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An insight into the phenomena involved in a multiple-function stationary phase for the capillary electrochromatographic separation of 2'-, 3'-, and 5'-monophosphorylated nucleoside isomers

The electrochromatographic separations of 2'-, 3'- and 5'-monophosphates of adenosine, guanosine, cytidine, and uridine were carried out with an open-tubular capillary column which was wall-coated with a highly selective reagent, 28-membered macrocyclic polyamine, 4, 8, 12, 18, 22, 26-hexaaza-1,15-dioxacyclooctaeicosane ([28]ane-N_6O_2). The effects of pH, composition and concentration of background electrolyte (BGE), applied voltage, column length, and the additive of the BGE, such as metal ions, borate, β-cyclodextrin and organic solvent on the separation of these monophosphorylated nucleotide isomers were investigated. The results suggested that the interactions between analytes and the bonded groups on the wall predominantly comprise anion coordination and anion exchange in addition to the electrophoresis. A well-resolved electrochromatogram was obtained with the capillary column of 100 cm (75 cm effective length) × 75 μm inside diameter (ID), citrate buffer (20 mM, pH 3.99), applied voltage of −22 kV and detection at 254 nm. Column efficiency was found with the average theoretical plate numbers of 119 500/m and a low detection limit of 0.01 μM level could be achieved for the separation of these isomers.

Keywords: Capillary electrochromatography / Macrocyclic polyamine / 2'-,3'-,5'-Monophosphorylated nucleoside isomers / Open-tubular column / Stationary phase
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1 Introduction

RNA and DNA with 2'-5'-linkages possess significantly different structural and chemical characteristics from the natural 3'-5'-linked nucleic acids [1]. A number of studies have been reported on the possible application of 2'-5'-linked RNA and DNA for antisense uses. Adah *et al.* [2] have published a review article relating chemistry and biochemistry of 2'-5'-oligoadenylate-based antisense strategy. It describes the application of a natural defense mechanism to develop effective agents for the post-transcriptional control of gene expression. Currently, Yu *et al.* [3] reported the immunostimulatory properties of phosphorothioate CpG DNA containing both 3'-5'- and 2'-5'-internucleotide linkages. These developments call for methods capable of high speed and reliable separation of 2'-, 3'- and 5'-phosphorylated nucleoside isomers.

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Abbreviation: [28]ane-N_6O_2, 4,8,12,18,22,26,-hexaaza-1,15-dioxacyclooctaeicosane

As a separation technique, capillary electrophoresis (CE) is among the methods with the highest speed and resolving power in a single dimension for charged molecules. However, due to their similar charge-to-size ratio, the separation of nucleotide isomers is very difficult by CE. In order to increase selectivity for these compounds, buffer additives must be employed. Huang *et al*. [4] used low-pH formate buffer, namely 50 mM, pH 3.7–4 or pH 3.8 with 0.1 mM CTAB to resolve the various 2'-, 3'- and 5'-nucleotide isomers. But the results exhibited poor resolution and reproducibility. Tadey and Purdy [5] employed complexation reactions with cyclodextrin and borate for the CE separation of nucleotide isomers. The method appears to be very effective to resolve all 12 nucleotide isomers in less than 15 min. Cahours *et al.* [6–8] have studied the complexation equilibrium between nucleotides and inorganic cations (Mg^{2+}, Ca^{2+}, Cd^{2+}, and Zn^{2+}) by CE. These studies would be useful for optimization of the separation of nucleoside mono-, di- and triphosphates by CE according to their different affinity for inorganic cations added to the buffer. Micellar electrokinetic chromatography with a mixture of Mg^{2+} (0.1 mM), β-CD (15 mM) and SDS (0.05 M) as a background electrolyte (BGE) has been employed for the separation of adenine, adenosine and nucleotide isomers of adenine by Kuwamura [9].

Capillary electrochromatography (CEC) is a hybrid separation technique of CE and high-performance liquid chromatography (HPLC) that has some of the best features of both techniques [10, 11]. For increasing the selectivity of the CEC, macrocyclic polyamine has been prepared and employed as the stationary phase of CEC in our lab [12–17]. The chemistry of polyazamacrocyclic ligands is of considerable interest since in addition to complexing cationic species, the protonated form of these ligands and their mononuclear and dinuclear metal complexes can form complexes with anionic substrates [18]. Some of the earlier examples of synthetic anion receptors were protonated or alkylated polyammonium macropolycycles. The nucleoside phosphate polyanions, AMP^{2-}, ADP^{3-}, ATP^{4-}, form complexes of much higher stability with the macrocyclic polyammonium structures than with the acyclic tetraammonium ligand spermine, which indicates a macrocyclic effect on anion binding, with respect to acyclic ligands [19, 20]. This topic continues to be an active area of research some 30 years later, as many examples of anion-directed assembly, sensors and receptors for anions or cation-anion pairs have been reported recently [21, 22].

Most macrocyclic polyammonium salts, based on the ethylenediamine pattern, require acidic pH for full protonation [20]. In the previous paper, a 1,3-propylene-diammonium unit macrocyclic polyamine, 4,8,12,18,22,26-hexaaza-1,15-dioxacyclooctaeicosane ([28]ane-N_6O_2) which has a protonated form even at neutral pH was prepared (Fig. 1), and applied as the stationary phase of an open-tubular CEC column for the separation of inorganic and organic anions [13], aromatic organic acids [14], and metal speciation [15]. Following our success in the separation of nucleoside monophosphates [16], we anticipated that this new bonded phase would be well suited for the analysis of a more difficult separation of nucleotide isomers.

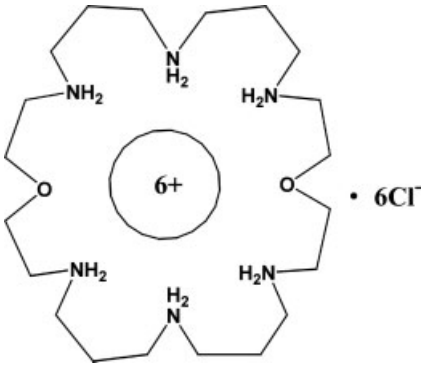

Figure 1. Structure of the macrocyclic polyamine [28]ane-N_6O_2 covalently bound to the inner wall of the fused silica.

