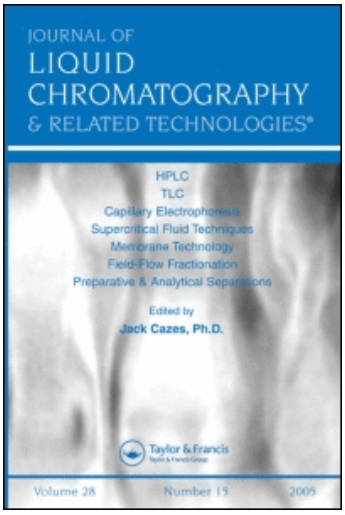

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SUB-MINUTE SEPARATION OF NUCLEOSIDE 5'-TRIPHOSPHATES WITH CO-ELECTROOSMOTIC CAPILLARY ELECTROPHORESIS

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ABSTRACT

Fast capillary electrophoretic separation of 5'-triphosphates of adenosine, guanosine, cytidine, and uridine was performed by using very short effective separation length. In order to get short run times the electroosmotic flow is reversed by flushing the fused-silica capillary with 0.2 % aqueous solution of polycationic surfactant (hexadimethrin bromide). An electrolyte containing magnesium cation as complexing agent was employed in order to increase the selectivity of nucleotides versus their affinity with inorganic cation.

A sub-minute separation of nucleoside triphosphates was obtained, below 50 s, between the regular capillary outlet and the detector (7 cm) using 10 mM citric acid/ 16 mM citrate buffer (pH 5) and 20 mM Mg^{2+}.

INTRODUCTION

Measurements of nucleoside triphosphate pools in mammalian cells are important aspects of nucleic acid synthesis and regulation and may be extremely helpful to reveal the mechanism of carcinogenesis and to monitor the treatment in many different types of cancer.[1-3] Capillary electrophoresis (CE) is currently undergoing rapid development owing to its efficiency, relative simplicity, speed and automation of separations, small buffer and sample consumption, and low operation cost.[4-5] As nucleotides are charged compounds showing strong UV absorption, many researchers attempted to develop CE methods for the separation of nucleotides.[6-16] However, baseline separation of nucleoside triphosphates was hardly achieved in a short separation time.

Takigiku[6] described an electrophoretic method for nucleoside triphosphate separation in the absence of electroosmotic flow, using 100 mM phosphate buffer (pH 2.5). The negatively charged nucleotides were detected at the anodic side applying negative high voltage. This CZE method separated 8 nucleoside triphosphates and deoxyribonucleoside triphosphates in 18 min. Geldart et al.[15] separated four 5'- nucleoside triphosphates by CE in 35 min, by using 30 mM carbonate/bicarbonate buffer (pH 10). High pH buffers allowed fast EOF but the nucleotides migrated in the opposite direction of the EOF and thus are detected after the EOF signal.

Recently, separation of twelve common nucleotides has been described by Wang et al.[20] by using 10 mM phosphate ammonium buffer (pH 6.1). The common four nucleoside triphosphates were resolved in less than 10 min.

Here we report a high speed separation analysis of ribonucleoside-5'-triphosphates by capillary electrophoresis with reversed electroosmotic flow by using capillary with short effective length. Co-electroosmotic mode was obtained by flushing the capillary with an organic cationic polymer (hexadimethrin bromide) before each injection. By electrostatic and hydrophobic interactions between the polymer and the inner surface of the capillary, the silica surface becomes positively coated and induces a fast anodic electroosmotic flow.[17-18] Thus, the electroosmotic velocity and the electrophoretic velocities of nucleotides have the same direction, which induces faster separation than those observed in counter-electroosmotic mode.

Moreover, optimization of resolution for co-electroosmotic methods can be achieved by using electrolyte additives, including inorganic cations.[19] By this means the electrophoretic mobilities of various nucleotides can be selectively altered according to their affinity with inorganic cations.

EXPERIMENTAL

Apparatus

Capillary electrophoresis separation was performed on a P/ACE 2100 apparatus (Beckman Instrument, Fullerton, CA, USA), using a fused-silica capillary of 47 cm x 50 μm (40 cm to the detector). Separations were carried out at constant temperature (25°C) by immersion of the capillary in a cooling liquid circulating in the cartridge. Direct UV detector was fixed at 254 nm (close to the maximum absorbance wavelength of the nucleotide UV absorption spectrum). The detection window was set at 7 cm from the end of the capillary and the detection aperture was 100 μm x 800 μm. The pH of each solution was checked on a Beckman pH meter (Model ϕ 10, Fullerton, CA, USA).

