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# Separation of ribonucleotides by capillary electrophoresis with multifunctional electrophoretic media of phosphate ammonium salts

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**Abstract**

Baseline separation of all common ribonucleotides by capillary electrophoresis (CE) was accomplished with bare fused-silica capillaries of 30 cm effective length and multifunctional electrophoretic media of simple composition, i.e., only phosphate ammonium salts, of moderate pH values in less than 10 min. A separation efficiency of $8 \cdot 10^5$/m in terms of theoretical plate number and a resolution between all adjacent peaks greater than 2 were obtained in capillaries of 75.0 cm effective length. Buffers containing phosphate anions resulted in good peak shape in the CE separation of the ribonucleotides. Among the phosphate buffers with different cations, potassium phosphate buffer showed better resolution than sodium phosphate buffer, but ammonium phosphate buffer gave the best resolution for the ribonucleotides. Inorganic ammonium cations affected the mobilities of ribonucleotides and enhanced their resolution during separation by ionic interaction and hydrogen-bonding. The migration behavior of the ribonucleotides was examined over the pH range from 5.5 to 9.0. In addition, the effects of organic solvents, such as acetonitrile, on the migration order of the ribonucleotides were studied. The CE method developed has proven to be a fast and simple means for the separation of the ribonucleotides with high efficiency and resolution. The results obtained demonstrated that buffers of phosphate ammonium salts have unique characteristics and may be excellent electrophoretic media for the separation of the ribonucleotides and other biochemical compounds by CE. 

*Keywords:* Buffer composition; Ribonucleotides; Nucleotides

## 1. Introduction

Nucleotides constitute an important class of compounds in the life sciences. They are best known as building blocks of nucleic acids. These compounds participate in a wide variety of biochemical processes, such as cellular metabolism, cell bioenergetics and signal transduction pathways [1,2]. Separation, identification and determination of nucleotides have been of great interest to researchers in the fields of modern separation technologies [2–23].

As capillary electrophoresis (CE) has the advantages of high separation efficiency, short analysis time, small sample consumption and low operation cost [24–26] and ribonucleotides are charged compounds showing strong UV absorption, many researchers attempted to develop CE methods for the separation of nucleotides [2–9,11–13,16,17, 20,23,27]. However, baseline separation of nucleotides, especially all the common ribonucleotides, was hardly achieved in a short separation time by using

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either bare or coated fused-silica capillaries. It is recognized that there are two difficulties in developing a successful CE method for the separation of nucleotides. One of the difficulties is the poor peak shape which significantly deteriorates efficiency and resolution in CE separation of nucleotides [3,16]. The other is the requirement for high selectivity to achieve acceptable resolution since some of the common nucleotides have similar mass-to-charge ratios and therefore similar mobilities in many reported electrophoretic media [13,17,23].

Improved peak shape in the CE separation of nucleotides has been obtained using fused-silica capillaries with "permanent" coatings in many reports [9,11,13,16,17]. In addition, the coatings also played a role in suppressing electroosmotic flow (EOF). Of the many "permanent" coatings, Ucon 75-H-90 000 [poly(ethylene–propylene)glycol] was the only one reported so far to be able to separate all the common ribonucleotides in capillary zone electrophoresis (CZE), although the run time was over 40 min [16]. Capillaries with polyacrylamide coating were used for the separation of ribonucleotides in isotachophoresis [9], in which zone sharpening might also play a role in the separation. Nevertheless, it is generally known that generation of "permanent" coatings on the capillaries typically involves laborious and time-consuming procedures. Alternatively, Tsuda et al. [3] reported a dynamic coating in which 0.5% ethylene glycol was added to a phosphate buffer of sodium and potassium salts to improve peak symmetry and consequently resolution in the CE separation of ribonucleotides. However, baseline separation of the ribonucleotides was not obtained.

To increase selectivity and to enhance resolution in the separation of nucleotides, many researchers have attempted to use micellar electrokinetic chromatography (MEKC). Both anionic and cationic surfactants were used to separate ribonucleotides in MEKC, but none were able to achieve baseline separation of all the common ribonucleotides [2,5,6]. The unsatisfactory resolution might be due to limited elution ranges of the MEKC systems, and to the insignificant differences in partition behavior of ribonucleotides between the micellar pseudo-stationary phase and the aqueous buffer phase.

Most recently, Uhrova et al. [23] reported a simple phosphate–borate buffer for the CE separation of ribonucleotides by using bare fused-silica capillaries without suppressing EOF. But only eleven of the common ribonucleotides were involved in their investigation and at least one pair of the studied compounds was not baseline resolved under the reported conditions. Simple CE buffers which can enhance selectivity and improve resolution of all the common ribonucleotides within relatively short separation times will be of great interest.