2 Materials and methods

2.1 Apparatus

All experiments were performed on a high-voltage power supply with a 30 kV capacity (model 890-CE; Jasco, Tokyo, Japan) and a variable-wavelength UV/Vis detector (Jasco 870-CE). Electrochromatograms were recorded and processed with a PC SISC-Lab data acquisition system (Scientific Information Service, Taiwan). Fused-silica capillaries with an external coating of polyimide were from Restek (Bellefonte, PA, USA). The modified capillaries were of 75 μm ID and the total length of the capillary was 70 and 100 cm, with a distance of 50 cm between the injection end and the detection window.

2.2 Reagents and chemicals

Most chemicals were of analytical reagent grade from Merck (Darmstadt, Germany). Purified water (18 MΩ cm) from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare all solutions. 2'-Adenosine monophosphate (2'-AMP), 3'-AMP, 5'-AMP, 2'-cytidine monophosphate (2'-CMP), 3'-CMP, 5'-CMP, 5'-guanosine monophosphate (5'-GMP), 2'-uridine monophosphate (2'-UMP), 3'-UMP, 5'-UMP, phosphoric acid, and sodium phosphate were purchased from Sigma (St. Louis, MO, USA). γ-Glycidoxypropyltrimethoxysilane was obtained from Aldrich (Milwaukee, WI, USA). Citric acid, acetic acid, benzyl alcohol, and dimethylformamide were obtained from Merck. All liquid reagents and solvents used in moisture-sensitive reactions were distilled and collected over type 4 Å molecular sieves. Stock solutions (5 mg mL^{-1}) of the nucleotide were prepared in pure water and diluted appropriately prior to use. All solvents and solutions for CEC analysis were filtered through a 0.45 μm cellulose ester membrane (Advantec MFS, Pleasanton, CA, USA).

2.3 Preparation of the capillary column

The column was prepared as described in [13]. Prior to any column modifications, fused-silica capillaries (75 μm ID, 70 cm long) were first flushed with 1 M NaOH (30 min), then pure water (15 min), 1 M HCl (30 min), and pure water (15 min). The capillaries were purged with nitrogen for 20 min, then dried at 110°C overnight. For coating, the capillary was filled with a 10% w/v solution of γ-glycidoxypropyltrimethoxysilane in toluene under the pressure of 30 psi. The filling rate was about 0.1 mL min^{-1}. The capillary was kept for 3 h at 110°C for silylization. After purging with toluene for several minutes to remove unreacted reagent, the capillaries were dried in a vacuum oven. The

capillary was then filled with a 1% w/v solution of the macrocyclic compound, [28]ane-$N_6O_2 \cdot 6$ HCl in *N*,*N*-dimethylformamide. After standing for 10 h at 120°C for functionalization, the dried capillaries were purged with ethanol and pure water for several minutes before equilibration with buffer solution. They were then ready for use.

2.4 Capillary electrochromatographic conditions

Samples were introduced electrokinetically at the cathodic end of the capillary column. The appropriate BGE was phosphate buffer. Benzyl alcohol was used as the neutral marker. The analytes were detected by monitoring their absorbance at 254 nm.

3 Results and discussion

For the macrocyclic polyamine-bonded phase under neutral or acidic conditions, the direction of EOF was reversed in comparison with that of an untreated fused-silica column [13]. This suggested that a bonded group with a positively charged surface had been formed. The prepared column was evaluated for the feasibility of the separation of nucleotide isomers. Figure 2 depicts the structure of these compounds.