The capillary was conditioned daily by rinsing with 1 M sodium hydroxide (5 min), then water (5 min), and finally with hexadimethrin bromide (2 min). Between two consecutive injections, the capillary was flushed for 0.5 min with 0.2 % (w/w) aqueous solution of hexadimethrin bromide and equilibrated for 1 min with the electrolyte buffer in order to improve the electroosmotic flow (EOF) and solute migration time reproductibilities. Electrolyte preparation was achieved with the help of Phoebus software (Sedere Co, Franklin MA, USA).

Chemicals

Ammonium citrate, citric acid, and hexadimethrin bromide were of analytical grade and obtained from Sigma (St. Louis, MO, USA). The water used for the preparation of electrolytes was of HPLC quality obtained from Elgastat UHQ II system (Villeurbanne, France).

Each nucleotide was under sodium salt form (99 % purity) and purchased from Sigma. Magnesium cation was under chloride salt form and obtained from Sigma. All electrolytes and rinsing solutions were filtered before use through a polypropylene filter with 0.22 μm porosity (Prolabo, Paris, France).

RESULTS AND DISCUSSION

In this study, a standard mixture of four common nucleoside 5'-triphosphate was selected (Figure 1). The electrophoretic buffer was slightly acidic (pH 5) because studied nucleotides are readily hydrolyzed in neutral and

