Analysis of Free Intracellular Nucleotides Using High-Performance Capillary Electrophoresis

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High-performance capillary electrophoresis (HPCE) with UV absorbance detection (254 nm) has been applied for analyzing intracellular free ribonucleotides. The nucleotide profiles obtained from peripheral blood lymphocytes differ from those obtained from Molt4 human leukemic cells. With a 140 mM borate buffer, pH 9.4, a nearly complete profile can be obtained in 25 min. HPCE has comparable resolution to that of high-performance liquid chromatography (HPLC) but is faster in terms of time per sample run (25 min vs 45 min) and requires much less sample (nanoliter range for HPCE vs microliter range for HPLC).

INTRODUCTION

Intracellular nucleoside metabolism has become an important aspect in the therapeutics of acquired immunodeficiency syndrome (AIDS). Azidothymidine (AZT, Zidovudine), 2',3'-dideoxyinosine (ddI), and other dideoxynucleoside analogs have been shown to block human immunodeficiency virus (HIV) replication. These prodrugs are activated by using intracellular host enzymes that convert these nucleosides to their triphosphate forms which then inhibit viral reverse transcriptase.[1-5] Since the triphosphate form is the active species, conditions that can maximize its intracellular concentration will increase the efficacy of the drug against HIV. Finding a method to increase the efficacy of nucleoside analogs without administering higher doses is an important area of research because it is believed that some drug side effects are related to the concentration of the parent drug. AZT toxicity leads to anemia and neutropenia[6] and ddI toxicity leads to a painful, peripheral neuropathy and pancreatitis.[7]

Previous studies that have examined the intracellular metabolism of nucleoside analogs have relied upon using radiolabeled nucleosides and high-performance liquid chromatography (HPLC) to provide an intracellular nucleotide profile of the cell.[1-5] While the resolving power of HPLC in analyzing intracellular nucleotides has been established,[8,9] its main drawback is the lengthy analysis time, typically about 45 min.[8,9] Intracellular nucleotide profiles with shorter run times have been obtained using HPLC[10-12] but usually at the expense of separating and identifying fewer nucleotides.

High-performance capillary electrophoresis (HPCE) has demonstrated its capacity to resolve nucleotides and oligonucleotides[13-15] and its applicability to quantitate three nucleotides present in fish tissues.[16] Here, we describe a method for obtaining an intracellular nucleotide profile using HPCE. Two cell lines were analyzed: human peripheral blood lymphocytes and Molt4 human leukemic cells. Nine nucleotide species were identified, all eluting within 25 min. Distinct differences in the nucleotide pools of the human peripheral blood lymphocytes and the Molt4 cells can be detected. The reproducibility and reliability of this proposed technique is also presented and discussed.

MATERIALS AND METHODS

Human peripheral blood lymphocytes were obtained from a healthy volunteer. A 25-mL aliquot of blood was drawn in heparinized Vacutainer tubes (Becton-Dickinson, Rutherford, NJ), and the lymphocytes, along with a small number of monocytes, were separated from the other blood constituents by using LeucoPrep cell separation tubes (Becton-Dickinson, Lincoln Park, NJ). Approximately 30 million cells were obtained, as determined by a hemocytometer count. After washing twice with 1 N phosphate-based saline (PBS) (Sigma, St. Louis, MO), the lymphocytes and monocytes were then lysed in 60% methanol[17] at 4 °C. The methanol extract was passed through a Bakerbond SPE C_{18} column (J. T. Baker, Phillipsburg, NJ) to remove any debris. The effluent was evaporated to dryness on a Savant SpeedVac concentrator (Farmingdale, NY). The cell extract was then taken up in deionized water.

Molt4 cells are a line derived from human acute T-cell lymphoblastic leukemia[18] and were a gift from Dr. Ronald Levy,

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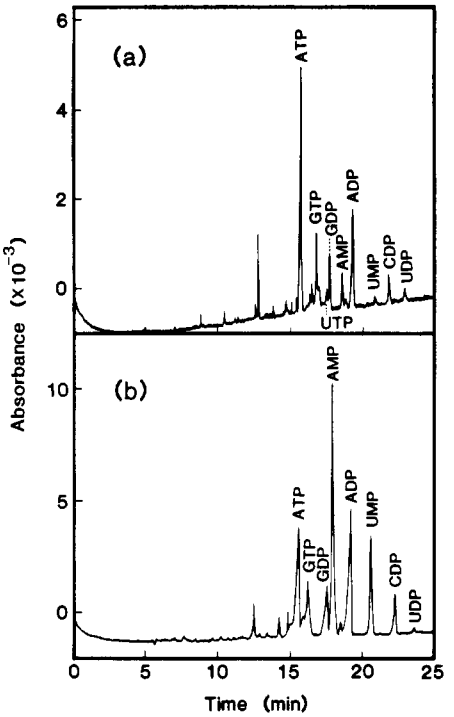

Figure 1. HPCE electropherograms of the nucleotide pool in (a) human peripheral blood lymphocytes and (b) Molt4 leukemic cells. See text for separation conditions.