This paper demonstrates that buffers of phosphate ammonium salts may be the right choice. Results of CE separation of all common ribonucleotides using bare fused-silica capillaries and multifunctional electrophoretic media of phosphate ammonium salts of moderate pH values with high efficiency and resolution are presented. The effects of organic solvents, such as acetonitrile, on the migration order of the ribonucleotides are studied. The effect of pH – over the moderate pH range from 5.5 to 9.0 – on the migration behavior of the ribonucleotides is examined as well.

## 2. Experimental

### 2.1. Apparatus

Experiments were performed on an $HP^{3D}$ capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany) and a laboratory-built CE system [28]. The $HP^{3D}$ CE system was operated at 30°C and data acquisition and manipulation were carried out by an $HP^{3D}$ CE ChemStation. The laboratory-built CE system consisted of a Spellman CZE1000R Power Supply (Plainview, NY, USA), a Linear Instruments UVIS 200 detector (Reno, NV, USA) and an HP 3390A Integrator (Hewlett-Packard, Avondale, PA, USA). Capillaries with polyimide coating were products of Polymicro Technologies (Phoenix, AZ, USA).

### 2.2. Chemicals

Adenosine, cytidine, guanosine and uridine 5′-mono-, di- and triphosphates were purchased from Sigma (St. Louis, MO, USA). The buffers were prepared using reagent grade phosphate sodium salts

(Fluka, Buchs, Switzerland), acetic acid and phosphate potassium salts (Merck, Darmstadt, Germany), phosphate ammonium tribasic (BDH, Poole, UK), phosphate ammonium dibasic (Hopkins and Williams, Essex, UK), phosphate ammonium monobasic (Judex, Sudbury Middlesex, UK), ammonium acetate (Hayashi, Osaka, Japan) and acetonitrile (Fisher Scientific, Pittsburgh, PA, USA). Water purified with a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout the study.

### 2.3. Procedure

The fresh capillaries were rinsed with water for 5 min, followed by 0.1 *M* NaOH for 10 min, water for 3 min, and finally by the run buffer for 10 min in the forward direction (from injection end to detection end). Benzyl alcohol was used as a marker to determine the EOF in part of the study. Otherwise, the EOF was characterized by the peak due to difference in the refractive indices of the sample matrix and the run buffers. Sample injection at the anode side was performed at 25 mbar for 2–6 s on the $HP^{3D}$ CE system and at 4.5 cm for 20 s on the laboratory-built CE system. Detection was performed at 260 nm. Separation was conducted at an electric field strength of 340 V/cm and at 30°C, unless otherwise stated.

## 3. Results and discussion

### 3.1. Effect of cations in buffer on separation

The effect of different inorganic cations in phosphate buffers on the CE separation of the ribonucleotides was examined. Fig. 1a–c shows the results of the separation of ribonucleotides in 100 m*M* phosphate buffers at pH 6.1 containing $Na^+$, $K^+$ and $NH_4^+$ cations, respectively. Table 1 lists the separation efficiencies of different buffer systems in terms of theoretical plates for GMP (an early migrating peak in this study) and ATP (a late migrating peak). It was noted by comparing the last peak pair of CTP and UTP in Fig. 1a and b that the phosphate buffer of potassium ions achieved a slightly better resolution of the ribonucleotides than the phosphate buffer of sodium ions. These results seemed to be contradictory to the trends of the separation efficiencies of the two kinds of phosphate buffers as shown in Table 1. However, it should be noted that the reduced EOF in the potassium phosphate buffer, which was attributable to strong binding of potassium ions to silanoate groups on the wall of the capillaries [29,30], might have made a more significant contribution to improve resolution. The best result in terms of resolution was obtained with phosphate ammonium buffer as shown in Fig. 1c and the best separation efficiency in terms of the theoretical plate number as shown in Table 1. The different characteristics of the ammonium phosphate buffer and the phosphate buffers of potassium and sodium salts might arise from the capability of ammonium ions to form hydrogen bonding besides the ionic interaction and result in considerable changes in the mobilities of the ribonucleotides. Resolution in the separation of the ribonucleotides in the ammonium phosphate buffer was significantly enhanced as a consequence of both improved selectivity and increased efficiency. The reproducibility with respect to migration time was 0.5% for GMP and 1.1% for UTP in terms of relative standard deviation (R.S.D.) for run-to-run triplicate measurements, and 0.8% for GMP and 3.8% for UTP for day-to-day triplicate measurements. No deterioration in performance of the capillaries was observed within two months of testing.

