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High-performance liquid chromatography method for the determination and quantitation of arabinosylguanine triphosphate and fludarabine triphosphate in human cells

Carlos O. Rodriguez Jr.[a,b], William Plunkett[a,b,d], Melanie T. Paff[c], Min Du[a], Billie Nowak[a], Prameen Ramakrishna[a], Michael J. Keating[b,d], Varsha Gandhi[a,b,d,*]

[a]*Department of Experimental Therapeutics, Box 71, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 71, Houston TX 77030, USA*

[b]*The University of Texas Graduate School of Biomedical Sciences, Houston TX, USA*

[c]*Glaxo Wellcome, Inc., Research Triangle Park, NC, USA*

[d]*Department of Leukemia, The University of Texas M. D. Anderson Cancer Center, Houston TX, USA*

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Abstract

A gradient anion-exchange high-performance liquid chromatographic assay was developed for the simultaneous determination and quantitation of the cytotoxic triphosphates of arabinosylguanine (ara-GTP) and fludarabine (F-ara-ATP). The method was validated with respect to selectivity, recovery, linearity, precision, and accuracy using authentic standards. To test this assay in a more complex biological matrix, perchloric acid extracts of circulating human leukemia cells spiked with known concentrations of ara-GTP and F-ara-ATP were examined. Finally, to assess the clinical utility of our method, perchloric acid extracts of circulating human leukemia cells isolated from patients treated with fludarabine and nelarabine were analyzed. The range of quantitation was 0.0125–10 nmol for the ara- and native NTPs in cellular extracts. This assay should be helpful in establishing the mechanistic rationales for drug scheduling and combinations of nelarabine and fludarabine, and for correlating the therapeutic efficacy and levels of the cytotoxic triphosphates in target cells.

Keywords: 506U78; Arabinosylguanine; Fludarabine; Nelarabine

1. Introduction

Nelarabine (known before as compound 506U78), a pro-drug of arabinosylguanine (ara-G), has proven effective in the treatment of patients with acute [1,2] and indolent [3] leukemias. As predicted by in vitro investigations [4–6], the drug was selective for T-lymphoblasts. In the first Phase I investigation, there was a >70% response in adult patients with T-cell acute lymphoblastic leukemia. Interestingly, additional Phase I studies demonstrated the efficacy of this drug for patients with indolent lymphoid diseases [3]. In contrast to patients with these diagnoses, patients with B-acute lymphoblastic leukemia or non-lymphoid leukemias did not respond to treatment with nelarabine.

*Corresponding author. Tel.: +1-713-792-2989; fax: +1-713-794-4316.

E-mail address: vgandhi@notes.mdacc.tmc.edu (V. Gandhi).

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Nelarabine is a 6-methoxy analog that is demethoxylated to ara-G by adenosine deaminase [4]. The drug, ara-G, is phosphorylated to ara-G 5′-monophosphate (ara-GMP) by cytosolic deoxycytidine kinase [4,5,7,8] and mitochondrial deoxyguanosine kinase [7,9,10]. Ara-GMP is then converted to the diphosphate and triphosphate species. The 5′-triphosphate of ara-G, ara-GTP, is the only cytotoxic metabolite [6]. The initial Phase I trial in patients with refractory hematological malignancies demonstrated that the clinical response to nelarabine was strongly correlated with peak intracellular levels of ara-GTP in circulating leukemia cells during therapy [2]. As the knowledge of ara-GTP levels in circulating leukemia cells is likely to have prognostic value, the development of a formally validated assay for the quantitation of ara-GTP in the target cells is desirable.

In-vitro and ex-vivo experiments using primary leukemia cells from patients with acute myelogenous leukemia (AML) or chronic lymphocytic leukemia (CLL) have demonstrated that the addition of 2′-fluoro-9-β-D-arabinosyladenine (F-ara-A) prior to ara-G incubations increases the accumulation of ara-GTP by biochemical modulation [11]. Based on this data, a clinical trial was designed to infuse F-ara-A monophosphate (fludarabine) 4 h before nelarabine administration. Similar to ara-G, fludarabine is metabolized to its 5′-triphosphate, F-ara-ATP [12]. When treated with the fludarabine–nelarabine couplet, circulating leukemia cells accumulate both F-ara-ATP and ara-GTP, suggesting a need for a high-pressure liquid chromatography (HPLC) assay to simultaneously separate and quantitate these clinically relevant analog triphosphates. The present report describes and validates an HPLC method that can be used for the quantitation of ribonucleotides alone, ribonucleotides with ara-GTP, or ribonucleotides with ara-GTP in combination with F-ara-ATP in human leukemia cells.

