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Analysis of nucleotide pools in human lymphoma cells by capillary electrophoresis

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Abstract

Capillary electrophoresis is applied to determine nucleotide pool levels in human Burkitt lymphoma cells. The analysis was performed on a 65 cm × 50 μm I.D. Ucon-coated column with on-column UV detector. The method requires only nanoliters of sample and a simple sample preparation procedure. Over 12 nucleotides were separated and quantitated with high resolution and reproducibility. The whole capillary electrophoretic separation time was only 35 min. These results demonstrate that capillary electrophoresis provides a useful and easy way to analyze nucleotide pools in cells.

1. Introduction

Capillary zone electrophoresis is rapidly being applied in both research and clinical settings. In this paper, the technique was used to measure the nucleotide pool levels in human tumor cells. The analysis of nucleotide pools in tumor cells may be extremely helpful, both in revealing the mechanism of carcinogenesis and in monitoring the treatment of the disease [1–5]. The nucleotides not only act as energy donors but also control the progression of the cell through the cell cycle. Furthermore, they are direct precursors of nucleic acid synthesis [2,6–9]. The biological significance of the intracellular nucleotide pools necessitates the development of a method to make it possible to measure a full range of nucleotides. The traditional technology, such as high-performance liquid chromatography (HPLC) and enzymatic analysis, are time consuming and unable to measure a broad range of nucleotides at the same time. With the application of capillary electrophoresis, we were able to obtain data efficiently and conveniently. This paper presents the analysis of nucleotide standards on a 65 cm × 50 μm I.D. Ucon-coated column. Over 12 nucleotides were analyzed with only nanoliters of sample and microliters of buffer reagent at pH 5.28. The analysis showed highly reproducible results, with mean migration times (within one standard deviation), and good sensitivity with detectable concentrations in micromoles. The total separation time was only 35 min. The method was further applied successfully to separate the nucleotide pools in the human Burkitt lymphoma cell lines. With the development of capillary electrophoresis, we are able to apply this technique in measuring nucleotide

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pools in cells conveniently and efficiently. In this article, standard nucloetides were first used to develop the separation methodology [10]. We further applied the developed technology to separate the nucleotide pools in the human lymphoma cell extract.

2. Experimental

2.1. Chemicals and Apparatus

Chemicals

All nucleotide standards and Tris·HCl were obtained from Sigma (St. Louis, MO, USA). Diabasic sodium phosphate was from Mallinckrodt (St. Louis, MO, USA) and monobasic potassium phosphate was from EM Science (Cherry Hill, NJ, USA). Ucon 75-H-90 000 [poly(ethylene–propylene)glycol] was obtained from Alltech (Deerfield, IL, USA). Dicumyl peroxide and hexamethyldisilazane were purchased from Aldrich (Milwaukee, WI, USA). RPMI 1640 and 10% fetal calf serum was from Hyclone (Logan, UT, USA).

Apparatus

A Model CES-1 capillary electrophoresis system (Dionex, Sunnyvale, CA, USA) was used in this study. Sample introduction was performed by electromigration injection for a certain time period at a fixed electrical potential. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. × 360 μm O.D. were used. The nucleotides were detected by on-column UV absorbance at 254 nm. Data were collected using a Model SP 4290 integrator (Spectra-Physics, San Jose, CA, USA).

2.2. Capillary column preparation

The details of this procedure have been reported elsewhere [11–14]. The coating solution consisted of a mixture of Ucon (1–8 mg ml^{-1}), hexamethyldisilazane (1–3 mg ml^{-1}) and dicumyl peroxide (0.1 mg ml^{-1}) in methylene chloride. After static coating, the capillaries were cross-linked by heating at the rate of 4°C/min, and holding at the final temperature of 150°C for 1 h.

2.3. Identification and quantitation of the nucleotides

The nucleotides were identified by comparing the migration times of the unknown peaks with those of standards eluted under the same conditions. The calibration curves of peak area vs. concentration of nucleotides were obtained by eluting the standards dissolved in the running buffers with the same conditions as above. The amount of each nucleotide in the cell extract was determined by reading the concentration from its respective standard curve.