### 3.2. Effect of anions in buffer on separation

The effect of anions in buffers on the CE separation of the ribonucleotides is shown in Fig. 2a–c. Deformed peak shape, low efficiency and poor resolution were observed in acetic acid–ammonium acetate buffer for the ribonucleotides, especially for ribonucleoside diphosphates and triphosphates. Similar deformed peak shape and deteriorated resolution of the ribonucleotides were observed in phosphate–Tri-HCl (30:50 m*M*) buffer of pH 5.2 inside bare silica capillaries [16]. These results might indicate, as suggested by McCormick [31] and supported by Tran et al. [32], that phosphate groups bind strongly to the silica surface of the capillary wall. Huang et al. noted the existence of analyte–wall interaction of adenosine and adenosine monophosphate with the fused-silica capillaries when the effective diffusion

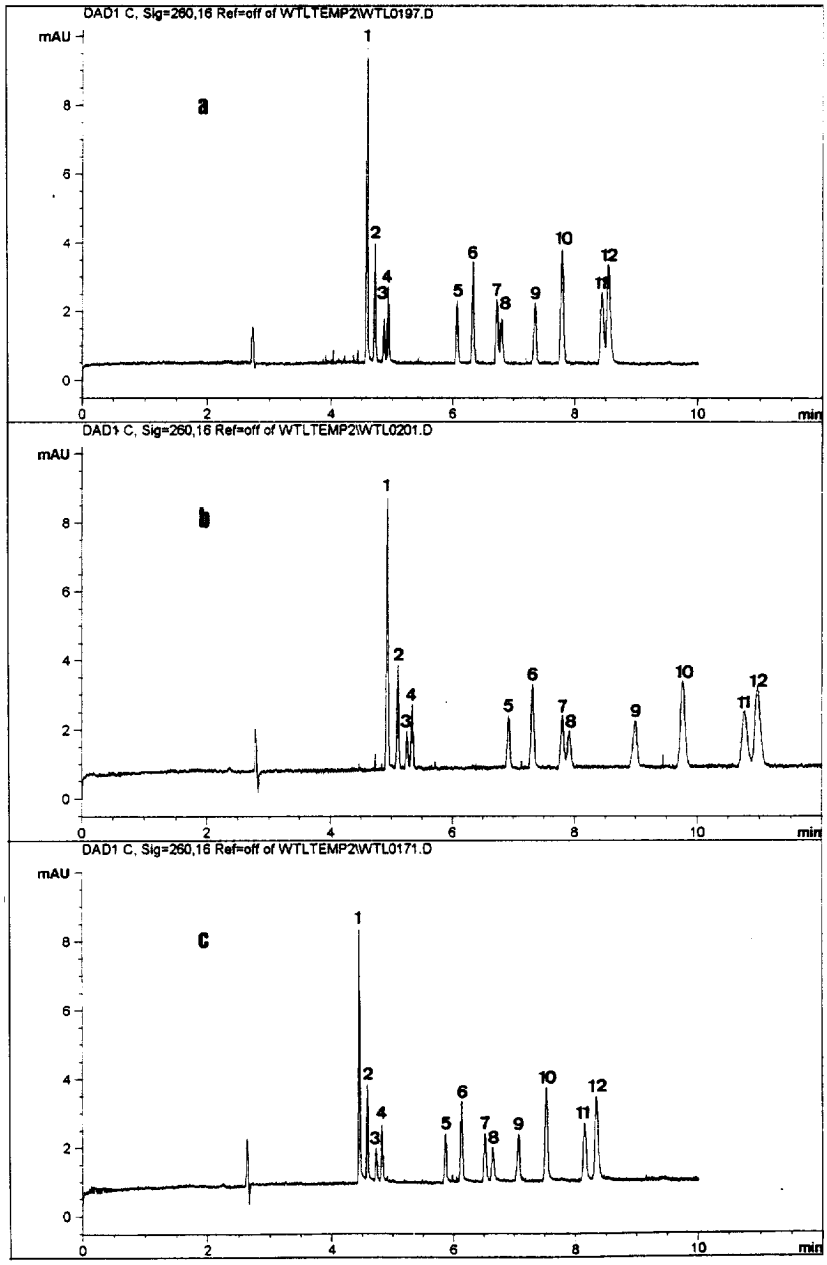


Fig. 1. Separation of common ribonucleotides in buffers of different phosphate salts. Operating conditions: total capillary length 38.5 cm (effective capillary length 30.0 cm)×45 μm I.D.; injection at 25 mbar for 2 s; applied voltage, 13.1 kV; temperature, 30°C. (a) 100 m*M* sodium phosphate (pH 6.1); (b) 100 m*M* potassium phosphate (pH 6.1); (c) 100 m*M* ammonium phosphate (pH 6.1). Peaks: 1=guanosine 5′-monophosphate (GMP) (0.16 m*M*), 2=adenosine 5′-monophosphate (AMP) (0.096 m*M*), 3=cytidine 5′-monophosphate (CMP) (0.091 m*M*), 4=uridine 5′-monophosphate (UMP) (0.091 m*M*), 5=guanosine 5′-diphosphate (GDP) (0.075 m*M*), 6=adenosine 5′-diphosphate (ADP) (0.16 m*M*), 7=cytidine 5′-diphosphate (CDP) (0.12 m*M*), 8=uridine 5′-diphosphate (UDP) (0.12 m*M*), 9=guanosine 5′-triphosphate (GTP) (0.096 m*M*), 10=adenosine 5′-triphosphate (ATP) (0.091 m*M*), 11=cytidine 5′-triphosphate (CTP) (0.21 m*M*), 12=uridine 5′-triphosphate (UTP) (0.10 m*M*). See Section 3 for details.