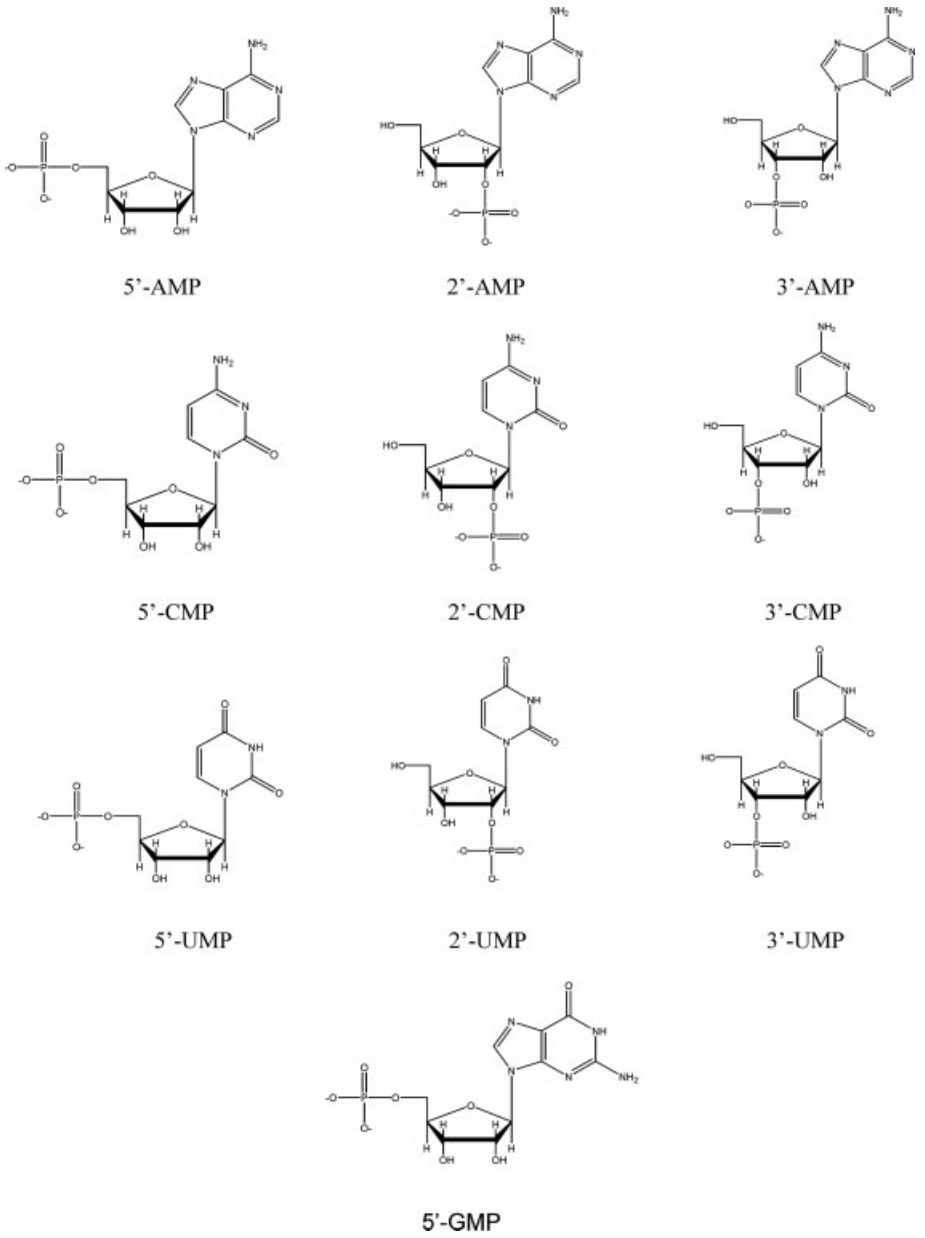

Figure 2. Structures of the monophosphorylated nucleoside isomers.

3.1 Effect of pH

In CE, the migration of the nucleotide isomers depends on their net electrical charge. Cahours *et al.* [8] reported that the phosphate chain of the nucleotide isomers studied has the same apparent negative charge in the weak acidic medium and migration of these nucleosides monophosphate depends upon the nature of the base on the nucleotide. Therefore, the separation of nucleotides is difficult to predict. Since the protonated forms of the macrocycle polyamine can bind anions through hydrogen bonding and coulombic forces, a competitive polyanion, citrate buffer, was first chosen as the BGE.

Figure 3 shows the electrochromatograms at pH values over the range from 2 to 5. A slower migration for the analytes was observed when the pH was increased. This is due to reduced protonation of the bonded group and results in a smaller EOF. Detailed examination of the electrochromatograms suggested that resolution would be improved when the pH was increased. Only three peaks at pH 2.27 and five peaks at pH 3.10 were shown when nine compounds were injected. Seven peaks were obtained at pH 3.75. A pH of 3.99 was adequate to distinguish the isomers of 3'-CMP, 2'-CMP and 5'-CMP. Baseline separations for the nine compounds injected were demonstrated at pH 4.5, except 3'-UMP and 2'-UMP. Under these conditions, all isomers were eluted in the order of their relative mobility (Table 1), namely 3'-UMP = 2'-UMP $>$ 5'-UMP $>$ 5'-GMP $>$ 3'-AMP $>$ 5'-AMP $>$ 2'-AMP $>$ 3'-CMP $>$ 5'-CMP $>$ 2'-CMP. But the difference in the relative mobility is only slight. In other words, the results reflected the interaction between the analytes and the bonded group which may contribute to separation in addition to electrophoresis.

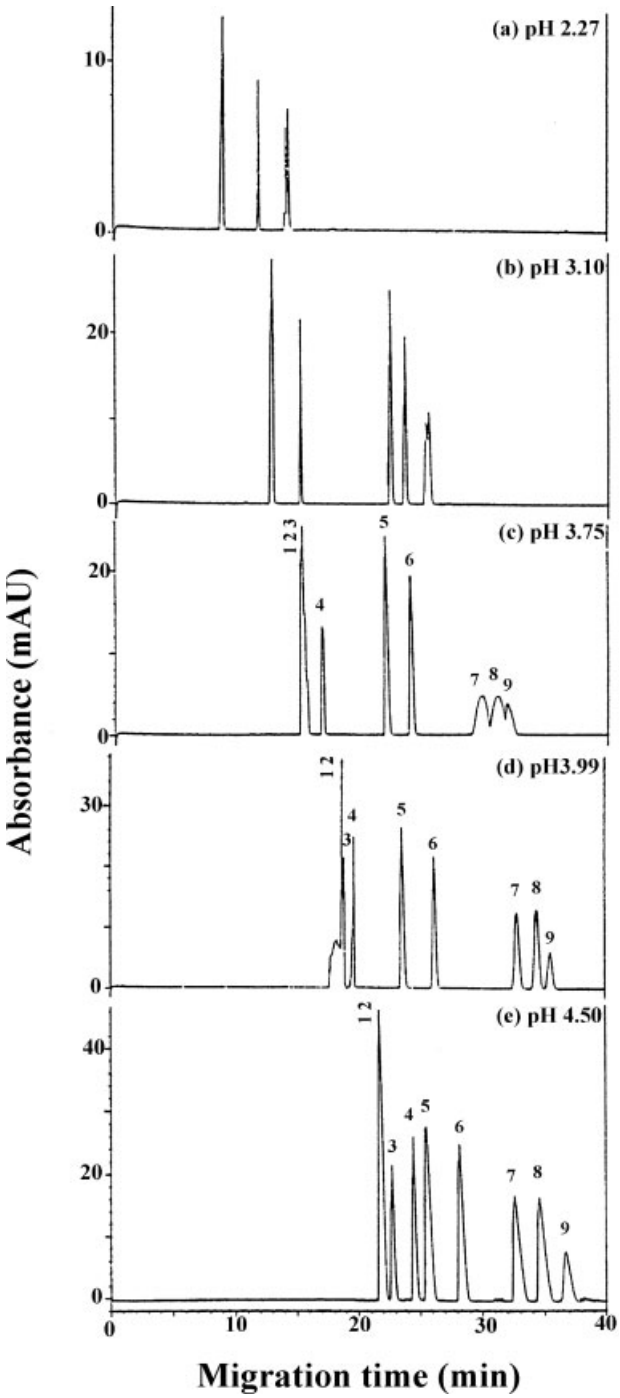

Figure 3. Electrochromatographic separation of monophosphorylated nucleoside isomers at different pH values. Conditions: Column, [28]ane-N_6O_2 bonded phase fused-silica capillary, 70 cm (50 cm) $\times$ 75 μm ID; applied voltage, −15 kV; sample concentration, 0.025 mg/mL; sample injection, electrokinetically (−5 kV, 10 s); detection, 254 nm; BGE, 30 mM citrate buffer (a) pH 2.27; (b) pH 3.10; (c) pH 3.75; (d) pH 3.99; (e) pH 4.50. Peak identification: 1, 3'-UMP; 2, 2'-UMP; 3, 5'-UMP; 4, 5'-GMP; 5, 3'-AMP; 6, 5'-AMP; 7, 3'-CMP; 8, 2'-CMP; 9, 5'-CMP.