2.4. Nucleotide pool extraction from human tumor cells

Raji cells, a human Burkitt lymphoma cell line, were used in these experiments. Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 50 μg/ml gentamicin before cell extraction. Cells were cultured until they reached the exponential phase and were then harvested. Cells were washed three times in Tris–phosphate buffer pH 7.4 and then adjusted to 10^7 cells per sample. The nucleotide pools were extracted with cold perchloric acid for 15 min from the cells using a modified procedure of Smee et al. [15]. Perchloric acid should be handled in the hood with care. The extraction was centrifuged at 5500 *g* for 5 min at 4°C. The supernatant containing the nucleotides was collected and neutralized to pH 5.28 with 1 *N* potassium hydroxide.

3. Results and discussion

3.1. Separation of the standard nucleotides

In this study, a standard mixture of 15 common nucleotides was selected. Operating buffer

at a pH of 5.28 was chosen because the nucleotides were relatively stable at a low pH and readily hydrolyzed at a high pH. The buffer composition and pH were critical for separation. High efficiency and resolution of the solutes were obtained on coated columns from a buffer containing the desired combination of phosphate and Tris–HCl [16]. The Ucon-coated fused-silica columns were used for the separation of various nucleotides. Fig. 1 demonstrates the capillary electrophoretic separation of standard nucleotides. The conditions used were electromigration injection for 6 s at −26 kV followed by −20 kV applied voltage. The eluting sequence was in an organized pattern showing triphosphate, diphosphate, diphosphate with sugar, and monophosphate nucleotides. High resolution was achieved in the separation as is shown in Fig. 1. The Ucon-coated column was very inert to biological samples, which increased the reproducibility of the analysis. Table 1 presents the mean migration times of the nucleotides and their relative standard deviations (R.S.D.s). The migration time R.S.D. is less than 1%. The optimal pH of separation for a Ucon-coated column is 3.0–8.0.

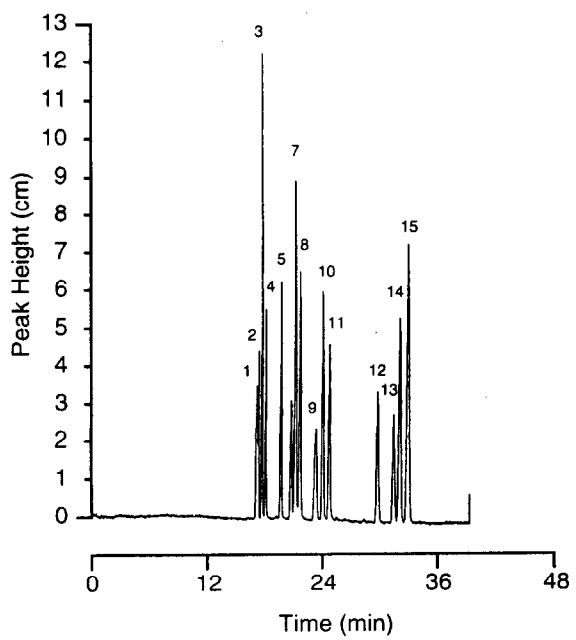

Fig. 1. Capillary electrophoretic elution of ribonucleotide standards on a Ucon-coated column. Conditions: 65 cm × 50 μm I.D. column, 30 m*M* phosphate–50 m*M* Tris·HCl buffer, pH 5.28; electromigration injection for 6 s at −23 kV; −20 kV applied voltage. Peaks: 1 = UTP; 2 = CTP; 3 = ATP; 4 = GTP; 5 = UDP; 6 = CDP; 7 = ADP; 8 = GDP; 9 = XMP; 10 = UDP-glucose; 11 = ADP-ribose; 12 = UMP; 13 = CMP; 14 = AMP; 15 = GMP.

Table 1
CE measurement of ribonucleotides in the cells

	Mean migration time (min)	R.S.D. (%)[a]	Per 10^7 cells
UTP	17.50	0.25	3.0
CTP	17.60	0.23	1.4
ATP	18.50	0.22	10.2
GTP	18.41	0.21	5.5
UDP	20.02	0.27	0.4
CDP	21.10	0.34	Trace
ADP	21.53	0.29	1.3
GDP	22.02	0.29	0.4
XMP	23.80	0.65	0.6
UMP	30.24	0.50	1.3
CMP	31.96	0.48	Trace
AMP	32.58	0.52	0.7
GMP	33.43	0.48	Trace

CE conditions as in the figures.
[a] $n = 9$.