2. Experimental

2.1. Chemicals

Commercially available ribonucleotides (CTP, UTP, ATP, and GTP) were used as standards (Sigma Chemical Co., St. Louis, MO, USA). Fludarabine and ara-G triphosphates were chemically synthesized by Sierra Bioresearch (Tucson, AZ, USA). All other chemicals were reagent grade.

2.2. Preparation of matrix

For the preparation of matrix, a 50-ml blood sample was obtained from a patient with T-prolymphocytic leukemia (T-PLL) with a count of 9.3×10^5 white blood cells/μl. The sample was collected into tubes containing heparin. The tubes were placed in an ice–water bath before being transported to the laboratory for processing. After removal of plasma, the cell pellet was resuspended in phosphate-buffered saline (8.1 g of NaCl, 0.22 g of KCl, 1.14 g of Na_2HPO_4, and 0.27 g of KH_2PO_4 per liter of H_2O, pH 7.4). The mononuclear cells were then isolated by density-gradient centrifugation as previously described [13]. Control studies have demonstrated that under these conditions leukemia cells are stable for at least 15 h with respect to size, membrane integrity, and cellular nucleotide content [13]. Normal nucleotides were extracted by using a perchloric acid (PCA) extraction method, and the extracts were neutralized with KOH as described [14], and then stored at −70°C until analyzed. Extraction of replicate samples resulted in less than 10% variation in nucleotide levels. Similarly, greater than 95% of nucleotides added to cell extracts were recovered [14].

2.3. Determination of injection volume

Because the number of cells in the peripheral blood varies among patients, a different volume of PCA extract needed to be injected for each patient. Generally, PCA extracts from the equivalent of 2×10^7 cells were injected for HPLC analyses. Review of the data from 18 patients on a previous Phase I clinical study [2] revealed that the mean leukemia cell extract equivalent to 2×10^7 cells was about 800 μl. Based on this data, injection volumes for standard nucleotide (NTP) preparation were kept constant at 800 μl. Similarly, quality control (QC) samples were prepared so that the 800 μl of extract would contain an equivalent of 2×10^7 cells.

2.4. Preparation of standard NTPs

A solution of each NTP, F-ara-ATP, and ara-GTP was made in water. The concentration of each stock was determined at pH 2.0 (0.01 *M* HCl) using a spectrophotometer. The wavelength and m*M* extinction coefficient for NTPs were 280 nm and 12.8 for CTP, 262 nm and 10.0, for UTP, 257 nm and 14.7, for ATP, 256 nm and 12.4 for GTP, 262 nm and 13.2, for F-ara-ATP, and 256 nm and 12.9, for ara-GTP. From these stock solutions a 10-ml solution of standards containing 10 nmol each of all four NTPs, F-ara-ATP, and ara-GTP in a volume of 800 μl was prepared. Hence, the final concentration of each triphosphate in this stock solution was 12.5 nmol/ml. This stock was diluted serially to make standards containing 10, 5, 2.5, 1.0, 0.5, 0.25, 0.1, 0.05, and 0.025 nmol each of all four NTPs, F-ara-ATP, and ara-GTP in an 800-μl volume. All standard solutions were stored at −70°C until analyzed.

2.5. Quality control samples

Three types of QC samples were prepared and stored at −70°C until analyzed. In the blank samples, (QC B) the analyses focused on the naturally occurring NTPs (CTP, UTP, ATP, GTP) from an equivalent of 2×10^7 cells in 800 μl of matrix. The 0.25 samples (QC 0.25) were blank QC samples (matrix) spiked with 0.25 nmol each of F-ara-ATP and ara-GTP. The 2.5 samples (QC 2.5) were blank QC samples (matrix) spiked with 2.5 nmol each of F-ara-ATP and ara-GTP. The volume of injection (800 μl) was kept constant for all three types of QC samples. The 0.25 and 2.5 nmol amounts were determined by UV absorbance of the stock solution as described above in Section 2.4. The 0.25 nmol of F-ara-ATP or ara-GTP in 2×10^7 cells in the QC 0.25 sample represented an intracellular concentration of 25–50 μ*M*. Similarly, QC 2.5 represented an intracellular concentration of 250–500 μ*M*. Based on our previous in vivo and in vitro investigations [2,11,12] the concentrations of F-ara-ATP and ara-GTP in the circulating leukemia cells were expected to fall between 25 and 100 μ*M* and 25 and 500 μ*M*, respectively. Hence, QC 0.25 and QC 2.5 were chosen because they were within the expected lower and upper limits of F-ara-ATP and ara-GTP in leukemia cells of patients treated with fludarabine and nelarabine.