3.2 Composition and concentration of the buffer solutions

Separations were further performed in buffers of different composition and concentration to establish how these parameters influence a CEC separation. In addition to the citrate buffer, phosphate, acetate (all are 30 mM, pH 3.99) and citrate/phosphate mix buffer (20 mM, pH 3.99) were also examined. The electrochromatograms of the separation are shown in Fig. 4. With benzyl alcohol as the neutral marker, the migration times and the corresponding μ_{EOF} are shown in Table 2. In CE, a greater ionic strength would result in a smaller EOF. Since the analytes were coeluted with the EOF, by considering the effective charges of acetate (−0.150), phosphate (−0.986) and citrate (−1.027), a slower migration was expected in the latter two cases. But there was no significant difference between the BGE of phosphate and citrate. It can

Table 1. Molecular mass, pK_a values, and relative mobility ($Q/M^{2/3}$) of the monophosphorylated nucleoside isomers[a)]

Analyte	M_r	pK_a[b)]	$Q/M^{2/3}$				
			pH 2.27	pH 3.10	pH 3.75	pH 3.99	pH 4.50
3'-UMP	324.2	9.43, 1.02, 5.88	−0.0212	−0.0212	−0.0212	−0.0212	−0.0212
2'-UMP	324.2	9.43, 1.02, 5.88	−0.0212	−0.0212	−0.0212	−0.0212	−0.0212
5'-UMP	368.1	9.5, 6.4	−0.0195	−0.0195	−0.0195	−0.0195	−0.0195
5'-GMP	407.2	2.4, 9.4, 6.1	-7.75×10^{-3}	−0.0152	−0.0174	−0.0177	−0.0181
3'-AMP	347.2	3.74, 5.92	-6.68×10^{-4}	-3.77×10^{-3}	−0.0102	−0.0130	−0.0172
2'-AMP	347.2	3.81, 6.17	-5.67×10^{-4}	-3.30×10^{-3}	-9.41×10^{-3}	−0.0122	−0.0168
5'-AMP	347.2	3.9, 6.2−6.4	-4.66×10^{-4}	-2.77×10^{-3}	-8.38×10^{-3}	−0.0112	−0.0162
3'-CMP	323.2	4.16−4.31, 6.04	-2.76×10^{-4}	-1.70×10^{-3}	-5.94×10^{-3}	-8.58×10^{-3}	−0.0146
2'-CMP	323.2	4.3−4.3, 6.19	-1.91×10^{-4}	-1.25×10^{-3}	-4.67×10^{-3}	-6.99×10^{-3}	−0.0130
5'-CMP	367.2	4.5, 6.3	-1.17×10^{-4}	-7.41×10^{-4}	-2.94×10^{-3}	-4.62×10^{-3}	-9.75×10^{-3}

a) Relative mobilities are calculated from Offord's empirical equation, $\mu_{rel} = Q/M^{2/3}$, where Q is the effective charge and M is the molecular mass (g/mol) (Kuhn, R., Hoffstetter-Kuhn, S., *Capillary Electrophoresis: Principles and Practice*, Springer-Verlag, Berlin 1993).

b) The data are from Fasman, G. D., Sober, H. A., *Handbook of Biochemistry and Molecular Biology*, 3rd Ed., Vol. 1, CRC Press, Boca Raton, FL 1976.

Table 2. Effect of different BGEs on migration time and electrophoretic mobility of the monophosphorylated nucleoside isomers[a)]

Analyte	Phosphate buffer[b)]		Citrate buffer[b)]		Acetate buffer[b)]		Citrate-phosphate buffer[c)]
	t_m (min)	μ_{ep} (cm^2/Vs)	t_m (min)	μ_{ep} (cm^2/Vs)	t_m (min)	μ_{ep} (cm^2/Vs)	t_m (min)
Benzyl alcohol	25.19	1.54×10^{-4} [d)]	63.82	6.09×10^{-5}	17.24	2.26×10^{-4}	
3'-UMP	11.80	-1.76×10^{-4}	16.94	-1.69×10^{-4}	9.61	-1.79×10^{-4}	16.66
2'-UMP	11.80	-1.76×10^{-4}	17.40	-1.62×10^{-4}	9.61	-1.79×10^{-4}	16.66
5'-UMP	12.07	-1.68×10^{-4}	17.58	-1.60×10^{-4}	9.61	-1.79×10^{-4}	17.19
5'-GMP	12.74	-1.51×10^{-4}	18.35	-1.50×10^{-4}	10.23	-1.54×10^{-4}	18.64
3'-AMP	14.57	-1.13×10^{-4}	21.79	-1.17×10^{-4}	11.66	-1.08×10^{-4}	22.52
5'-AMP	15.72	-9.30×10^{-5}	24.26	-9.91×10^{-5}	12.29	-9.00×10^{-5}	25.32
3'-CMP	17.88	-6.30×10^{-5}	30.57	-6.61×10^{-5}	13.77	-5.60×10^{-5}	32.82
2'-CMP	18.45	-5.70×10^{-5}	32.13	-6.01×10^{-5}	14.05	-5.10×10^{-5}	35.40
5'-CMP	19.04	-5.00×10^{-5}	33.21	-5.61×10^{-5}	14.28	-4.60×10^{-5}	37.78

a) Column: [28]ane-N_6O_2 bonded phase capillary; effective length, 50 cm, 75 μm ID, total length, 70 cm; applied voltage, −15 kV; sample injection, electrokinetically (−5 kV, 10 s); sample concentration, 0.025 mg/mL; detection at 254 nm

b) 30 mM, pH 3.99

c) 20 mM, pH 3.99

d) Marker of EOF mobility (μ_{eof})

be concluded that the replacement of analyte by phosphate seems greater than that by citrate. In other words, the affinity of phosphate toward the bonded group might be stronger than that of citrate. The results agreed with the property shown in the previous paper, that the phosphate moiety and not the nucleoside and the base, is the predominant active center for nucleotide binding to the macrocyclic polyamine [16]. Moreover, the highest separation efficiencies including number of theoretical plate and resolution were provided in citrate buffer and not the citrate-phosphate buffer.

