Journal of Chromatography, 559 (1991) 247–256
Elsevier Science Publishers B.V., Amsterdam

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Reproducibility and quantitation of separation for ribonucleoside triphosphates and deoxyribonucleoside triphosphates by capillary zone electrophoresis

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ABSTRACT

Capillary zone electrophoresis has been evaluated for the separation and quantitation of ribonucleoside and deoxyribonucleoside triphosphates. For adequate resolution, capillaries were treated to reduce electroosmotic flow and capillary zone electrophoresis was performed with negative high voltage. Results from both a fully automated and a manual instrument are found to have comparable performance characteristiscs. The described method is linear with a minimum concentration detection limit of approximately 0.001 mg/ml per nucleotide.

INTRODUCTION

Capillary electrophoresis has been used to analyze a diverse array of molecules, including proteins and peptides [1–4], nucleosides, nucleotides and oligonucleotides [5–11] and drugs and drug metabolites [12–15]. The most commonly used mode of capillary electrophoresis is performed with open tubes and is termed capillary zone electrophoresis (CZE). When surfactants are added to the buffer at concentrations exceeding their critical micellar concentration, both neutral and charged analytes can be resolved. This mode of capillary electrophoresis is termed micellar electrokinetic capillary chromatography (MECC, or MEKC). Both CZE and MECC have been previously described for the analysis of various nucleotide species [5–10,16]. This paper describes our efforts at resolving and quantitating ribonucleoside triphosphates (NTPs) and deoxyribonucleoside triphosphates (dNTPs) by CZE.

Measurements of NTP and dNTP pools in mammalian cells are of importance in studying aspects of DNA and RNA synthesis and regulation [17,18]. Several methods exist for the measurement of NTP and/or dNTP precursor pools, including enzymatic assays [19,20], gas chromatography [21], and HPLC [22]. Of these, only HPLC has been described to measure the common 8 NTPs and dNTPs (ATP, dATP, CTP, dCTP, GTP, dGTP, dTTP and UTP) simultaneously, but requires *ca.* 60 min to elute all components [22].

0021-9673/91/$03.50

Because CZE offers ease, rapid analysis times and impressive resolving power, we sought to develop a CZE method for the separation and quantitation of NTPs and dNTPs. In CZE, selectivity is a function of the relative differences in the electrophoretic mobilities of the analytes. Mobility is related to molecular charge, size, shape and solution viscosity. Additionally, in uncoated fused-silica capillaries, an electric-field-induced solvent flow occurs within the capillary. This flow is termed electroosmosis. Normally, electroosmotic flow is toward the cathode (CZE with positive high voltage, or "normal polarity"). Hence, while anions would normally migrate towards the anode, electroosmosis is often of sufficient magnitude to force them towards the cathode. Therefore, both cations and anions can be analyzed simultaneously. However, because we were interested in rapidly resolving only strongly anionic compounds, we describe a method for nucleotide separation in the absence of electroosmotic flow, wherein anion migration is permitted to proceed from the cathode to the anode (CZE with negative high voltage, or "reverse polarity"). This CZE method is capable of resolving the common 8 NTPs and dNTPs with UV detection (254 nm) within an 18 min separation time. Reproducibility and quantitation are assessed.

Although the absolute amount of detectable nucleotide in CZE can be very small in comparison to HPLC, the minimum detectable concentration is only comparable, being on the order of 0.001 mg/ml (*ca.* 10^{-6} *M*).

EXPERIMENTAL

Reagents

All ribonucleoside triphosphates, deoxyribonucleoside triphosphates, EDTA and γ-methacryloxypropyltrimethoxysilane were obtained from Sigma (St. Louis, MO, USA), and were used without further purification. All buffer components, N,N,N′N′-tetramethylethylenediamine (TEMED), and electrophoresis-grade acrylamide were obtained from Bio-Rad (Richmond, CA, USA). Methanol was obtained from J. T. Baker, Inc. (Phillipsburg, NJ, USA). Sample solutions were prepared by dissolving (deoxy)nucleotides in 0.1 *M* phosphate buffer at pH 2.5, and stored frozen. The operating buffer was composed of 0.05 *M* phosphate at pH 2.7 with 0.002 *M* EDTA, and was made fresh daily.