2.6. HPLC instrumentation

An Alliance HPLC instrument (Waters Corporation, Milford, MA, USA) was used for all analyses. This instrument contained the 2690 Separation Module, an integrated solvent and sample management platform. To assure the stability of cell extracts during sample injection time, the 2690 Module was attached to a sample heater–cooler system that was maintained at a constant temperature of 4°C. While the maintenance of this temperature is not necessary during sample injection time, the acid extracted samples should be stored at −20°C prior to HPLC injection. The system also contained a Waters 486 Tunable Absorbance Detector for detection of ultraviolet (UV) absorbance of the eluants. The instrument was connected to a Compaq personal computer. The data were acquired and processed to generate reports using Millennium Chromatography Manager Software (version 2.1, Waters Corporation). The reports and the chromatograms were printed on an NEC Pinwriter P6200 printer (NEC Technologies, Inc., Boxborough, MA, USA).

2.7. Separation and quantitation of nucleotides

Three separate sets of standards and QC samples were analyzed on three different days using HPLC. For each set, the known concentrations of standards (eight concentrations, each in duplicate) and the QC samples (3 concentrations, each in sextuplicate) were applied to a 10-SAX Partisil anion-exchange column (4.6×250 mm, Whatman, Clifton, NJ, USA). The samples were eluted at a flow-rate of 1.5 ml/min with a 50-min concave gradient (curve #8) on a Waters 2690 Separation Module from 60% 0.005 *M* $NH_4H_2PO_4$ (pH 2.8) and 40% 0.75 *M* $NH_4H_2PO_4$ (pH 3.6) to 100% 0.75 *M* $NH_4H_2PO_4$ (pH 3.6). The first two sets of standards and QC samples were analyzed on one column, and a new column was used for third set. The column eluate was monitored by UV absorption at 256 nm on the Waters 486 Tunable Absorbance Detector, and the NTPs were quantitated by electronic integration with reference to the injected standards. This wavelength was

selected because it was the absorption maximum for ara-GTP.

2.8. Calibration curves

With each set of standards, a calibration curve was generated by using the eight concentrations of the standards. Calibration curves were prepared separately for each NTP, ara-GTP, and F-ara-ATP by using Millennium software. The experimental data points (concentrations versus peak area) were fitted by weighed least-squares linear regression analysis method to an equation of $y = ax + b$, where a represents the slope and b represents the intercept of the calibration curve at $x = 0$. The ara-GTP and F-ara-ATP in the QC samples were identified by comparisons of their retention profiles and absorption spectra with those of the authentic samples. The concentrations of nucleotides in the QC samples were calculated by substitution of the peak area into the equation of the calibration curve.

2.9. Calculations and statistical analysis

The linearity of the calibration curves for each data set was calculated by using Millennium software associated with the HPLC system. Linear regression analyses for the calibration curve from all three data sets were obtained by using GraphPad Prism software (version 2.01, GraphPad Software, Inc., San Diego, CA, USA). The coefficient of variation and standard deviation analyses for the QC samples were calculated using Microsoft EXCEL software (version 7.0, Microsoft Corporation, Redmond, WA, USA) by using the preprogrammed formulas.

3. Results and discussion

3.1. Standard calibration curves

Serially diluted standard samples in buffer that contained all four NTPs and both ara-NTPs were used. Three separate sets of calibration curves were generated on three separate days and analyzed. Each set of standards had between 0.025 and 10 nmol of each triphosphate. Two chromatographic analyses were completed on one column. The third analysis was completed on a second column. There were a total of 90 peaks evaluated for each of the ara-NTPs and more than 100 peaks for each of the native nucleotides. For illustrative purposes, a representative chromatogram of a 0.25-nmol standard run is shown in Fig. 1. Baseline resolution was achieved between each of the six peaks representing the four native NTPs and the two ara-NTPs. The peaks eluted between 15 and 50 min; specifically CTP, UTP, ATP, F-ara-ATP, GTP, and ara-GTP eluted at 20±0.4,