A good separation was found at pH 4.5 (Fig. 3), but considering both the peak shape and the analysis time, a pH of 3.99 was chosen for the subsequent work. The effect of the citrate buffer concentration on these separations was

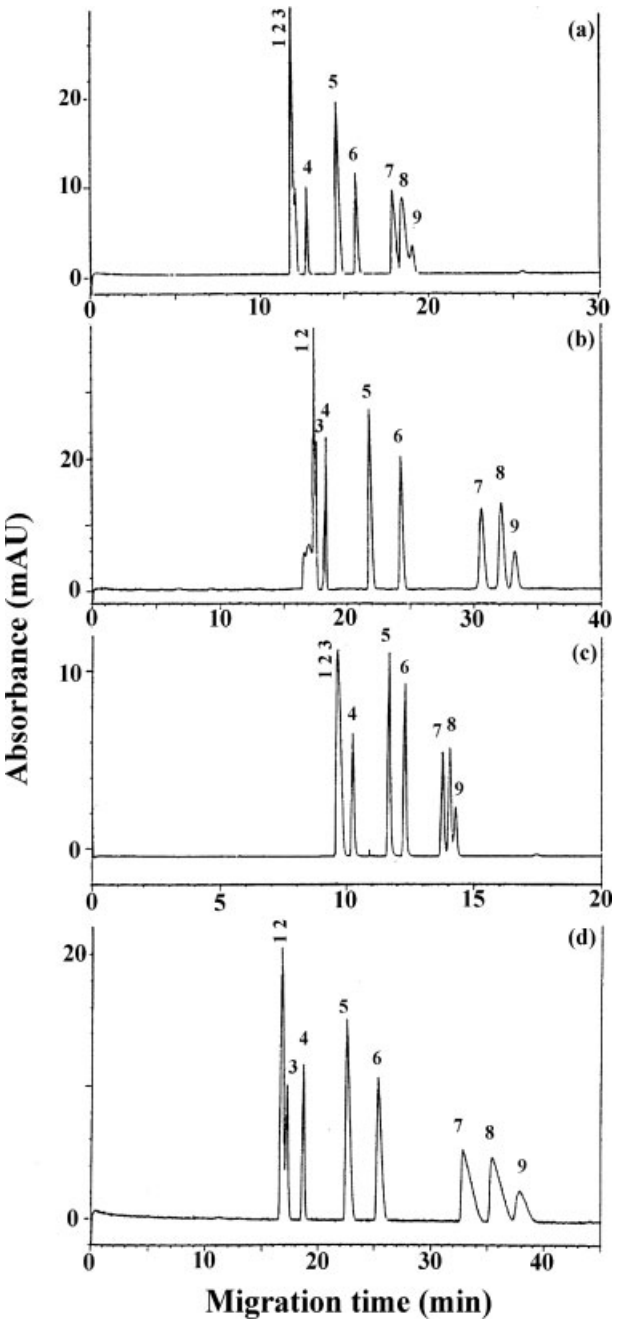

Figure 4. Electrochromatographic separation of monophosphorylated nucleoside isomers at different BGEs. Conditions as in Fig. 3, except sample concentration, 0.025 mg/mL (a–c); 0.0125 mg/mL and BGE, pH 3.99 (a) 30 mM phosphate; (b) 30 mM citrate; (c) 30 mM acetate; (d) 20 mM citrate-phosphate buffer. Peak identification as in Fig. 3.

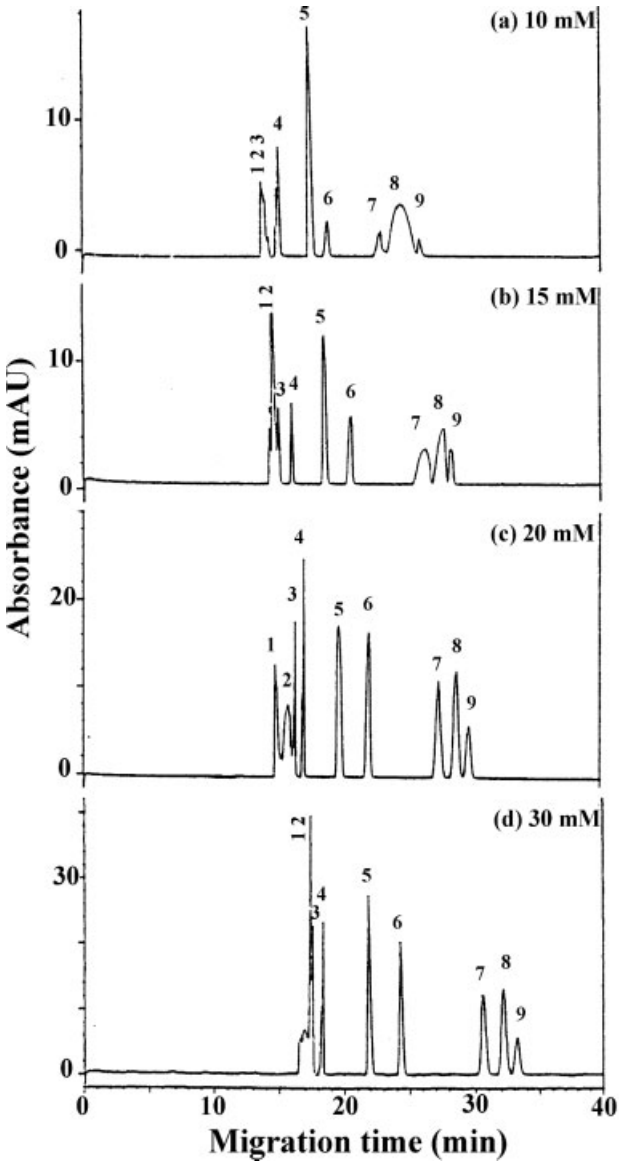

Figure 5. Electrochromatographic separation of monophosphorylated nucleoside isomers at different buffer concentrations. Conditions as Fig. 3, except BGE, citrate buffer (pH 3.99) (a) 10 mM; (b) 15 mM; (c) 20 mM; (d) 30 mM. Peak identification as in Fig. 3.

examined over concentration levels of 10–30 mM. Resolution improved when the buffer concentration was increased (Fig. 5). But in the 30 mM buffer, the separation of the UMP isomer deteriorated. One possible explanation of this fact might be the fast, simultaneous replacement of the analytes by the high concentration of citrate buffer.