Stanford University. The cells were grown in RPMI 1640 media with 2 mM glutamine supplemented with 10% fetal bovine serum and 2% streptomycin/penicillin (200 units/mL). All three items were purchased from Applied Scientific (San Francisco, CA). Approximately 30 million cells were obtained, as determined by a hemocytometer count. The Molt4 cells were washed once with 1 N PBS, and the cellular extract was obtained using the method described above.

High-performance capillary electrophoresis was performed on a PACE System 2000 (Beckman Instruments, Palo Alto, CA[19]). The instrument is controlled by PACE software (Beckman Instruments) in a WINDOWS (Microsoft, Redmond, WA) environment. System Gold (Beckman Instruments) software was used to integrate the peaks. The runs were performed under the following conditions: T = 25 °C, λ = 254 nm, V = 16 kV, I = 58 μA, 140 mM borate buffer, pH 9.4. A 75-μm diameter and 57.0-cm length (50 cm to the detector) fused silica capillary column was used. The column was washed with 0.1 N NaOH for 3 min, deionized water for 3 min, and 140 mM borate buffer, pH 9.4, for 2 min before each injection. Injections were made by applying a slight head pressure to the sample solution for 2 s (delivering approximately 2 nL).

Peaks on the electropherogram were identified by spiking with pure samples of the standard nucleotides (Sigma, St. Louis, MO). Concentrations of the nucleotides were determined by using the peak areas of known nucleotide concentrations. Losses during a typical sample workup were estimated by adding known concentrations of standard nucleotides (approximately 20 times the intracellular concentration) to the cell suspension before lysis and calculating the recovery after passing through the Bakerbond SPE C_{18} column. Recovery was found to be 65–70%.

RESULTS AND DISCUSSION

Electropherograms of the intracellular nucleotide content of human peripheral blood lymphocytes and Molt4 human leukemic cells were obtained (Figure 1) and the major intracellular nucleotides were identified by spiking experiments with standard nucleotides. In this spiking experiments we coinject authentic nucleotides and observe comigration. Nine nucleotides were identified, all eluting within 25 min. The migration order of the nucleotides, with triphosphate nucleotides eluting before the monophosphate and diphosphate nucleotides, was reproducible under the conditions described above. Several other peaks were not identified and may be attributable to other intracellular compounds or to residual extracellular debris. There tended to be more extracellular debris associated with the isolation of the peripheral blood lymphocytes than with the Molt4 cells, mostly arising from lysed red blood cells and to LeucoPrep tube column matrix. This may account for the sloping baseline seen in the peripheral blood lymphocytes electropherograms. Electropherograms were readily quantifiable. The PACE unit has very good reproducibility with consecutive runs in terms of migration time and individual peak area. Table I summarizes the variability in ATP, ADP, and AMP peak areas over five consecutive runs. These results show that HPCE produces reliable and reproducible data.

Table I

	concn (mM)						
nucleotide	1	2	3	4	5	av	sd
ATP	0.208	0.208	0.227	0.217	0.235	0.219	0.012
ADP	0.066	0.074	0.073	0.073	0.076	0.072	0.00378
AMP	0.020	0.020	0.018	0.019	0.022	0.020	0.00148

Table II

nucleotide	concn to area ratio (mM/arbitrary area units)	nucleotide	concn to area ratio (mM/arbitrary area units)
GTP	0.265	ATP	0.251
UTP	0.322	ADP	0.219
CTP	0.409	AMP	0.212

Nucleotide concentrations were calculated by injecting known concentrations of each nucleotide, performing the separation under the conditions described above, and calculating the area under each peak. The ratios of concentration to peak area are shown in Table II, and the profiles of the nucleotide standards are shown in Figure 2. The elution pattern of the nucleotide standards is slightly different from the elution pattern of the intracellular nucleotides. For example, the difference in migration time between ATP and AMP is much greater than the difference in migration time between AMP and ADP in the intracellular nucleotide electropherograms (Figure 1), but in the electropherogram of the nucleotide standards (Figure 2) the difference in migration time between ATP and AMP and the difference in migration time between AMP and ADP are equivalent. This behavior may be accounted for by the presence of extracellular debris in the intracellular nucleotide samples which can coat the capillary column and alter elution patterns.

Using the concentration to peak area ratios to calculate nucleotide concentrations, distinct differences can be seen in the nucleotide ratios of the human lymphocytes and of the Molt4 cells (Table III). As shown in this table, the ATP, ADP, and AMP concentrations (averaged over consecutive runs of the same sample) are different in the two cell lines, indicating differences in cellular metabolism. One index of the energy status of a cell is the energy charge[20] defined as

$$\text{energy charge} = \frac{[\text{ATP}] + 0.5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

Thus, the higher the energy charge, the higher is the energy status of the cell. The energy charge of most cells is in the range 0.80–0.95. From our data we find that the energy