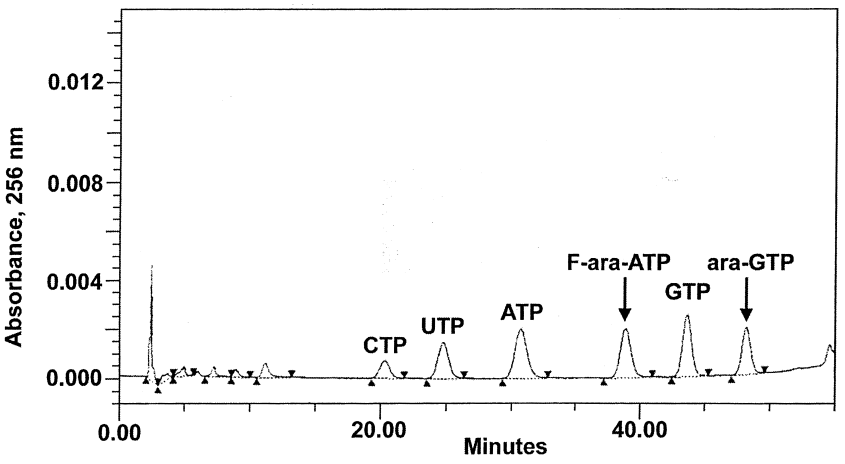

Fig. 1. Representative chromatogram of a standard nucleotide mixture. A buffered solution containing 0.25 nmol each of CTP, UTP, ATP, GTP, F-ara-ATP, and ara-GTP was injected and analyzed by HPLC as described in the Experimental section.

25±0.5, 31±0.6, 39±0.8, 44±0.5, and 48±0.5 min, respectively.

Similar HPLC analyses were performed with different concentrations of the standard sample. Data from all three sets were combined and plotted to determine the linearity of the HPLC elution profile for each concentration of ara-GTP and F-ara-ATP (Fig. 2). The data demonstrated a linear relationship between area under the curve and nmol of ara-NTP for concentrations between 0.025 nmol and 10 nmol for both F-ara-ATP ($r^2=0.9994$) and ara-GTP ($r^2=0.9998$). Similar results (data not shown) were obtained in the same concentration range for CTP ($r^2=0.9991$), UTP ($r^2=0.9997$), ATP ($r^2=0.9997$), and GTP ($r^2=0.9999$). The values for both the regression parameters (values of slopes and intercepts) and goodness of linear fit (regression coefficient, r^2; deviation of the residuals from the regression line, $S_{y.x}$) clearly demonstrate that the determinations were linear for CTP, UTP, ATP, GTP, F-ara-ATP, and ara-GTP at these concentrations.

At the lowest concentration tested (0.025 nmol), the intra-day variation (in duplicate samples at three separate times) for quantitation was less than 1.0% for both F-ara-ATP and ara-GTP. The inter-day variation was less than 1.0% for these six data points for each analog triphosphate (Table 1). Similar data were obtained for CTP, UTP, ATP, and GTP (data not shown). The concentration range used (0.025 to 10 nmol) represented concentrations of analog triphosphates (2×10^7 cell equivalents) in leukemia cells in the range of 2.5 μ*M* to 2.0 m*M*, and fell within the linear range of our assay. This range covers the expected intracellular concentrations of NTPs and ara-NTPs found in circulating leukemia cells from patients receiving nelarabine (20–60 mg/kg/d) and fludarabine (20–50 mg/m^2/d) [2].

3.2. Limit of integration/detection

Standards were serially diluted and then used to identify the lower limits of integration and detection. The limits of integration were 0.0125 nmol for UTP, ATP, GTP, and ara-GTP; 0.025 nmol for F-ara-ATP; and 0.05 nmol for CTP. The limit of detection was 0.003 nmol for each of the four NTPs, ara-GTP, and F-ara-ATP (data not shown).