Instrumental

CZE was performed with a Beckman P/ACE System 2000 controlled with an IBM PS/2 Model 55SX with Beckman P/ACE v1.1 software; and a Bio-Rad HPE 100. In both instruments, the inlet was held at negative high voltage with respect to the grounded outlet. Both units were used with fused-silica capillaries treated to reduce electroosmosis. The polymer-coated capillary (20 cm total length × 25 μm I.D.; P/N 148-3011) used in the Bio-Rad instrument was obtained from Bio-Rad and was used without further modification.

The fused-silica capillary used in the Beckman was obtained from Polymicro Technologies (75 μm I.D. × 260 μm O.D.; Phoenix, AZ, USA) and was fitted into a Beckman capillary cartridge (P/N 338463). Total capillary length was 69.5 cm. The capillary was treated by the method described by Hjertén [23] with the following modifications (all reactions were performed with the capillary held at 50°C). Capillaries were first rinsed with 0.1 *M* NaOH for 10 min, followed by methanol for 10

min, followed by distilled water for 10 min. Capillaries were then silylated with γ-methacryloxypropyltrimethoxysilane by aspirating a 0.5% solution in distilled water (pH was adjusted to 3.5 with glacial acetic acid) for 30 min. Silylated capillaries were rinsed with distilled water for 10 min, followed by dry nitrogen gas. A polyacrylamide coating was then applied by aspirating a 20 ml deaerated 3% (w/v) acrylamide solution containing 8 μl of TEMED and 10 mg ammonium persulfate for 30 min. Finally, capillaries were rinsed with distilled water for 10 min, followed by dry nitrogen gas.

Two different injection methods were used. Electrokinetic injection was used exclusively with the Bio-Rad and all samples were injected under the same conditions, *i.e.*, 2 kV for 10 s (unless otherwise indicated). Pressure injection was used with the Beckman. Briefly, an inlet pressure of 3.45 mPa pressure was applied for a specified time interval (5 s, unless otherwise indicated) to introduce sample into the capillary.

At least 5 column volumes of operating buffer was used to rinse the capillaries between runs. All other operating conditions are as described in Results and Discussion.

Data collection and processing were accomplished on a IBM PS/2 Model 70 with Turbochrome II software (v. 2.0) and PE Nelson Series 900 Interface units (Cupertino, CA, USA).

RESULTS AND DISCUSSION

Fig. 1 shows an electropherogram of a standard solution of ATP, dATP, CTP, dCTP, GTP, dGTP, dTTP, UTP and ITP (0.6 ng each; ITP at 1.25 ng). The rare

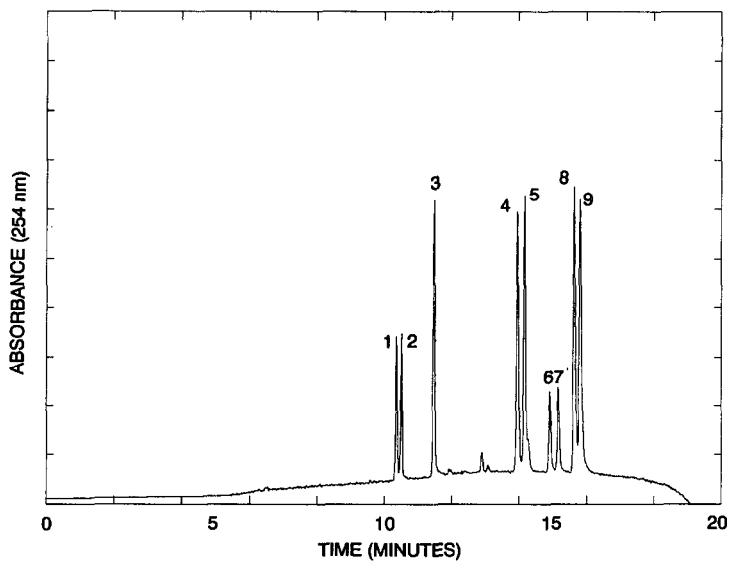

Fig. 1. Electropherogram of NTP and dNTP mixture. Concentration of original mixture was 0.031 mg/ml per nucleotide, ITP was 0.063 mg/ml. All other details are as described in Table I for the Beckman instrument. Peaks: 1 = UTP; 2 = dTTP; 3 = ITP; 4 = GTP; 5 = dGTP; 6 = dCTP; 7 = CTP; 8 = dATP; 9 = ATP.