Table 1
Separation efficiencies of different electrophoretic systems

| Buffer | Dimension of capillary | N/m[a] Test compound: GMP | N/m Test compound: ATP |
|---|---|---|---|
| 100 m*M* Sodium phosphate (pH 6.1) | $L_t$=38.5 cm[b] $L_e$=30.0 cm[b] 45 μm I.D. | $6.7 \cdot 10^5$/m | $7.0 \cdot 10^5$/m |
| 100 m*M* Potassium phosphate (pH 6.1) | As above | $5.7 \cdot 10^5$/m | $4.3 \cdot 10^5$/m |
| 100 m*M* Ammonium phosphate (pH 6.1) | As above | $7.0 \cdot 10^5$/m | $7.7 \cdot 10^5$/m |
| 100 m*M* Ammonium phosphate (pH 6.1) | $L_t$=83.5 cm $L_e$=75.0 cm 45 μm I.D. | $8.0 \cdot 10^5$/m | $6.5 \cdot 10^5$/m |
| 100 m*M* Ammonium phosphate (pH 8.0) | As above | $7.6 \cdot 10^5$/m | $8.4 \cdot 10^5$/m |
| 50 m*M* Ammonium phosphate containing 24% acetonitrile (pH 8.0) | As above | $8.7 \cdot 10^5$/m | $4.9 \cdot 10^5$/m |

[a] N/m: theoretical plate number per meter.
[b] $L_t$: total capillary length; $L_e$: effective capillary length.

coefficients of the analytes were determined by CE in a buffer of 20 m*M* sodium phosphate at pH 7 [33]. The physical character of the interaction was not clear.

McCormick [31] modified fused-silica capillaries with phosphate moieties to improve the separation of peptides and proteins. Chen and Calif [34] documented a dynamic coating buffer of sodium phosphate to suppress adsorption of proteins on to the fused-silica capillaries in a patent. Our previous work [28] and those of others [35] demonstrated that buffers containing ribonucleotides could be successful in the separation of phosphates and polyphosphates. All the studies gave us an impetus to explore the potential use of phosphate buffers to improve peak shape and resolution in the CE separation of ribonucleotides. By comparing Fig. 2c with Fig. 2a and Fig. 2b, one can clearly observe the effect of the phosphate anions on the improvement of peak shape and enhancement of resolution of the ribonucleotides. The experimental study of the effect of the concentration of phosphate on the separation of the ribonucleotides showed that a phosphate concentration of 50 m*M* or higher was necessary to maintain good peak shape.

Ionic strength and buffer capacity of the buffers used for Fig. 2a–c were calculated and the results are tabulated in Table 2. The calculation of buffer capacities was based on the following equation which can be applied to an arbitrary acid–base mixture in aqueous system. The equation was reported with all details by one of the authors previously [36,37].

$$\beta = 2.303[H^+] + [OH^-] - \sum_{j=0}^{L1} M1(j) \cdot \{ \sum_{i=1}^{N1(j)} (i \cdot (N1(j) - 1) \cdot \delta 1(j,i)) - \sum_{i=1}^{N1(j)} (i \cdot \delta 1(j,i)) \cdot \sum_{i=0}^{N1(j)} ((N1(j) - 1) \cdot \delta 1(j,i)) \} + \sum_{j=0}^{L2} M2(j) \cdot \{ \sum_{i=1}^{N2(j)} (i \cdot i \cdot \delta 2(j,i)) - \sum_{i=1}^{N2(j)} (i \cdot \delta 2(j,i)) \cdot \sum_{i=0}^{N2(j)} (i \cdot \delta 2(j,i)) \} \quad (1)$$

where $L1$ is the number of acids in the mixture; $N1(j)$ the number of dissociable protons of an acid of $H_{N1(j)}A$; $\delta 1(j,i)$ the distribution coefficient of the species of $H_{N1(j)-i}A^{i-}$ from the acid of $H_{N1(j)}A$; and $M1(j)$ the analytical concentration of the acid of $H_{N1(j)}A$. The same designation is valid for bases in