3.3 Effect of applied voltage

The effect of varying the working voltage from −10 kV to −25 kV on the migration times of the analytes was investigated. An increase in the applied voltage decreased the migration time. A potential of −15 kV yielded the best

compromise in terms of run time, current generated and efficiency of separation, especially for the UMP isomer. This potential was used in subsequent stages of method development.

3.4 Additives in the background electrolyte

Because resolution of 3'-UMP, 2'-UMP and 5'-UMP isomers cannot be improved by the factors studied, we adopted another strategy to improve the separations.

3.4.1 Interaction of nucleotides with borate

Influence of borate complexation on the electrophoretic behavior of carbohydrates in CE has been studied by Hoffstetter-Kuhn *et al.* [23]. The results indicated that borate could form a complex with the glucose in the open-chain form but, under conditions of very high borate concentrations, the pyranose form also complexes with borate. Recently, D'Acunto *et al.* [24] also systematically studied the potential binding of borate ions to mono- and oligonucleotides.

To improve resolution of nucleotide mixtures, borate as additive over the concentration range from 0.05 mM to 3 mM was tested. The addition of borate into the citrate buffer (20 mM, pH 3.99) would result in the formation of boric acid. But it will not affect the pH, since boric acid is a rather weak acid ($K_a = 5.81 \times 10^{-10}$). The EOF change as the borate concentration increased was due to the ionic strength increase. The sequence for the elution was similar to that without the borate additive. The migration time differences between them, in decreasing order are 5'- > 2'- > 3'- for each isomer (Fig. 6). The 5'-isomer forms the strongest complex with borate, since the vicinal hydroxyl groups with *cis*-configuration can form stable complexes [23]. The 2'-isomer represents the intermediate case since the phosphate group is not in a position to sterically hinder complex formation, and that of 3'-isomer, containing hydroxyl groups at the 2'- and 5'- position, is the least.

3.4.2 Interaction of nucleotides with metal ions

Cahours *et al.* [6–8] reported the addition of inorganic cations to the buffer could improve the resolution of nucleotide mono-, di- and triphosphates in CZE separation [6–8]. In this work, either magnesium chloride or zinc acetate was tested to see whether the resolution of UMP isomer would be improved. The results showed that a faster migration for the analytes was indicated with the addition of 0.1 M magnesium chloride (Fig. 7). The phenomenon can be explained as follows. In this work, all nucleotides are monophosphates. Mg^{2+} coordinates only at the phosphate group and has a very low affinity toward N donors of the nucleotide [25]. Meanwhile, both the bonded macrocyclic polyammonium and Mg^{2+} can potentially interact simultaneously with the phosphate group of the nucleotide. The mentioned result is thus explicable. However, on further increasing the concentration, a slightly increase of the analysis time was found. One explanation for this finding might be a greater ionic strength resulting in a decrease of EOF. With the addition of zinc acetate to the BGE, no improvement for the UMP isomer separation was indicated (Fig. 8). Although zinc ion has greater affinity with the nitrogen donor of the base, there was no significantly different migration behavior from that with the addition of magnesium chloride.

3.4.3 Effect of β-cyclodextrin

Tadey and Purdy [5] employed complexation reactions with CD and borate for the CE separation of nucleotide isomers. Kuwamura [9] used a mixture of Mg^{2+}, β-CD

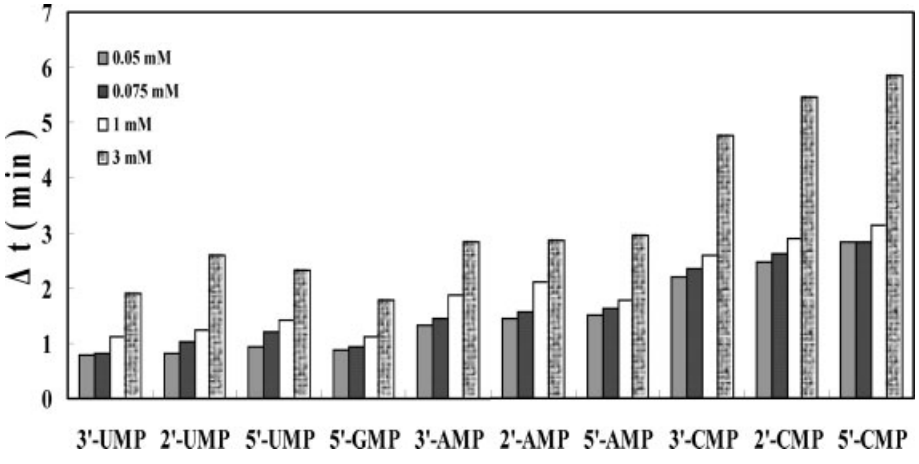

Figure 6. Effect of borate concentration on the migration time of monophosphorylated nucleoside isomers by comparison with those without the additive. Conditions as Fig. 5, except sample concentration, 0.0125 mg/mL and BGE, 20 mM citrate buffer (pH 3.99) with the addition of borate.

