIm-BF_4 from 2 to 35 mmol • L^{-1}. Figure 2B shows the separation chromatograms of nucleotides using 23 mmol • L^{-1} BMIm-BF_4 as the mobile phase. It is apparent that the presence of BMIm-BF_4 leads to better resolution, peak symmetry, and detection sensitivity. The resolution of GMP and IMP is not so good when the concentration of BMIm-BF_4 is above 35 mmol • L^{-1}. Considering the analysis time and resolutions of the analytes, the concentration of BMIm-BF_4 is suitable at about 25 mmol • L^{-1}.

Subsequently, the relationship between the concentrations of BMIm-BF_4 and the retention factors (k') of nucleotides was plotted. In Figure 3, log k' of nucleotides are plotted against the concentrations of BMIm-BF_4. Retention factors are calculated as $k'=(t_R-t_0)/t_0$; where t_R is the retention time of the analytes, and t_0 is the column hold-up volume or the void volume time, which is determined by the retention time of deuterium oxide. It can be seen that in the concentrations of BMIm-BF_4 ranging from 2 to 35 mmol • L^{-1}, the linearity is good ($R^2>0.995$). It shows that the analysis time of nucleotides can be changed by varying the con-

centration of BMIm-BF_4 without gradient elution and organic modifiers such as methanol, acetonitrile *etc.*, which are often used for the determination of nucleotides in RP-HPLC.

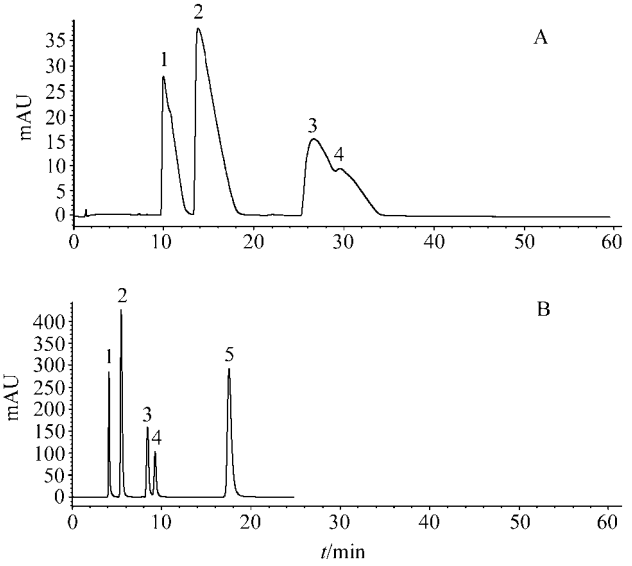

Figure 2 Effect of aq. BMIm-BF_4 conc. on the separation of nucleotides (A) water, and (B) 23 mmol • L^{-1} as mobile phases; 1═CMP, 2═UMP, 3═GMP, 4═IMP and 5═AMP. Detected at 260 nm.

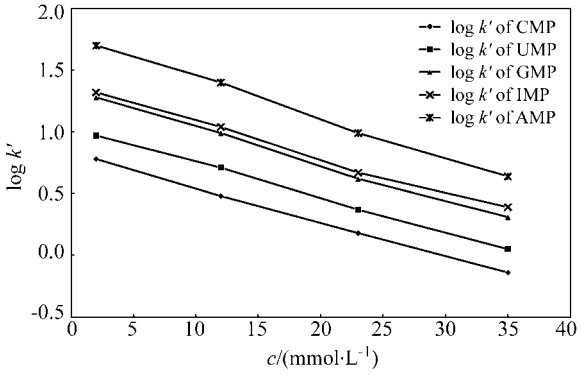

Figure 3 Plot of concentrations of BMIm-BF_4 vs. log *k*′.

Influence of different alkyl group on the imidazolium cations

On the basis of the above experiments, 1-ethyl-3-methylimidazolium tetrafluoroborate (EMIm-BF_4), 1-propyl-3-methylimidazolium tetrafluoroborate (ProMIm-BF_4), and 1-butyl-3-methylimidazolium tetrafluoroborate (BMIm-BF_4) were investigated to study the effect of different alkyl group on separation of these nucleotides (all with a concentration of 25 mmol • L^{-1}, pH ~6.5). The results show that the retention of nucleotides increases with the increase of alkyl chain length on cations except that of AMP.