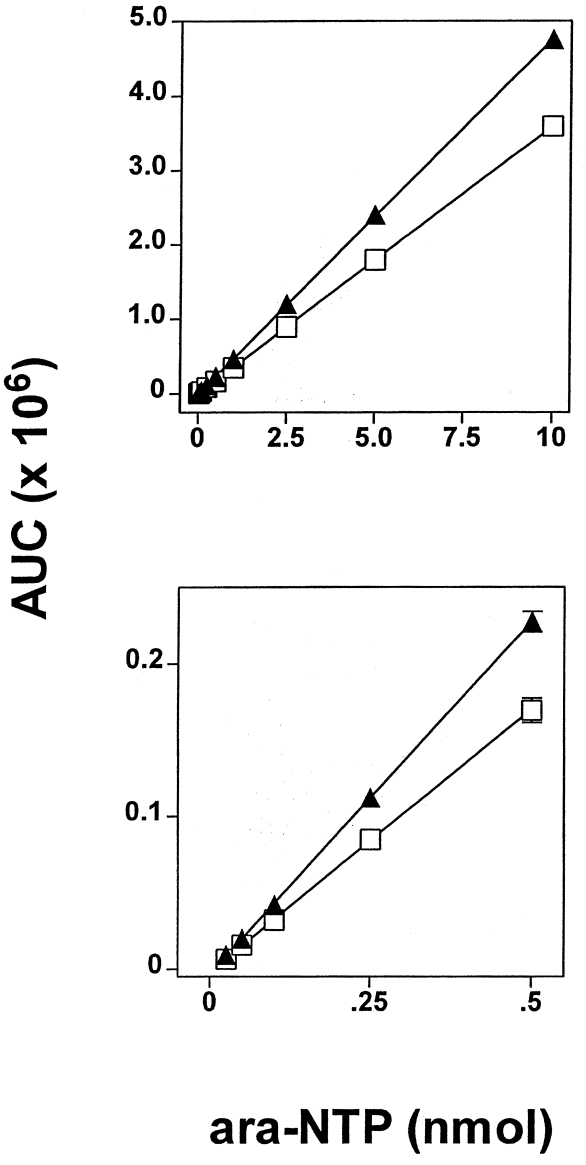

Fig. 2. The relationship between HPLC peak profile (area under the curve) and indicated concentrations of ara-NTPs. Linear regression analysis for F-ara-ATP (▲, $r^2=0.9994$) or ara-GTP (□, $r^2=0.9998$) were calculated as described in the Experimental section. Similar results were obtained for the four native NTPs (data not shown). (A) All ten concentrations of ara-NTPs are shown. (B) The five lowest concentrations are expanded for clarity. The data points are means±SD of three separate runs completed in duplicate. Where error bars are not visible, they are covered by the symbols.

Table 1
Intra- and inter-day variation in ara-NTP quantitation in buffer at the lowest concentration

Nucleotide	Days	nmol		Intra-day variation		
		Analysis 1	Analysis 2	Mean	SD	% C.V.
F-ara-ATP	1	0.030	0.031	0.031	0.001	2
	2	0.028	0.028	0.028	0.000	0
	3	0.030	0.027	0.029	0.002	7
Inter-day variation	Mean	0.029	0.029	–	–	–
	SD	0.001	0.002	–	–	–
	%C.V.	0.039	0.073	–	–	–
Ara-GTP	1	0.039	0.036	0.038	0.002	6
	2	0.032	0.033	0.033	0.001	2
	3	0.030	0.031	0.031	0.001	2
Inter-day variation	Mean	0.034	0.033	–	–	–
	SD	0.005	0.003	–	–	–
	%C.V.	0.140	0.075	–	–	–

3.3. Quality control samples in cellular matrix

The data obtained by using authentic standards in buffer suggested that we had achieved the optimal separation and resolution of each of the four NTPs and ara-GTP and F-ara-ATP. To test whether we could achieve similar resolution in a more complex matrix and to test the clinical applicability of this method, we made PCA extracts from T-PLL lymphocytes and resolved the NTPs as described above in the Experimental section. Fig. 3A shows the endogenous nucleotides contained in QC B. As was the case for the standard controls, CTP, UTP, ATP, and GTP eluted at 20, 25, 31, and 44 min, respectively. Assuming that the nucleotides from a given number of cells of known volume are distributed uniformly in the total cell water, the areas under the curve for the nucleotides present in our QC samples were 78, 390, 2600, and 530 μM CTP, UTP, ATP and GTP, respectively. As with the standard control, there was baseline resolution between all peaks in QC B.