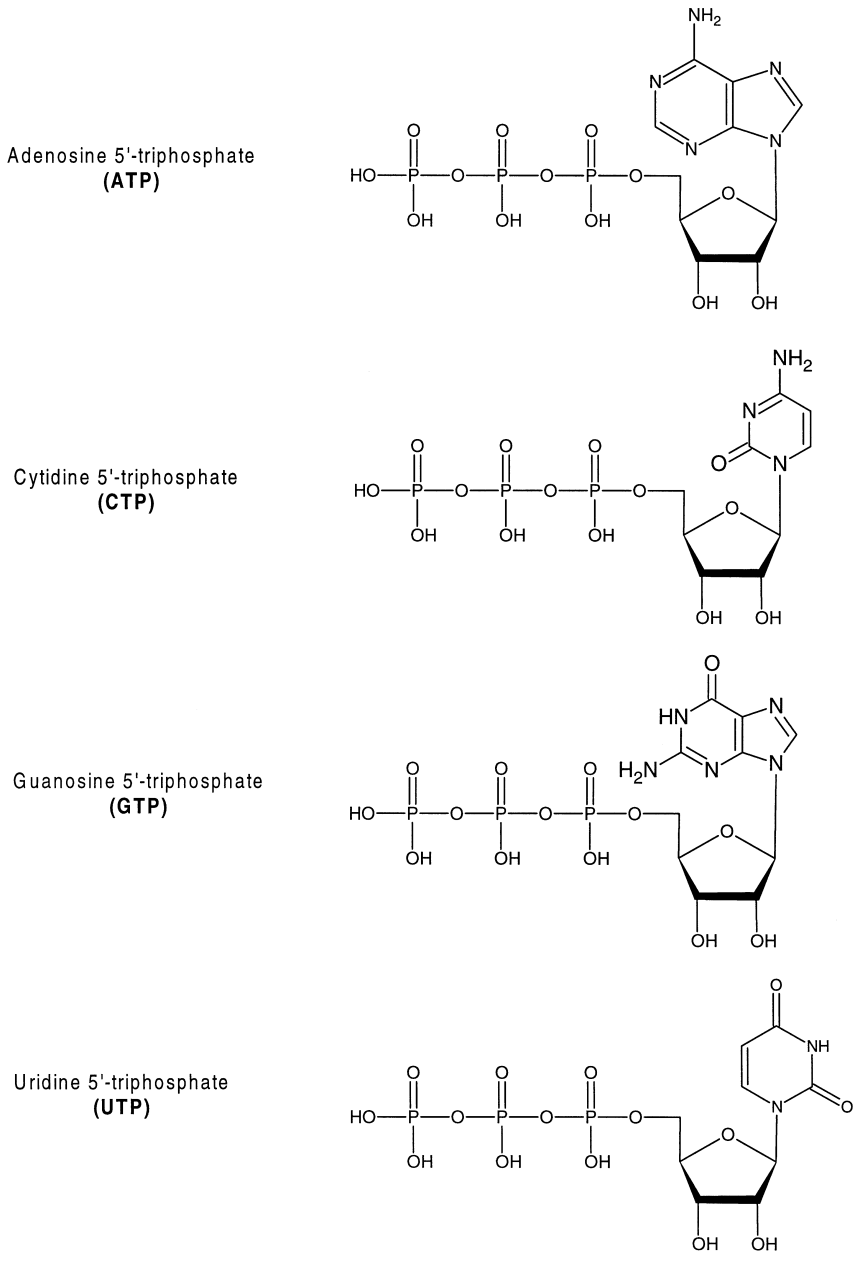

Figure 1. Structures of selected nucleoside 5'-triphosphates.

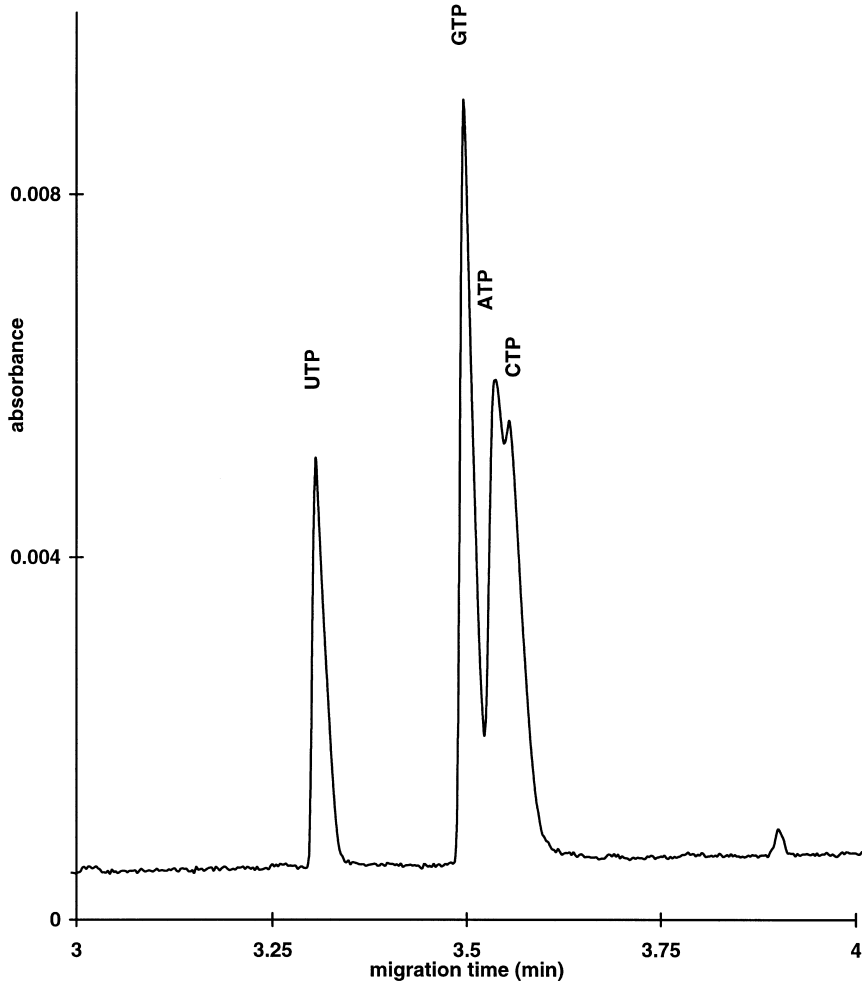

Figure 2. Separation of nucleoside 5'-triphosphate mixture by capillary electrophoresis. Electrolyte: 16 mM ammonium citrate/10 mM mM citric acid (pH 5); fused silica capillary dimensions: 47 cm (40 cm to detector) x 50 µm I.D.; direct UV detection: 254 nm applied voltage: - 20 kV temperature: 25°C; hydrodynamic injection: 5 s; nucleotide concentration: 100 $mg.L^{-1}$; capillary conditioned step: 1 min with 0.2 % hexadimethrin bromide aqueous solution before each run.