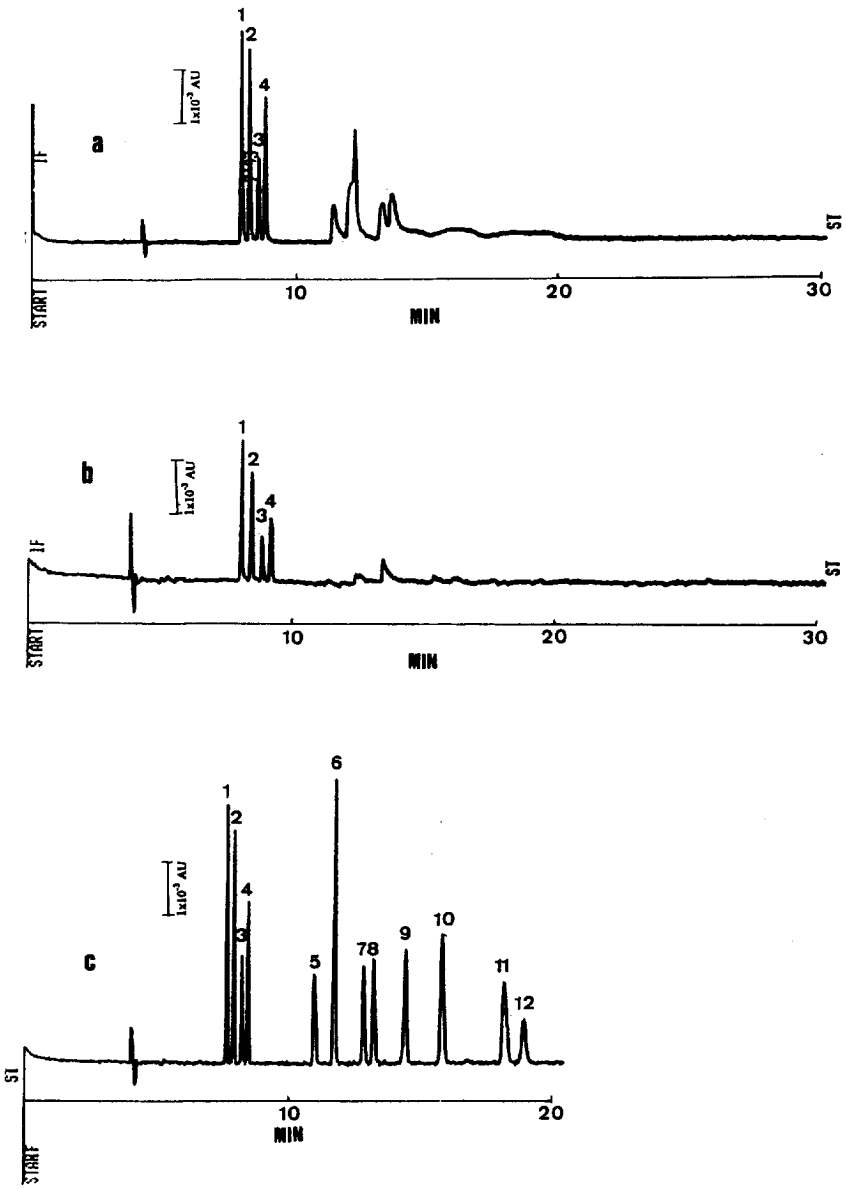


Fig. 2. Comparison of separation of common ribonucleotides in buffers of acetic acid–ammonium acetate with ammonium phosphate at pH 6.1. (a) A mixture of 100 m*M* acetic acid–ammonium acetate buffer, (b) a mixture of 160 m*M* acetic acid–ammonium acetate buffer, (c) a mixture of 100 m*M* ammonium phosphate buffer. Total capillary length 52.0 cm (effective capillary length 40.0 cm)×45 μm I.D.; injection at 4.5 cm for 20 s; applied voltage, 15 kV. A laboratory-built CE system was used at ambient temperature (25±1°C). For other conditions see Fig. 1.

the mixture but with 2 indicating the corresponding terms for a base. From Table 2, it follows that differences of the buffers in ionic strength and buffer capacity were not the main reason for different peak shape and resolution in Fig. 2a–c. In fact, ionic strength and buffer capacity of all the above buffers were sufficiently high because of high concentration of the buffer components. As a comparison, a

Table 2
Ionic strength and buffer capacity of the buffers used for Fig. 2a–c

| | Ionic strength | Buffer capacity |
|---|---|---|
| Buffer used for Fig. 2a | 0.096 | 0.0098 |
| Buffer used for Fig. 2b | 0.15 | 0.016 |
| Buffer used for Fig. 2c | 0.11 | 0.016 |

background electrolyte of 10 m*M* chromate commonly used in CE for separation of anions has a much lower buffer capacity, i.e., less than $5 \cdot 10^{-3}$ in its useful pH range.