nucleotide, ITP, was used as an internal standard. All components are clearly separated with the total separation time under 18 min. Interestingly, the elution order for the NTPs corresponds with that reported by Silver *et al.* [24] for an electrophoretic separation on paper as well as a previous CZE separation demonstrating indirect fluorescence detection [25]. CZE separations of the dNTPs have not previously been reported.

For the compounds studied here, the overall net charge of each nucleotide probably primarily affects its respective migration rate. In the pH range of the buffers used (<3), the uracil, thymine and inosine moieties are essentially neutral, while guanine is predominantly positive, and cytosine and adenine are virtually completely positive [26]. Under these experimental conditions, we would expect that the most anionic components would exhibit the largest migration rate, and indeed that is what is observed. Interestingly, the migration rates of the deoxyribonucleotides closely paralled those of their ribonucleotide analogues, which probably further indicates that the overall net charge of the molecules predominantly influences their migration rates. Additionally, size may also play an important role as evidenced by the migration order of the least anionic components (cytidine and adenine nucleotides), where the larger purines, ATP and dATP, exhibited the lowest migration rates.

Reproducibility of the electropherograms

Reproducibility of migration time. Average migration times and relative standard deviations (R.S.D.) for the nucleotides are listed in Table I. Because we used coated capillaries, migration times were not influenced by electroosmotic flow. Hence,

TABLE I

REPRODUCIBILITY OF MIGRATION TIME AND MOBILITY

Conditions: operating buffer, 0.05 *M* phosphate (pH 2.7) with 0.002 *M* EDTA. Beckman instrument capillary 69.5 cm × 75 μm I.D., derivatized; length to detector 62.8 cm; pressure injection for 5 s; 20 kV applied voltage. Bio-Rad instrument capillary 20 cm × 25 μm I.D., derivatized; length to detector 17 cm; electrokinetic injection for 10 s at 2 kV; 2 kV applied voltage.

Instrument	Component	Migration time (n = 5, min)		Mobility
		Mean	R.S.D. (%)	
Beckman	UTP	10.55	0.039	20.69
	dTTP	10.70	0.417	20.40
	ITP	11.65	0.013	18.74
	GTP	14.11	0.088	15.46
	dGTP	14.33	0.013	15.23
	dCTP	15.08	0.017	14.48
	CTP	15.33	0.131	14.24
	dATP	15.74	0.575	13.86
	ATP	16.02	0.601	13.62
Bio-Rad	dTTP	8.35	1.43	20.37
	dGTP	11.01	1.49	15.45
	dCTP	11.58	1.50	14.68
	dATP	12.08	1.47	14.07

electrophoretic mobilities (μ) could be directly calculated by eqn. 1 and are included in Table I.

$$\mu = \frac{L_d L_t}{tV} \tag{1}$$

Here L_d is the length of the capillary from the injection end to the detector, L_t is the total length of the capillary, t is the migration time and V is the applied voltage.

The observed migration order is a direct function of each nucleotide's electrophoretic mobility. Mobility is primarily influenced by analyte size and charge (characteristics which can be influenced by solvent properties such as pH and viscosity), but hydrophobicity [27] and molecular conformation [28] can also affect mobility. Notably, the electrophoretic mobilities as determined from the two instruments used in this study are very similar, indicating comparable performance despite differing capillary lengths, diameters and coating chemistries. However, there are important experimental differences between the two systems. We required the longer (69.5 cm total length) capillary to resolve adequately all 9 nucleotides. The shorter capillary (20 cm total length) used on the Bio-Rad permitted only resolution of a smaller number of components (for these experiments we chose the 4 dNTPs as model compounds). It is reasonable to expect that a longer capillary would perform in an analogous manner to the (longer) capillary we used with the Beckman instrument. Indeed, an application note from Bio-Rad (P/N 1575-14) illustrates a separation of the 8 common NTPs and dNTPs on a 50 cm × 50 μm I.D. coated capillary, using a 0.1 M phosphate buffer at pH 2.5 with 0.002 M EDTA.