Table 1

Apparent Charges and Electrophoretic Mobilities of Studied Nucleotides at pH 5[a]

	ATP	CTP	GTP	UTP
Apparent Charge	-2.86	-2.60	-3.00	-3.00
Electrophoretic Mobility ($x\ 10^5\ cm^2.V^{-1}.s^{-1}$)	-35.4	-35.1	-35.7	-38.5

[a] The apparent charge of each nucleotide has been calculated, with Phoebus software. Experimental conditions for electrophoretic mobilities determination: electrolyte, 16 mM ammonium citrate/10 mM citric acid (pH 5); UV detection: 254 nm; applied voltage: - 20 kV; temperature: 25°C; hydrodynamic injection: 5 s; nucleotide concentration: 100 mg L^{-1}; capillary conditioned step: 0.5 min with 0.2 % hexadimethrin bromide aqueous solution and equilibrated for 1 min with the electrolyte buffer before each run.

alkaline media.[7] In citrate buffer (pH 5) without magnesium (Figure 2), UTP migrated prior than GTP, ATP and CTP. At pH 5, the phosphate chain of nucleotides has the same negative charge (- 3.0), so the difference of electrophoretic mobility is generated by the charge of the base moiety. If uracil and guanine are neutral at pH 5, adenine is slightly positive (+ 0.14) and cytosine is the most positively charged (+ 0.40). Additionally, the size of base may also play an important role on the migration order of the least anionic compounds (UTP and GTP) (Table 1).

However, selectivity between GTP, ATP and CTP was not sufficient at this pH. To improve the resolution of nucleoside triphosphate mixture, a complex-forming reaction, employing magnesium cation, has been used. It is well known that ribonucleoside triphosphates interact with magnesium cations.[20-21] Recently, we studied the effect of the inorganic cation addition on the electrophoretic mobilities of AMP, ADP, ATP.[22] Figure 3 shows the effect of magnesium concentration on the electrophoretic mobilities of nucleotides.

The mobility difference between each nucleotide increases with an increase of magnesium concentration, but the migration order was not changed. The addition of magnesium cation decreases the negative charge of phosphate