### 3.3. *Effect of pH on separation*

Riboucleotides are compounds with both acidic and basic groups. pH should be an important parameter for optimization of their separation. Ribonucleotides are more stable in a mild pH range around the physiological pH [38,39]. Therefore, optimization of pH was carried out in a limited pH range (5.5 to 9.0).

Fig. 3 plots the mobilities of ribonucleotides measured in the pH range from 5.5 to 9.0. Ribonucleotides of the same phosphate chain length showed similar changes in their mobilities with pH. It means that it is the phosphate groups that dominate the mobility changes of the ribonucleotides with pH in the pH range studied. Mobilities of the ribonucleoside monophosphates increased most significantly with pH from 5.5 to 9.0. Mobilities of the ribonucleoside diphosphates also increased with pH, but much less significantly. Mobilities of ribonucleoside triphosphates were almost constant. These observations might be attributed to different acidities of mono-, di- and triphosphate groups. Ribonucleotides of cytidine showed a slightly different pattern in mobility change with pH compared with ribonucleotides of the other bases studied. Fig. 3 shows that the ribonucleotides can be well separated in buffers of phosphate ammonium salts of a rather broad pH range as the differences in their mobilities are sufficiently large. Fig. 4a,b shows electropherograms obtained both in a weakly acidic buffer and in a weakly basic buffer of ammonium phosphate, respectively. The resolutions between all adjacent

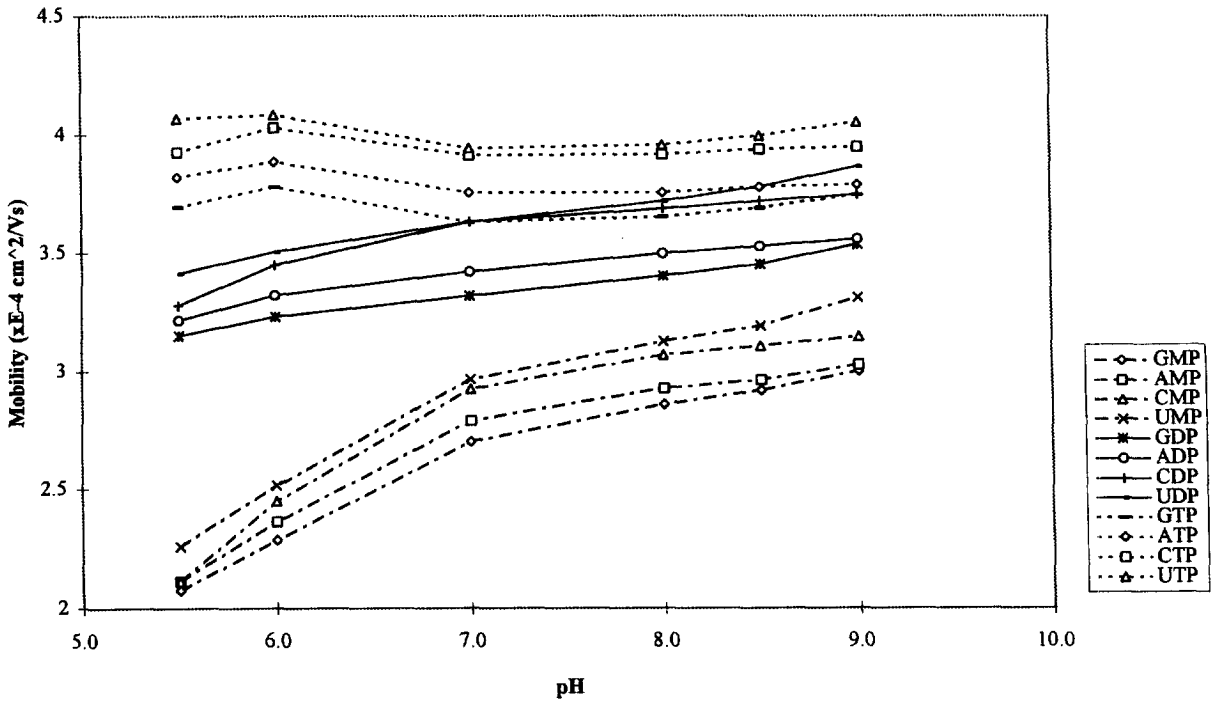


Fig. 3. Mobilities of ribonucleotides measured in 50 m*M* ammonium phosphate of pH range 5.5 to 9.0.