Influence of different ionic liquids

Four ionic liquids, such as 1-propyl-3-methylimidazolium chloride (ProMIm-Cl), 1-butyl-3-methylimidazolium chloride (BMIm-Cl), 1-pentyl-3-methylimidazolium chloride (PenMIm-Cl), and *N*-butyl-pyridinium tetrafluoroborate (N-B-P-BF_4), were chosen to investigate the effect of different RTIL on the chromatographic behavior of the analytes. The concentrations of the four ionic liquids are all at 25 mmol • L^{-1} (pH ~6.5). The representative chromatograms are shown in Figure 4. The ionic liquids with Cl^- as counterion provide the same good separation for these nucleotides at the expense of longer analysis time in comparison with the ionic liquids combined with BF_4^-. It is illustrated that different anions of RTIL play an important role in separating the nucleotides. In addition, the retention of the nucleotides increases with the increase of alkyl chain length on cations, which is the same as observed using imidazolium-BF_4 ionic liquids. Unfortunately, when N-B-P-BF_4 solution is used as the mobile phase, some peaks of nucleotides are superpositioned on the baseline noise at 260 nm (Figure 4B). IMP and AMP can not be detected probably because of the co-absorbance of the nucleotides and N-B-P-BF_4.

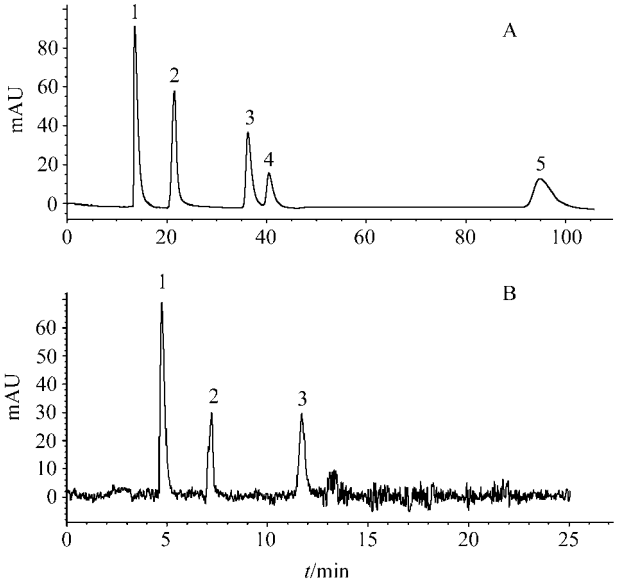

Figure 4 Chromatograms of nucleotides using (A) BMIm-Cl and (B) N-B-P-BF_4 as mobile phases; 1═CMP, 2═UMP, 3═GMP, 4═IMP and 5═AMP. Detected at 260 nm.

According to the references,[8,32-36] certain molecular interactions, including strong hydrogen bonding (the hydrogen on C-2 carbon of the imidazolium cation), hydrophobic interactions and ion-dipole or ion-induced-dipole, strong ion-pairing effects, *etc.* were found in RTIL. It is suggested that the retention time of nucleotides changes due to certain molecular interactions between nucleotides and ionic liquids. In view of the experimental phenomena above mentioned, we think that the changes of retention of nucleotides are attributed to these molecular interactions between the nucleotides and the RTIL in the mobile phase. On the other

hand, the cations of RTIL can preferentially adsorb on C_{18} stationary phase by covering active silanol sites,[8,11,12] which leads to the improvement of the peak symmetry.

Linearity, precision, and detection limit of nucleotides

Five nucleotides were separated using 25 mmol $\cdot L^{-1}$ BMIm-BF_4 as the mobile phase, and the linearity, precision, and detection limit are obtained, respectively. Calibration curve was obtained by plotting peak area vs. concentration of nucleotides as follows:

$A=16.137c+45.998$ ($n=6$, $r=0.9993$) for CMP at concentration of 2.5—500 mg $\cdot L^{-1}$;

$A=11.505c+48.584$ ($n=6$, $r=0.9998$) for UMP at concentration of 2.0—320 mg $\cdot L^{-1}$;

$A=15.116c+37.745$ ($n=6$, $r=0.9995$) for GMP at concentration of 2.2—220 mg $\cdot L^{-1}$;

$A=8.1308c+19.338$ ($n=6$, $r=0.9989$) for IMP at concentration of 2.0—200 mg $\cdot L^{-1}$;

$A=20.384c+72.132$ ($n=6$, $r=0.9990$) for AMP at concentration of 2.0—200 mg $\cdot L^{-1}$.

The detection limits of CMP, UMP, GMP, IMP, and AMP are 0.15, 0.17, 0.24, 0.50, and 0.34 mg $\cdot L^{-1}$, respectively.

Conclusion

In this study, successful separation of nucleotides has been achieved using 1-alkyl-3-methylimidazolium-based ionic liquids as the mobile phase additives. Many organic additives are less soluble in water, which limits their use as solvents in reversed-phase HPLC. Therefore the use of RTIL enlarges the number of water-miscible organic additives. The retention of the nucleotides can be changed by using an appropriate ionic liquid as additives, and satisfactory separation can be achieved by changing different alkyl substituents of cations, different anions or different concentrations of RTIL. Through the use of RTIL as mobile phase additives the amount of organic solvent (such as methanol or acetonitrile) required and environmental pollution can also be reduced.

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