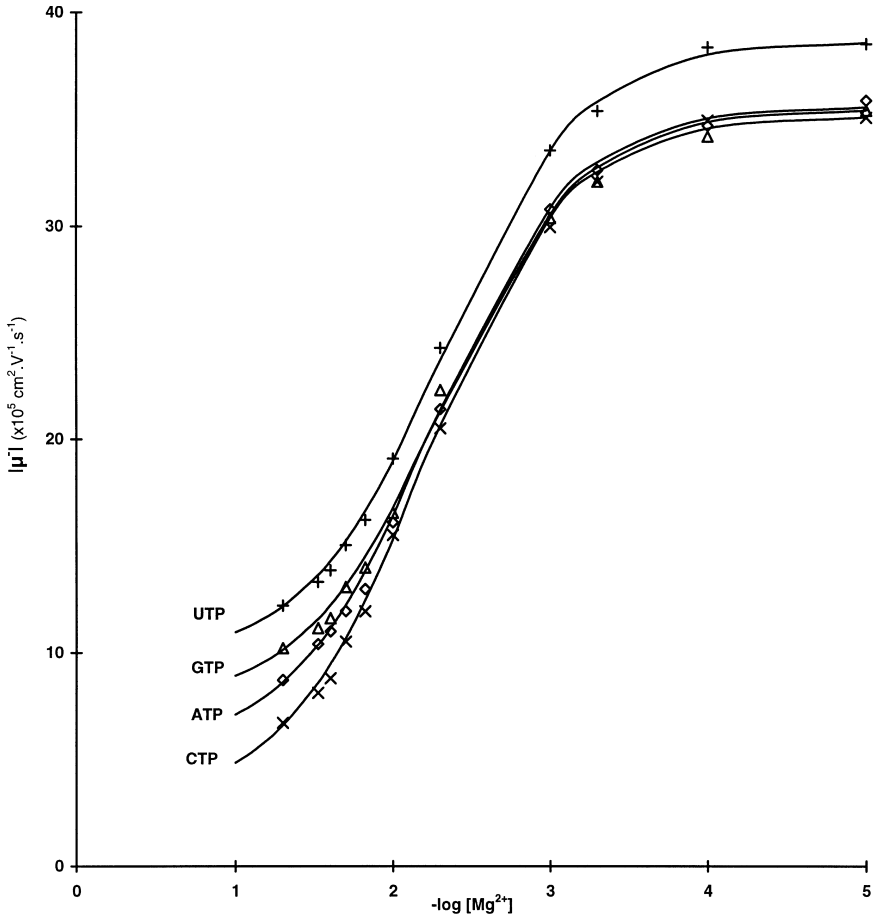

Figure 3. Effect of magnesium concentration upon electrophoretic mobilities of nucleoside 5'-triphosphates in capillary electrophoresis. Electrolyte 16 mM ammonium citrate/10 mM citric acid (pH 5); fused silica capillary dimensions: 47 cm (40 cm to detector) x 50 μm I.D.; UV detection: 254 nm; applied voltage: - 20 kV; temperature: 25°C; hydrodynamic injection: 5 s; nucleotide concentration: 100 mg L^{-1}; capillary conditioned step: 0.5 min with 0.2 % hexadimethrin bromide aqueous solution and equilibrated for 1 min with the electrolyte buffer before each run.

chain and thus increases the charge difference between each nucleotide leading to their baseline separation (Figure 4). The addition of magnesium cation (20 mM) in electrolyte buffer allowed obtaining a good resolution between the four nucleotides.

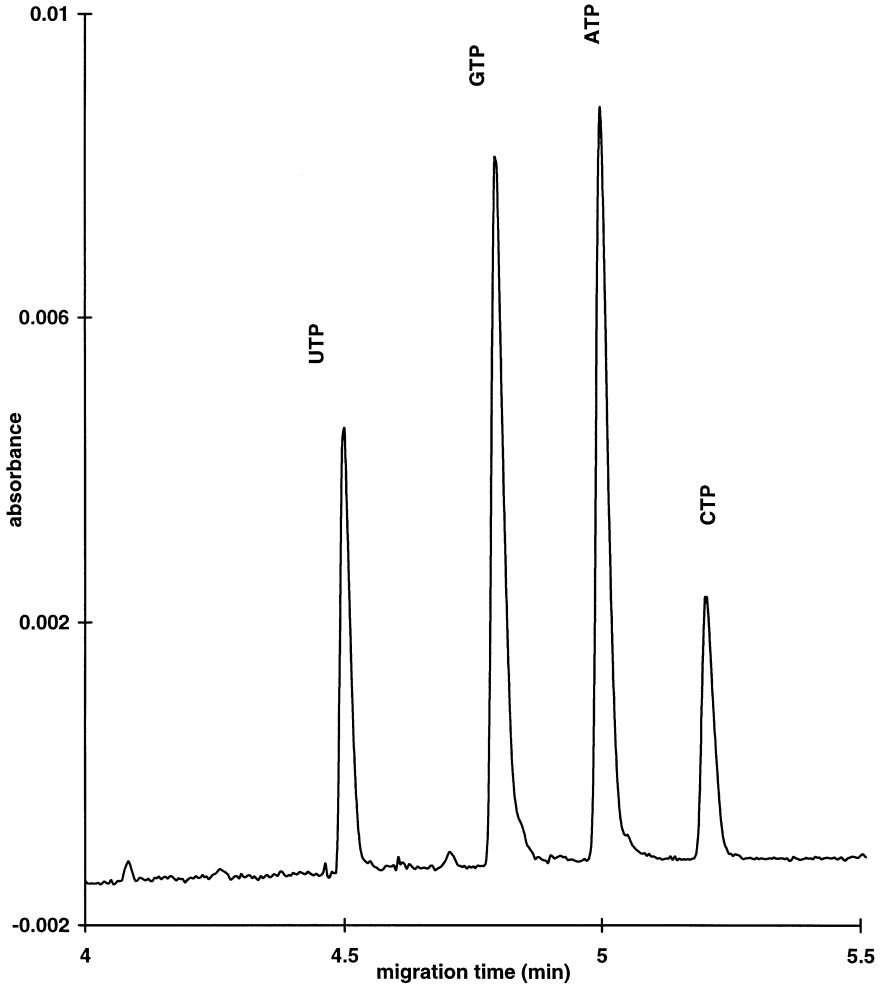

Figure 4. Influence of magnesium cation added to the electrolyte upon the separation of nucleoside 5'-triphosphates by capillary electrophoresis. Electrolyte : 16 mM ammonium citrate/10 mM mM citric acid (pH 5), 20 mM Mg^{2+} ; fused silica capillary dimensions: 47 cm (40 cm to detector) x 50 µm I.D.; direct UV detection: 254 nm ; applied voltage: - 20 kV; temperature: 25°C; hydrodynamic injection: 5 s; nucleotide concentration: 100 $mg.L^{-1}$; capillary conditioned step: 1 min with 0.2 % hexadimethrin bromide aqueous solution before each run.