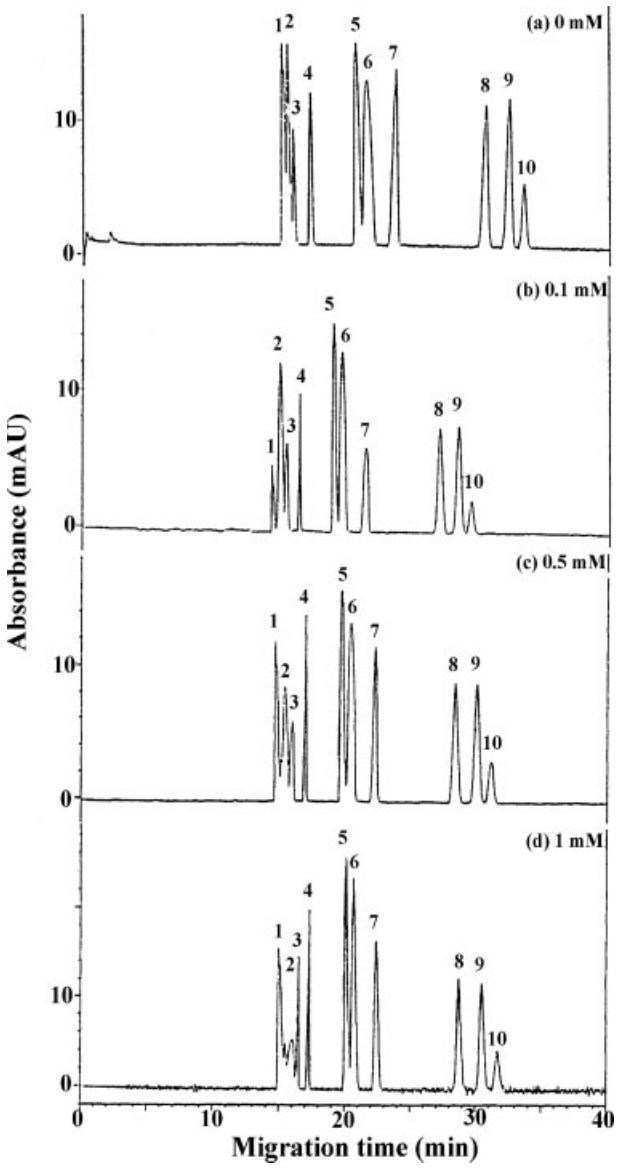

Figure 7. Electrochromatographic separation of monophosphorylated nucleoside isomers by the addition of magnesium chloride in the citrate buffer. Conditions as in Fig. 6, except BGE, 20 mM citrate buffer (pH 3.99) with the addition of (a) 0; (b) 0.1; (c) 0.5; (d) 1 mM $MgCl_2$. Peak identification: 1, 3'-UMP; 2, 2'-UMP; 3, 5'-UMP; 4, 5'-GMP; 5, 3'-AMP; 6, 2'-AMP; 7, 5'-AMP; 8, 3'-CMP; 9, 2'-CMP; 10, 5'-CMP.

and SDS as a BGE for the separation of nucleotide isomers. In this work, the addition of β-CD over the concentration range of 1–4 mM was also studied. Unfortunately, the host-guest and hydrogen bonding affinity with the analytes was not expected. Not only slower migration but also lower separation efficiency was demonstrated. Serious adsorption of a neutral marker on the inner wall of the column was also found. The EOF was gradually

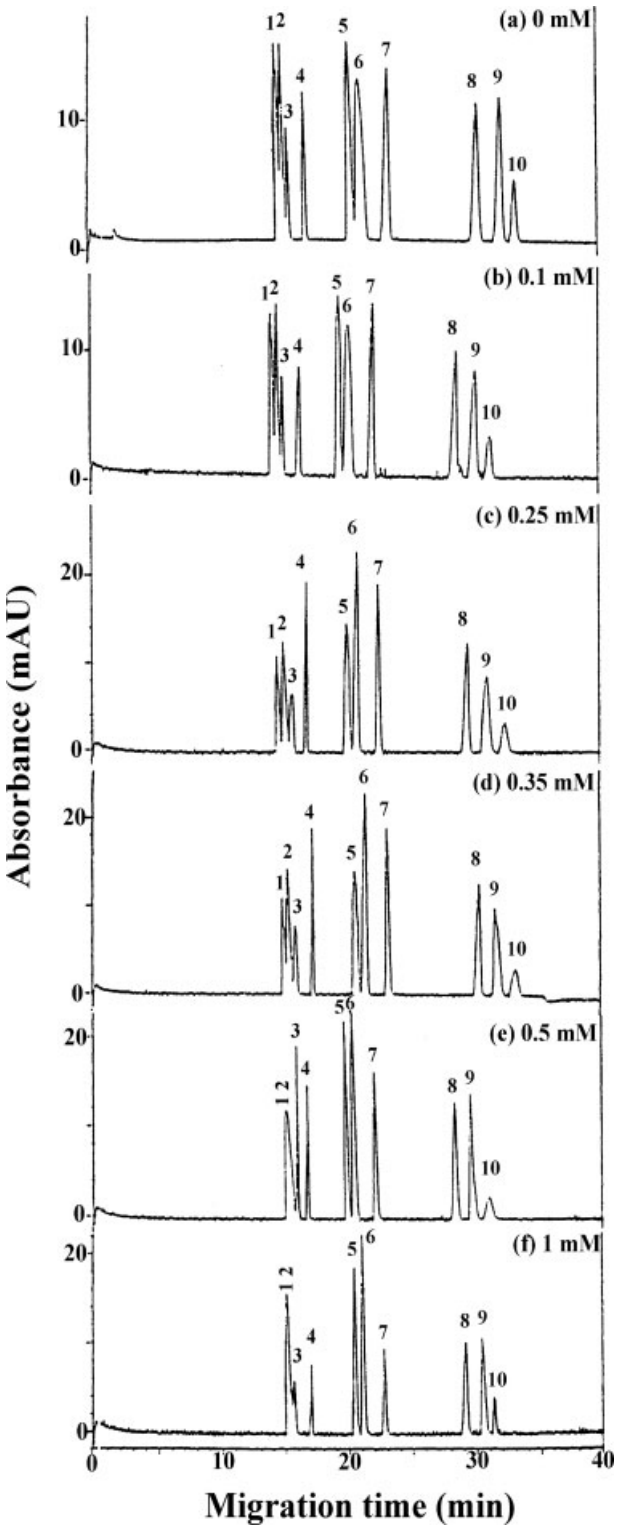

Figure 8. Electrochromatographic separation of monophosphorylated nucleoside isomers by the addition of zinc acetate in the citrate buffer. Conditions as in Fig. 6, except BGE, citrate buffer (20 mM, pH 3.99) with the addition of (a) 0; (b) 0.1; (c) 0.25; (d) 0.35; (e) 0.5; (f) 1 mM $Zn(OAc)_2$. Peak identification as in Fig. 7.

reduced and eventually the column was corrupted. This suggests that the binding sites for the analytes were masked by CD, due to the hydrophobic and hydrogen bonding interaction between the bonded macrocyclic polyamine and the CD.