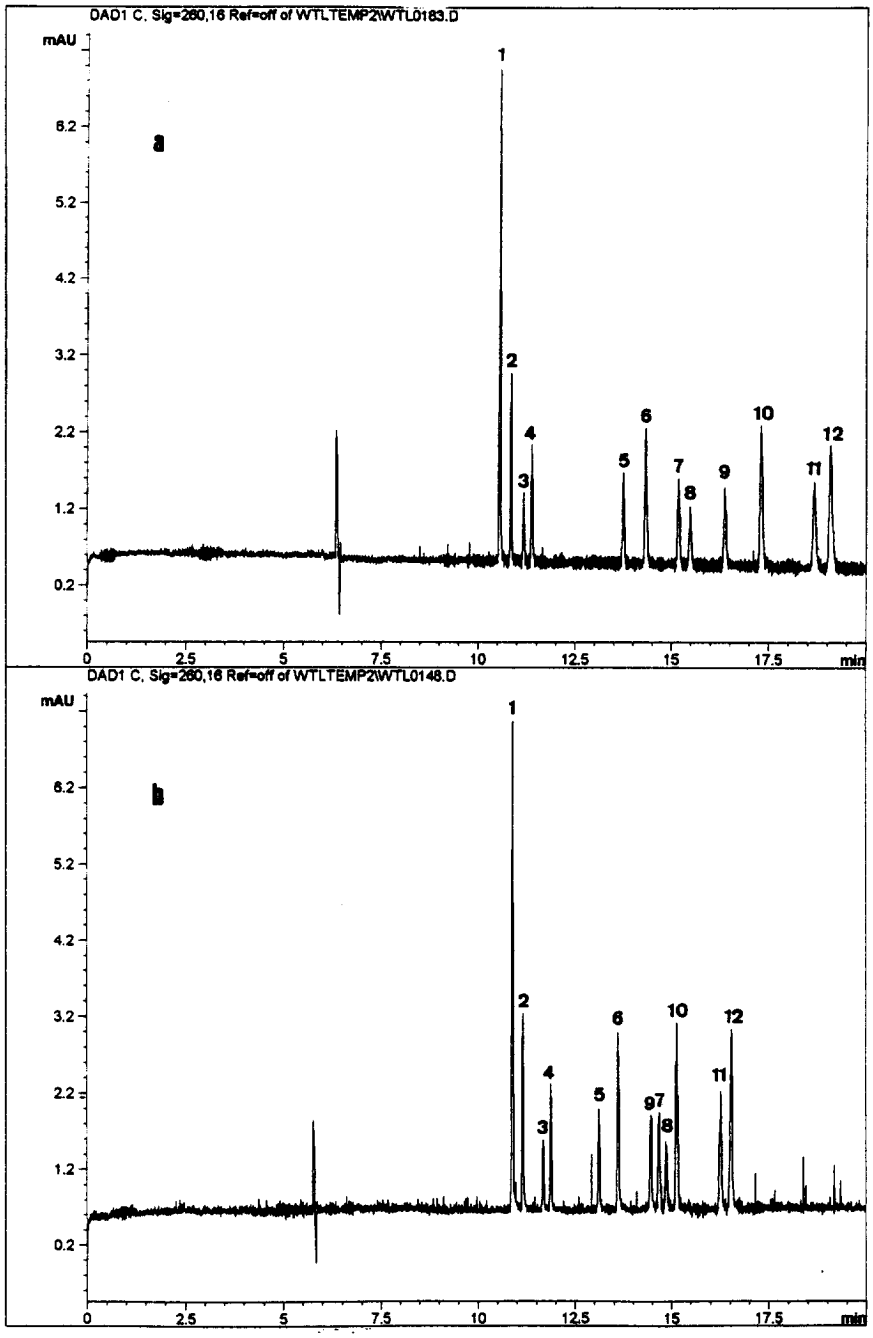


Fig. 4. Separation of ribonucleotides in weak acidic and weak basic buffers of ammonium phosphate. Total capillary length 83.5 cm (effective capillary length 75.0 cm)×45 μm I.D.; injection at 25 mbar for 6 s; applied voltage, 28.5 kV, temperature, 30°C. (a) 100 m*M* ammonium phosphate (pH 6.1), (b) 50 m*M* ammonium phosphate (pH 8.0). For other conditions see Fig. 1.