Several chromatographic and enzymatic approaches have been used to measure the ribonucleotides. Among these methods, HPLC is the most common and suitable for the separation of nucleotides [17–20]. There are several drawbacks of HPLC which can be overcome by capillary electrophoresis. First, HPLC typically takes about 45 min, not including the time spent conditioning and regenerating [21,22]. Capillary electrophoresis only takes about 35 min and it accurately separates more nucleotides. The Ucon-coated column usually requires less maintenance, and thus the time spent on HPLC conditioning and regenerating is saved. Second, HPLC requires the use of an organic solvent as the mobile carrier, which is more expensive. Capillary electrophoresis uses electrical force and, therefore, it does not need an organic solvent. Third, the injection volume is in the microliter range in HPLC, but in high-perform-

ance capillary electrophoresis only in the nanoliter range.

3.2. Peak identification and quantitation

The peaks were identified by (1) comparing retention times of the unknown peaks with those of the standard nucleotides eluted under the same conditions and (2) spiking with pure single nucleotide standard added to the sample. Since these analyses were carried out under carefully controlled conditions, and most other interfering biological materials were removed from the samples before analysis, few sample matrix problems were encountered. Under the buffer running condition of pH 5.28, no neutral marker peak was eluted within a continuous run time of 5 h. In addition, the column was coated with Ucon, which is relatively inert to biological samples [23]. No problems were encountered concerning the sample adsorption and modification of the column surface. The column was rinsed with 5 ml of running buffer after each run.

3.3. Application

Cells were cultured in the media until they reached the exponential growth phase. A good extraction method was crucial for the analysis. Through the extraction technique, we could not only selectively extract the compounds of interest from others with similar detection characteristics, but we could also precipitate all of the protein in order to avoid column modification. The complete extraction procedure should give good recoveries and the compounds should be stable in the final extract. Perchloric acid was used to extract the nucleotides. Recoveries of 80 to 100% and protein precipitation of greater than 98% were obtainable [16,26–28]. The supernatant was quite stable under frozen conditions. To verify this, we analyzed the same sample under the same conditions a month later. The result showed no differences either in migration time or concentration. Fig. 2 shows an electropherogram of ribonucleotides of a human Burkitt lymphoma cell extract under the same conditions. The quantitative results were obtained by comparing the peak areas to the calibration curve (Fig. 3). The results suggest very high resolution, leading to identification in the sample of the most common nucleotides. The concentrations of the individual pool size (Table 1) differed significantly. The adenine nucleotides comprised approximately 70% of the total nucleotide pool in the cells, whereas the cytosine nucleotides comprised only about 1%. This high difference may be closely associated with growth phases and metabolic characteristics of tumor cells. The tumor cells used in this research by their nature were rapidly reproducing without normal growth constraints. Therefore, they had a very high intracellular ATP level to provide energy to synthesize proteins and enzymes that

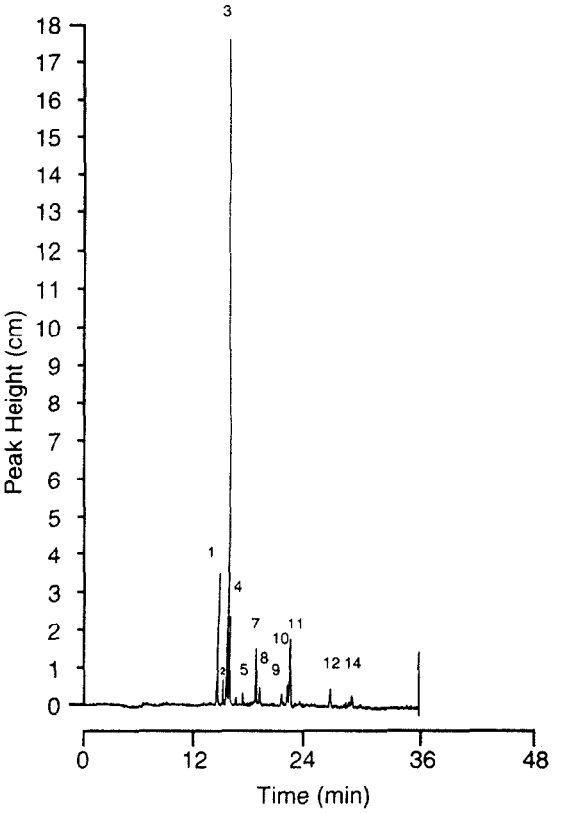

Fig. 2. CE separation of ribonucleotides of lymphoma cells run under the same conditions as the standards (Fig. 1). Peaks: 1 = UTP; 2 = CTP; 3 = ATP; 4 = GTP; 5 = UDP; 7 = ADP; 8 = GDP; 9 = XMP; 10 = UMP-glucose; 12 = UMP; 14 = AMP.