Table 2

Logarithms of the Formulation Constants (log K) and Stoichiometry of UTP-, ATP-, GTP-, GTP-Mg^{2+} Complexes at pH5[a]

Nucleotide	Log K	Stoichiometry
ATP	2.25	1 : 1
CTP	2.26	1 : 1
GTP	2.31	1 : 1
UTP	2.33	1 : 1

[a] Fused silica capillary dimensions: 47 cm (40 cm to detector) x 50 µm I.D; electrolyte, 16 mM ammonium citrate/10 mM citric acid (pH 5); UV detection: 254 nm; applied voltage: - 20 kV; temperature: 25°C; hydrodynamic injection: 5 s; nucleotide concentration: 100 mg L^{-1}; capillary conditioned step: 0.5 min with 0.2 % hexadimethrin bromide aqueous solution and equilibrated for 1 min with the electrolyte buffer before each run.

The variation of nucleotide electrophoretic mobility caused by the addition of magnesium cation to the running buffer can be used to estimate the formation constant (K) and the stoichiometry[22-23] of complexes formed between nucleotides and Mg^{2+} cation.

The formation constant and the stoichiometry of nucleotide-inorganic cation complexes can be deduced from the dependence of the electrophoretic mobility of each nucleotide on the negative logarithm of the inorganic cation concentration. Table 2 reports the stoichiometry and the constants for UTP-, ATP-, GTP-, CTP-Mg^{2+} complexes at pH 5.

We should mention that the use of the citrate/citric acid buffer for these experiments exhibits that formation constants determined in this study are more conditional rather true thermodynamic values due to the ability of citrate anion to form complexes with Mg^{2+} cations.[24]

Experimental log K-values are quite similar for all studied nucleotides. Indeed, magnesium cation preferably interacts with the oxygen sites of the phosphate groups and have only a low affinity for the base residues.[21]