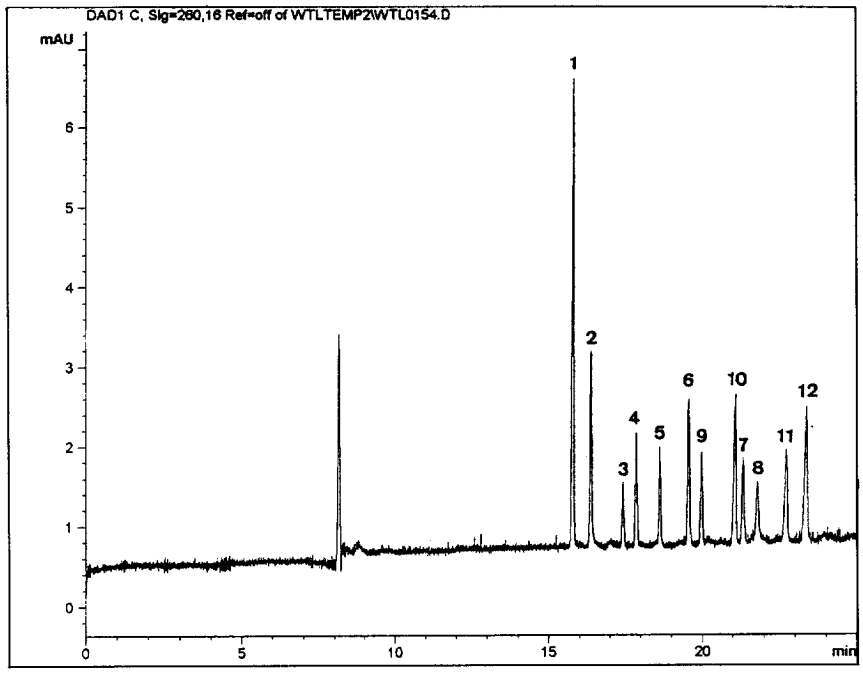


Fig. 5. Separation of common ribonucleotides in 50 m*M* ammonium phosphate containing 24% acetonitrile. The mixed water–acetonitrile buffer had a pH of 8.0 in its aqueous part. Total capillary length 83.5 cm (effective capillary length 75.0 cm)×45 μm I.D.; injection at 25 mbar for 6 s; applied voltage, 28.5 kV; temperature, 30°C. For other conditions see Fig. 1.

peaks of the ribonucleotides were larger than 2.7 under the conditions employed in Fig. 4a. These values were much higher than those in Fig. 1c. Such high resolutions were due to very high separation efficiencies (see Table 1) resulting from the longer effective capillary length which allowed use of a higher voltage in the separation. The resolutions between all adjacent peaks of the ribonucleotides were greater than 2.4 under the conditions used for Fig. 4b. It was also noted that there was a difference among migration orders for CDP, UDP and GTP in Fig. 4a and Fig. 4b.

It is worth noting that the nucleosides of monophosphates migrated the fastest away from the injection end of the capillaries (the anode side) toward the detector (the cathode side), then generally followed by the nucleosides of diphosphates and finally followed by the nucleosides of triphosphates in all the above phosphate buffers in the pH range studied. This behavior was due to the fact that the nucleosides of monophosphates have the shortest phosphate chain length and the least negative charges and therefore the slowest electrophoretic mobilities. The nucleosides of triphosphates have the longest chain length and the most negative charge and the fastest electrophoretic mobilities. The migration order in the present study was different from that reported by Uhrova et al. [23]. In their case the nucleosides of triphosphates migrated between monophosphates and diphosphates. Explanation for it was based on incomplete dissociation of nucleoside triphosphates. But one should not ignore interaction of 5′-ribonucleotides, which contain hydroxyl groups at the 2′- and 3′- position of the sugar moiety, with borate in the buffers used in their studies. Such interaction was pointed out when Tadey and Purdy reported CE separation of nucleotide isomers [40]. Among the nucleotides of the same chain length of phosphates, migration order is from nucleotides of guanosine, adenosine, cytidine, to uridine base. With the same phosphate chain length, nucleotides of purine bases have lower electrophoretic mobilities

than those of pyrimidine bases because purine bases possess greater mass than pyrimidine bases.

### 3.4. Effect of organic solvents on separation

Effects of organic solvents, such as acetonitrile, on the separation of ribonucleotides were also studied since it has not been yet reported. Different selectivity in CE separation may be obtained when mixed aqueous-organic media are used instead of aqueous media. Fig. 5 shows an electropherogram obtained in a 50 m*M* ammonium phosphate buffer containing 24% acetonitrile. The aqueous part of the buffer had a pH of 8.0. Although measurement of apparent pH in mixed aqueous–organic media was possible, we considered that the measurement of apparent pH in a series of mixed aqueous–organic media of different fraction of organic solvent was unnecessary since medium effect and the liquid junction potential could not be negligible and could not be constant. In these cases, the measured apparent pH value would not fall accurately on the conventional activity scale in water as defined by the pH standards [41]. Migration order of GTP, ATP, CDP and UDP in Fig. 5 was different from those in both Fig. 4a and Fig. 4b. Addition of organic solvents to the aqueous buffer would change the viscosity and the dielectric constant of the bulk solution, and consequently the mobilities of ionic solutes. Mobilities of ionic solutes in nonaqueous and mixed aqueous–organic media were also, even more profoundly in many cases, influenced by organic solvents due to the changes in the size of the solvated solutes and/or their dissociation constants ($pK_a$ values) [42]. When the fraction of acetonitrile decreased, peaks of ATP and CDP merged. But when the fraction of acetonitrile increased further, peaks of GTP and ADP merged.

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