Mobilities are also influenced by temperature fluctuations [29–32]. Good reproducibility can only be achieved by rigorous temperature control. We would expect better heat dissipation from the 25 μm I.D. column used in the Bio-Rad (relative to the 75 μm I.D. column used in the Beckman), but the Beckman instrument provides temperature regulation via a thermostatted heat transfer fluid. Based on migration time reproducibilities obtained for both instruments (Table I), both mechanisms appear effective. However, optimum temperature control is dependent on the combination of both factors, *i.e.*, efficient radial heat transfer through the capillary and precise thermostatting of the capillary itself.

Quantitation

Both detector linearity and reproducibility of sample introduction primarily affect quantitation. Detection of nucleotides was accomplished by on-column UV absorbance detection at 254 nm. For this study, we chose to measure peak areas (normalized to migration time), instead of peak heights. Other studies have noted the advantages of measuring peak areas [33,34].

Sample introduction. Pressure injection was used exclusively with the Beckman PACE instrument. The volume introduced into the capillary (V_{inj}) by this method can be calculated by the Poiseuille equation:

$$V_{inj} = \frac{\Delta P \pi t^4 t_{ap}}{128 \eta L_t} \tag{2}$$

where ΔP is the pressure difference across a capillary of length L_t and diameter d, t_{ap} is the total duration of applied pressure and η is the buffer viscosity. Although we did not verify calculated values, we determined linearity and reproducibility for a series of different injection times. A good linear correlation was found between peak area and injection time for these nucleotides. A representative plot of UTP peak area *vs.* injection time is shown in Fig. 2A. Each injection was performed in triplicate. The R.S.D. of the mean peak areas for each injection time were between 1 and 8% (represented as error bars in Fig. 2A). Electrokinetic injection was used exclusively with the Bio-Rad CE instrument. The amount of solute introduced into the capillary by this method is dependent on its electrophoretic mobility and on the ionic strengths of the sample solution and running buffer. Again, we determined linearity and reproducibility for a series of different injection times. Good linear correlations were found between peak area and injection time. A representative plot of UTP peak area *vs.* injection time is shown in Fig. 2B. The R.S.D. of the mean peak areas for each injection time was comparable, though somewhat higher (*ca.* 9%), to that observed for pressure injection.

In general, the linearity and reproducibility of peak area *vs.* injection time for pressure injection was slightly better than for electrokinetic injection. In particular, the longest electrokinetic injection time (20 s) seems to have resulted in a lower sample load than what might be expected based on smaller injection times (Fig. 2B). These results are analogous to those reported by Moring *et al.* [33] for similar experiments. Previous comparisons of injection methods [33,35] have generally shown hydrodynamic injections to be more linear than electrokinetic injection. This is primarily because of