Fig. 3B represents a QC 0.25 matrix run demonstrating, in addition to the four endogenous nucleotides, the addition of F-ara-ATP and ara-GTP which elute at 39 and 48 min, respectively. Again, baseline resolution was achieved between the four nucleotides in the sample and the added ara-NTPs. The quantitation of the native NTPs varied less than 5% between all runs (data not shown). Importantly, comparison of QC B with QC 0.25 demonstrated the absence of UV-absorbing peaks that may coelute at F-ara-ATP and ara-GTP elution times in the QC B matrix, which might confound quantitation of the arabinoside triphosphates.

Data regarding the intra- and inter-day variations in the concentrations (mean$\pm$SD) of all four NTPs, F-ara-ATP, and ara-GTP in each set are presented in Table 2. The data show that the precision (SD) and accuracy (%C.V.) of the method were good within each day and between 3 days when the samples were analyzed. For the intra-day variations, among NTPs, the greatest deviation was 2.7%C.V.; this was for CTP which is the endogenous cellular NTP present at the relatively lowest concentration and analyzed at suboptimal wavelength. The %C.V. values for intra-day analyses for F-ara-ATP and ara-GTP varied between 0.4 and 3, and 0.4 and 2, respectively. Similarly, for inter-day, low coefficients of variation were obtained for CTP (7%), UTP (1%), ATP (3%) and GTP (4%) (Table 2). Again, the data demonstrates that the variation was low, and the values for coefficients of variation for NTPs varied between 1 and 7% for the 54 QC samples. The low coefficient

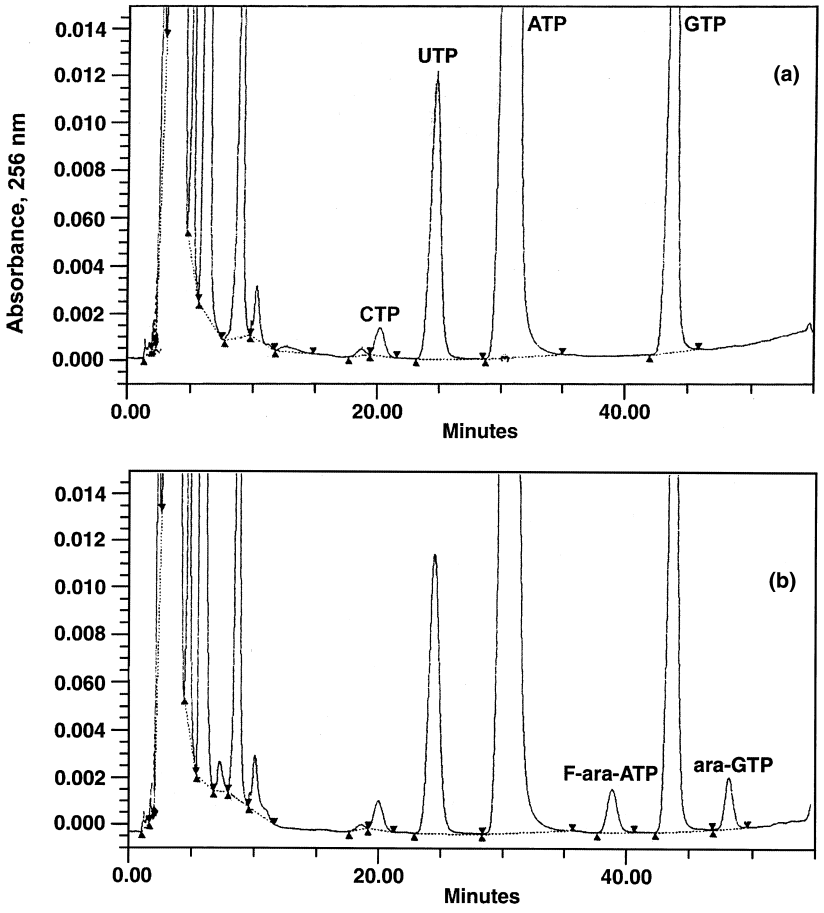

Fig. 3. Representative chromatogram of QC sample matrix from 2×10^7 cell-equivalent. (A) QC matrix blank or (B) QC matrix spiked with 0.25 nmol each of F-ara-ATP and ara-GTP. Each sample was prepared and the nucleotides quantitated as described in the Experimental section.

of variation was also true in the 24 QC samples analyzed for F-ara-ATP (4–5%) and ara-GTP (1–2%).