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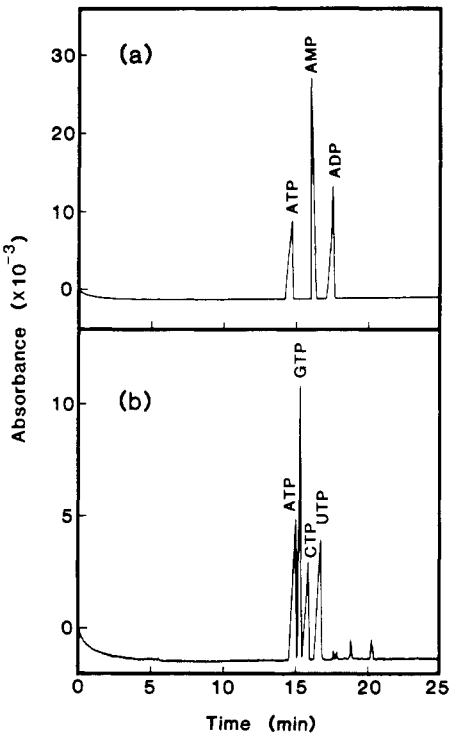

Figure 2. HPCE electropherograms of (a) adenosine mono-, di-, and triphosphates and (b) the triphosphates of the four nucleotide standards. See text for separation conditions.

Table III

	av concn (mM)		
	ATP	ADP	AMP
peripheral blood lymphocytes (N = 5 runs)	0.219	0.072	0.020
Molt4 leukemic cells (N = 3 runs)	0.244	0.199	0.304

charges of human peripheral blood lymphocytes and Molt4 cells are 0.82 and 0.46, respectively. Overall, when compared to normal human peripheral blood lymphocytes, Molt4 cells show an increase in the pool size of adenine nucleotides and an increased ratio of ADP and AMP to ATP. These phenomena have recently been shown in the lymphoblastic leukemic cells in children with acute lymphoblastic leukemia (ALL).[21] When intracellular ribonucleotide pools of lymphoblastic leukemic cells from untreated children and from relapsed children who had been treated with various chemotherapeutic agents were analyzed, the nucleotide pools from the relapsed children were expanded with an increased ratio of di- and monophospate nucleotides to triphosphate nucleotides. Since diphosphate pools can be directly converted into deoxyribonucleotides by ribonucleotide reductase,[22] high diphosphate concentrations may be indicative of rapid cell growth. Also, a monophosphate pools can be correlated with de novo and salvage pathways,[22] and an increased monophosphate pool size may reflect an increased need for ribonucleotides, both for replication and for cell metabolism. Thus, it appears that HPCE could play a role in cancer research.

HPCE has proven to be an effective method in separating different nucleotide species. Previously, it has been shown that HPCE has the power to resolve a mixture of 2′, 3′, and 5′ isomers of adenylate, guanylate, uridylate, and cytidylate[15] and to quantitate nucleotide concentrations in fish tissues.[16] Here, HPCE was used to approach the problem of resolving different intracellular nucleotide species. Nine different compounds were separated and identified. The resolution of HPCE is comparable to that of HPLC for this purpose.[8,9]

One of the unique aspects of HPCE is the small injection volume used for each run. Thus, HPCE is much more economical and more useful when the amount of sample is limited. While HPLC usually handles volumes in the microliter range, HPCE can inject volumes in the nanoliter range. Overall, in terms of the amount of compound, HPCE requires 100 times less than the quantity required by HPLC.[15] However, this low injection volume also presents a problem when the total amount of compound injected into the system is considered. For a given sample, HPLC can inject a larger quantity of the sample because of its capability for handling larger volumes. This may account for the many more peaks that can be identified in an intracellular nucleotide profile using HPLC.[8,9] This problem can be partially circumvented by concentrating the sample to a smaller volume so that a larger fraction of the sample is injected with each HPCE run.

In terms of separation time for intracellular nucleotides. HPCE is superior to HPLC. The run time using HPLC is about 45 min.[8,9] Here, analysis performed using HPCE takes less than 25 min. In addition, the fused-silica capillary column generally requires less maintenance than HPLC columns. Thus, time spent conditioning, regenerating, and washing HPLC columns can be saved using HPCE. Over the course of a large number of samples, HPCE can be a great time-saving method when compared to HPLC.

HPCE appears to be a promising approach in studying intracellular nucleotide pools. This method may be useful not only in the pharmacological study of anti-HIV nucleoside analogs, such as AZT and ddI, but also in other areas of cellular biology. Since intracellular nucleotide concentrations may be indicative of a cell's metabolic rate, its energy stores, and its rate of replication, a method that would quickly and easily measure these nucleotides could find many biological and clinical applications. The ability of HPCE to separate intracellular nucleotides with a short resolution time and with a limited injection volume would indicate that HPCE may be of use in studying many of these questions in cellular biology.

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Registry No. ATP, 56-65-5; ADP, 58-64-0; AMP, 61-19-8; GTP, 86-01-1; GDP, 146-91-8; UMP, 58-97-9; CDP, 63-38-7; UDP, 58-98-0; UTP, 63-39-8; CTP, 65-47-4.

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