3.4.4 Effect of organic solvent

The use of methanol as additive in the BGE (5–15% v/v) was also studied. This did not produce an improvement in the separation. The more methanol, the longer was the migration time. Since a high percentage of methanol would increase the viscosity, the phenomenon is rational.

3.5 Reproducibility

Except for the UMP isomers, excellent resolution with an average plate number of 10^5/m was indicated for the other seven isomers. The relative standard deviation of the migration time for the seven consecutive injections ranging from 0.63% to 1.55% was demonstrated (Table 3). The prepared column can be used longer than three months with injections more than 1000. Calibration curves for the determination of the nucleotide isomers are shown in Table 4. The detection limit defined by three times the signal-to-noise ratio for these separations was approximately $10^{-7}-10^{-8}$ M.

Table 3. Reproducibility in the CEC separation of 10 monophosphorylated nucleoside isomers[a)]

Analyte	Migration time (min) Mean value ± SD (RSD%)[b)]	Mobility (μ_{ep}, cm^2/Vs)
3'-UMP	21.95 ± 0.15 (0.69)	-1.61×10^4
2'-UMP	22.04 ± 0.16 (0.71)	-1.60×10^4
5'-UMP	22.15 ± 0.15 (0.68)	-1.59×10^4
5'-GMP	23.73 ± 0.15 (0.63)	-1.42×10^4
3'-AMP	27.58 ± 0.30 (1.08)	-1.09×10^4
2'-AMP	28.35 ± 0.31 (1.09)	-1.03×10^4
5'-AMP	30.04 ± 0.35 (1.16)	-9.16×10^5
3'-CMP	36.17 ± 0.52 (1.14)	-5.96×10^5
2'-CMP	37.56 ± 0.57 (1.52)	-5.38×10^5
5'-CMP	38.48 ± 0.60 (1.55)	-5.02×10^5

a) Column: [28]ane-N_6O_2 bonded phase fused-silica capillary,100 cm (75 cm) × 75 μm ID; BGE, citrate buffer (20 mM, pH 3.99); applied voltage: −22 kV; sample injection, electrokinetically (−7 kV, 10 s); sample concentration, 6.25×10^{-3} mg/mL (3'-UMP, 5'-GMP, 3'-AMP, 2'-AMP), 0.0125 mg/mL (5'-AMP, 3'-CMP, 2'-CMP), 0.025 mg/mL (5'-CMP); detection at 254 nm
b) Seven consecutive injections

4 Concluding remarks

As the similar electrophoretic mobility, no simple buffer system can be used for the CZE separation of monophosphorylated nucleotide isomers. The separation of

Table 4. Quantitation of monophosphorylated nucleoside isomers resolved by CEC[a)]

Analyte	Linear equation[b)]			Detection limit[b)]	
	Slope	Intercept	r^2	(mg/L)	(μM)
3'-UMP	4×10^9	2.09×10^3	0.9970	1.23×10^{-2}	3.79×10^{-2}
2'-UMP	4×10^9	3.41×10^3	0.9972	1.03×10^{-2}	3.18×10^{-2}
5'-UMP	4×10^9	1.91×10^3	0.9957	1.42×10^{-2}	3.85×10^{-2}
5'-GMP	5×10^9	3.23×10^3	0.9979	1.64×10^{-2}	4.03×10^{-2}
3'-AMP	7×10^9	3.15×10^3	0.9837	1.38×10^{-2}	3.97×10^{-2}
2'-AMP	1×10^{10}	4.90×10^3	0.9915	9.27×10^{-3}	2.67×10^{-2}
5'-AMP	6×10^9	6.05×10^3	0.9948	3.05×10^{-2}	8.87×10^{-2}
3'-CMP	4×10^9	2.57×10^3	0.9899	3.93×10^{-2}	1.22×10^{-1}
2'-CMP	5×10^9	5.46×10^3	0.9921	3.75×10^{-2}	1.16×10^{-1}
5'-CMP	2×10^9	4.13×10^3	0.9954	7.85×10^{-2}	2.14×10^{-1}

a) Conditions as in Table 3[a)]
b) Linear range was $1.0\times10^{-6}-6.0\times10^{-6}$ M for 2'-UMP, 5'-UMP, 3'-UMP, 5'-GMP, 3'-AMP, and 5'-AMP and $2.0\times10^{-6}-1.2\times10^{-5}$ M for 5'-AMP, 3'-CMP. 2'-CMP, and 5'-CMP. The results were based on 95% confidence level, $n = 5$.

y, peak area (μV·s); x, concentration (M)

2'-, 3'- and 5'- isomers is even more difficult. In this work, an open-tubular column coated with a 28-membered macrocyclic polyamine was employed for the CEC separation of these isomers. The competitive anions for the coordination, such as phosphate, citrate, acetate, and citrate/phosphate buffers have been tested as the BGE. It was shown that the nature of the BGE has a major influence on the separation among the several parameters investigated. Additionally, UMP isomer comprised the most difficult pair to separate. Even borate, metal ions, β-CD, or methanol as additives of the BGE failed to improve the resolution. All the results suggested that the contribution of the selectivity seems to stem mainly from the bonded group. The selectivity of the bonded group depends on electrostatic, hydrogen bonding and structural effects. Anion exchange as one of the mechanisms should also be considered since the macrocyclic polyamine existed as protonated form. Due to the high selectivity of the column, a simple composition of the BGE can be used for the separation of the nucleotide isomers. Additionally, the results were highly reproducible and a low detection limit was obtained. Thus, we can conclude that CEC separation of the monophosphorylated nucleoside isomers with macrocyclic polyamine-coated capillary column is a highly promising analytical method.

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5 References

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