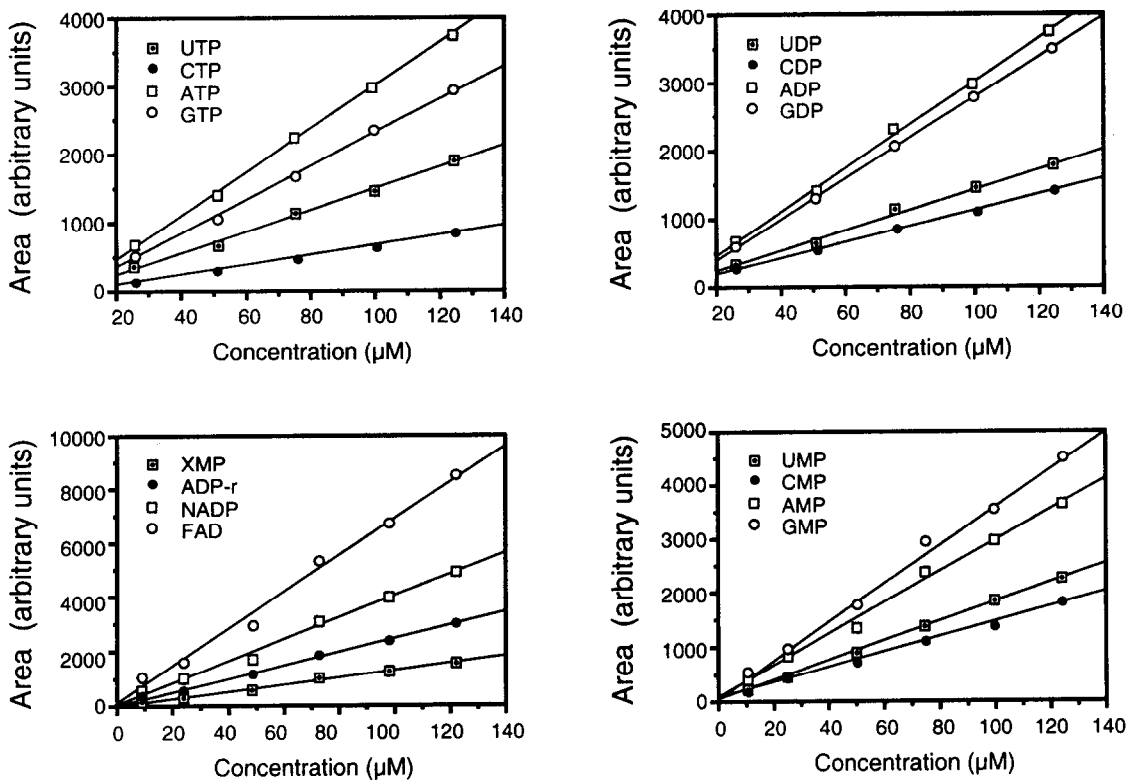

Fig. 3. Plots of peak areas vs. solute concentration of ribonucleotides. Conditions as in Figs. 1 and 2.

they need in the proliferation processes. The enzyme and protein synthesis were highly regulated by the nucleotide pools through transcription and translation. Our study indicates active nucleotide pools in these cells. Also, there were high levels of uracil nucleotides. UTP accounts for about 20% of the triphosphate pool which may be involved with the cell repair mechanism. Uracil nucleotides are actively involved in the cell salvage pathway to repair damaged DNA, cells may therefore need to maintain a high level of uracil nucleotides to carry out these biological functions [5,29]. The monophosphate nucleotides only accounted for 1% of the whole nucleotide pool in the Raji cells.

The results indicate that capillary electrophoresis will have broad application in the future because of its ability to measure the nucleotide pool levels with high resolution and low labor intensity. It will provide an efficient way to analyze nucleotide pools in biological samples.

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