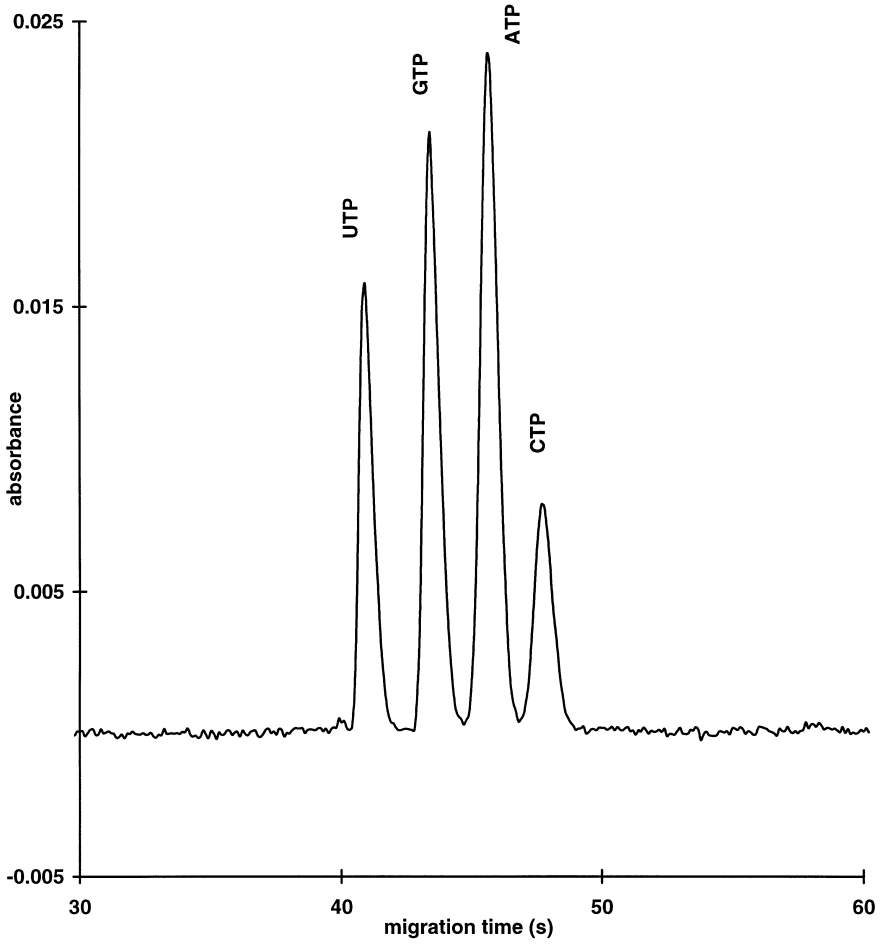

Figure 5. Sub-minute separation of nucleoside 5'-triphosphates by capillary electrophoresis. Fused silica capillary dimensions : 47 cm (7 cm to detector) x 50 µm I.D.; electrolyte: 16 mM ammonium citrate/10 mM citric acid (pH 5), 20 mM Mg^{2+}; UV detection: 254 nm; applied voltage: + 25 kV; temperature : 25°C; electrokinetic injection: 5 kV (1s) ; nucleotide concentration: 5 mg L^{-1} ; capillary conditioned step: 0.5 min with 0.2 % hexadimethrin bromide aqueous solution and equilibrated for 1 min with the electrolyte buffer before each run.

Sub-Minute Separation

Fast separations are important for automated analysis of many large samples. According to Eq. (1), the migration time (t_m) of a solute in CE depends on the length of capillary:

Table 3

Migration Time Repeatability During CE Separation of Nucleotides[a]

	Migration Time	
Nucleotide	**Mean Value**[b] **(s)**	**R.S.D. (%)**
ATP	46	0.53
CTP	48	0.57
GTP	44	0.46
UTP	41	0.43

[a] Fused silica capillary dimensions: 47 cm (7 cm to detector) x 50 µm I.D; electrolyte, 16 mM ammonium citrate/ 10 mM citric acid (pH 5), 20 mM $MgCl_2$; UV detection: 254 nm; applied voltage: + 25 kV; temperature: 25°C; electrokinetic injection: +5 kV (1s); nucleotide concentration: 5 $mg.L^{-1}$; capillary conditioned step: 0.5 min with 0.2 % hexadimethrin bromide aqueous solution and equilibrated for 1 min with the electrolyte buffer before each run. [b] Ten consecutive injections.

$$t_m = \frac{L_t . L_d}{(\mu_{ep} + \mu_{eo}).U} \qquad (1)$$

where L_t and L_d are the total and the effective length of the capillary, respectively, t_m is the migration time and U is the applied voltage. By shortening the effective separation length, a further reduction of the separation time can be achieved. Recently, Zemann[25] reported sub-minute separations of organic and inorganic anions using co-electroosmotic capillary electrophoresis. From a theoretical point of view, performing the CE separation on a 7 cm effective length capillary (applied voltage: + 25 kV) would induce a 7 times faster separation than a regular 40 cm effective length (applied voltage: - 20kV). Experimentally, the separation time gain was found to be 6.5. Figure 5 illustrates a fast co-electroosmotic baseline separation (50 s) of nucleoside triphosphates using citric acid/citrate buffer (pH 5) and 20 mM Mg^{2+}.

The separation was performed between the capillary outlet and the detector window (7 cm) with a positive applied voltage (+ 25 kV). Electrokinetic injection was carried out by dipping the capillary outlet end into the sample vial during 1s and by applying positive high voltage (+ 5 kV).

Repeatability

The repeatability of the CE method was determined by injecting ten times nucleotide mixture (5 mg.L^{-1}) in a capillary filled with the previously described buffer. Mean migration times and Relative Standard Deviations (R.S.D.) of 5'-triphosphates are reported in Table 3.

This CE method provided a good accuracy for the migration time with R.S.D values varying from 0.43 to 0.57 %.

CONCLUSION

High speed CE separations of nucleoside 5'-triphosphates have been performed in less than one minute by using short effective capillary length (7 cm). The selectivity has been managed by adding magnesium cation to ammonium citrate/ citric acid buffer (pH 5) in order to benefit the affinity of these biological molecules versus metal ions.

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