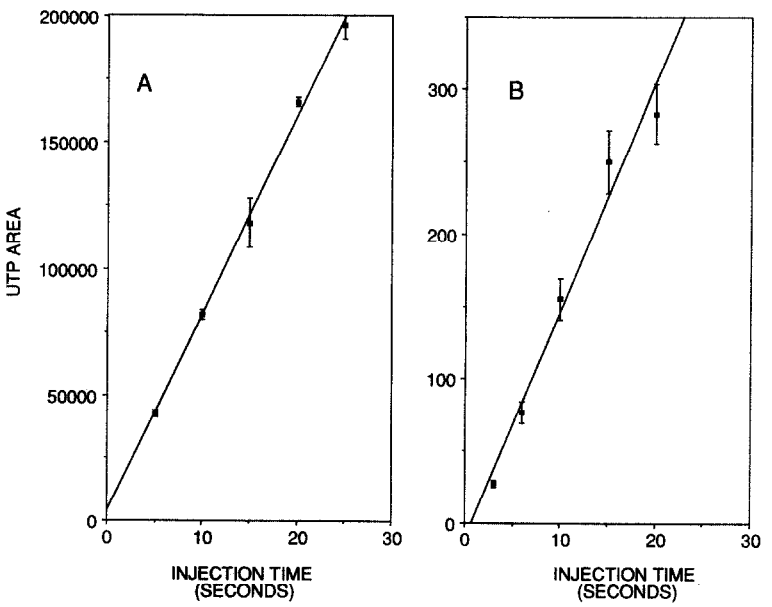

Fig. 2. Linearity of sample introduction as a function of injection duration: (A) represents pressure injection on the Beckman instrument and (B) represents electrokinetic injection on the Bio-Rad. All other details are as described in Table I. (A) $y = 3587.4 + 7836.2x$; $r^2 = 0.997$. (B) $y = -11.756 + 15.772x$; $r^2 = 0.974$.

fluctuating local electric fields at the capillary inlet during electrokinetic injection. This effect can be minimized by dissolving samples in a higher conductivity buffer (relative to the operating buffer) [33], as we have done here.

Peak area–concentration relationships

Nucleotide peak area–concentration relationships were determined for both instruments and the results are summarized in Table II. Table II also summarizes nucleotide peak area–concentration relationships when calculated using ITP (0.0625 mg/ml) as an internal standard. Fig. 3A (CTP sample concentration *vs.* normalized peak area) and B (CTP/ITP concentration ratio *vs.* CTP/ITP response ratio) contain representative plots demonstrating the linearity of the measurements on the Beckman instrument. The relationship between nucleotide peak areas and concentration was found to be linear through nearly three orders of magnitude for both instruments. Interestingly, use of the internal standard did not significantly improve this linearity. Of course, use of an internal standard would be most valuable in a multi-stage sample preparation and extraction process, which was not attempted for this report. The minimum detectable concentration (based on a 3:1 signal-to-noise ratio on the electropherograms) are given in Table III. These figures would change under differing injection conditions. For example, sample stacking during electrokinetic injection would improve the minimum detectable concentration [36,37].

TABLE II

LINEARITY OF PEAK AREA *VS.* SAMPLE CONCENTRATION

Conditions: operating buffer, 0.05 *M* phosphate (pH 2.7) with 0.002 *M* EDTA. Beckman instrument capillary 69.5 cm × 75 μm I.D., derivatized; length to detector 62.8 cm; pressure injection for 5 s; 20 kV applied voltage; individual nucleotide concentrations: 0.0078 mg/ml, 0.0156 mg/ml, 0.0312 mg/ml, 0.0625 mg/ml, 0.125 mg/ml and 0.25 mg/ml; ITP at 0.0625 mg/ml. Bio-Rad instrument capillary 20 cm × 25 μm I.D., derivatized; length to detector 17 cm; electrokinetic injection for 10 seconds at 2 kV; 2 kV applied voltage; individual nucleotide concentrations at 0.0156 mg/ml, 0.0313 mg/ml, 0.0625 mg/ml, 0.125 mg/ml and 0.25 mg/ml. All samples run in triplicate.

Instrument	Component	Equation of the line (concentration *vs.* peak area)			Conc. *vs.* peak area normalized to ITP
		r^2	slope	*y*-intercept	r^2
Beckman	UTP	0.985	$2.2 \cdot 10^5$	−322	0.960
	dTTP	0.996	$1.9 \cdot 10^5$	−286	0.973
	GTP	0.993	$3.9 \cdot 10^5$	−2238	0.990
	dGTP	0.989	$4.1 \cdot 10^5$	−3863	0.995
	dCTP	0.995	$1.1 \cdot 10^5$	−482	0.983
	CTP	0.994	$1.2 \cdot 10^5$	−559	0.986
	dATP	0.998	$4.0 \cdot 10^5$	−856	0.967
	ATP	0.989	$4.9 \cdot 10^5$	−2900	0.990
Bio-Rad	dTTP	0.994	$2.1 \cdot 10^4$	233.9	
	dGTP	0.999	$2.5 \cdot 10^4$	45.5	
	dCTP	0.995	$8.8 \cdot 10^3$	91.7	
	dATP	0.998	$3.0 \cdot 10^4$	303	

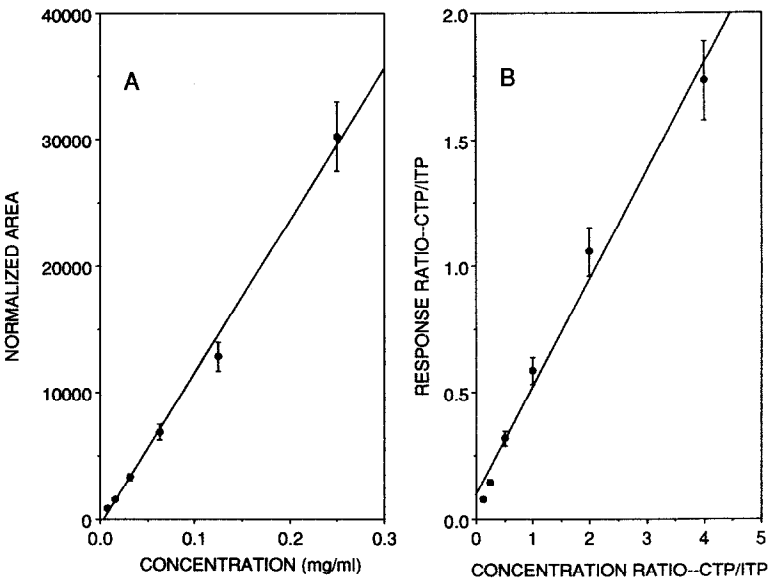

Fig. 3. Linearity of peak area as a function of sample concentration. (A) represents CTP sample concentration *vs.* normalized peak area and (B) represents those same values normalized against the internal standard, ITP (0.063 mg/ml). All other details are as described in Table II. (A) $y = -559 + 1.2 \cdot 10^5 x$; $r^2 = 0.994$. (B) $y = 9.4 \cdot 10^{-2} + 0.43x$; $r^2 = 0.986$.

TABLE III

MINIMUM DETECTABLE CONCENTRATION AT 254 nm

Conditions: operating buffer, 0.05 *M* phosphate (pH 2.7) with 0.002 *M* EDTA. Beckman instrument capillary 69.5 cm × 75 μm I.D., derivatized; length to detector 62.8 cm; pressure injection for 5 s; 20 kV applied voltage. Bio-Rad instrument capillary 20 cm × 25 μm I.D., derivatized; length to detector 17 cm; electrokinetic injection for 10 s at 2 kV; 2 kV applied voltage. Minumum detectable concentration determined where signal-to-noise ratio = 3 (0.01 a.u.f.s., rise time 0.1 s).

Instrument	Component	Minimum detectable concentration	
		pmol/ml	mg/ml
Beckman	UTP	7500	0.0036
	dTTP	6400	0.0031
	ITP	3200	0.0053
	GTP	3000	0.0015
	dGTP	850	0.0004
	dCTP	800	0.0004
	CTP	650	0.0003
	dATP	230	0.0001
	ATP	120	0.0001
Bio-Rad	dTTP	18200	0.0136
	dGTP	3700	0.0019
	dCTP	3500	0.0016
	dATP	1000	0.0005

These experiments indicate that efficient, sensitive and quantitative measurements of nucleotide triphosphates are possible by CZE. We believe this technique is a viable alternative to more traditional high-performance liquid chromatographic [22,38] and enzymatic [19,20] methods and capitalizes on many CZE strengths, including small sample size, simplicity and speed. To assess the quantitative potential of the technique further we are currently conducting a series of comparative experiments with cultured mammalian cell extracts.

ACKNOWLEDGEMENT

We thank Lisa Kalish, Dave Hansen and Tim Wehr for method development efforts and many useful discussions.

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