3.4. Clinical utility of the assay

To test the clinical usefulness of our method, we isolated and analyzed 2×10^7 circulating peripheral leukemia cell equivalents from a patient with B-cell chronic lymphocytic leukemia (Fig. 4A–C). Patients received 1.2 g/m^2 intravenously over 2 h of nelarabine on day 1 and 30 mg/m^2 intravenously over 30 min of fludarabine followed by 1.2 g/m^2 of nelarabine on day 3. Peripheral blood samples were obtained before treatment (Fig. 4A), at the end of the first nelarabine infusion (Fig. 4B), and at the end of the second nelarabine infusion (Fig. 4C). The pre-treatment sample showed that the baseline resolution of CTP, UTP, ATP, and GTP was similar to those obtained for the standards. Fig. 4B shows the end of a 2-h infusion of 1.2 g/m^2 of nelarabine. Again, there is baseline resolution of the native nucleotides and ara-GTP at the expected times. Fig. 4C shows the intracellular concentrations of native and ara-nucleotides after intravenous administration of the fludarabine–nelarabine couplet. As was the case for the standards, QC, and pretreatment samples, in clinically derived samples, our method resolved and

Table 2
Intra- and inter-day variation in NTP and ara-NTP quantitation of 0.25 and 2.5 nmol-spiked QC samples

	NTP (nmol)				0.25 nmol		2.5 nmol	
	CTP	UTP	ATP	GTP	F-ara-ATP	ara-GTP	F-ara-ATP	ara-GTP
Intra-Day								
Day 1								
Mean	0.370	2.035	13.243	2.639	0.218	0.285	2.273	2.698
SD	0.01	0.01	0.07	0.01	0.002	0.002	0.01	0.01
%C.V.	2.7	0.5	0.5	0.4	0.9	0.7	0.4	0.4
Day 2								
Mean	0.424	2.056	13.379	2.655	0.235	0.282	2.319	2.739
SD	0.01	0.02	0.17	0.02	0.002	0.001	0.03	0.01
%C.V.	2	1	1	1	1	0.4	1	0.4
Day 3								
Mean	0.432	2.036	12.408	2.449	0.224	0.296	2.108	2.678
SD	0.01	0.01	0.12	0.04	0.006	0.006	0.02	0.01
%C.V.	2	0.5	1	2	3	2	1	0.4
Inter-Day								
Mean	0.409	2.043	13.010	2.581	0.226	0.288	2.233	2.705
SD	0.03	0.02	0.45	0.10	0.01	0.01	0.10	0.04
%C.V.	7	1	3	4	4	2	5	1

allowed quantitation of the native and ara-NTPs. The areas under the curves for CTP, UTP, ATP, F-ara-ATP, GTP, and ara-GTP were equivalent to cellular concentrations of 106, 780, 4200, 43, 500, and 690 μM, respectively. The quantitation of the native nucleotides varied less than 10% in the three runs. It is important to note that in Fig. 4A and B, there was no UV-absorbing peak that might confound quantitation of the ara-NTPs. It is also interesting that in this patient, there was also baseline resolution of dATP and dGTP at 36 and 46 min, respectively (Fig. 4A–C). The deoxynucleotides were not quantitated, however, dATP and dGTP were consistently present in low amounts in other leukemia cell extracts (data not shown). The peak visible at 55 min in Figs. 1, 3A and B, and 4A–C represents the buffer equilibration front.

In conclusion, our data demonstrated the ability of this HPLC elution scheme to simultaneously separate the major ribonucleoside 5′-triphosphates, from the active drug metabolites, ara-GTP and F-ara-ATP, in both simple aqueous solutions and in a more complex patient sample-derived matrix. The triphosphates of fludarabine and ara-G are the cytotoxic metabolites, hence the present HPLC method is suitable for clinical application. A separate method, however, is available for the simultaneous detection of the mono-, di-, and tri-phosphates of arabinosyl analogs [15]. The present chromatographic technique for the detection and quantitation of ara-GTP and F-ara-ATP is directly applicable to the biochemical pharmacology studies in the cells from patients treated with nelarabine alone, or in combination with fludarabine. As we have demonstrated, the response to therapy is strongly correlated with accumulation of ara-GTP in leukemia cells during therapy [1–3]. Therefore, this separation method may be useful for establishing the mechanistic rationales for drug scheduling and combinations of nelarabine and fludarabine. Furthermore, this assay should be helpful for evaluating the therapeutic efficacies of these schedules and combinations.

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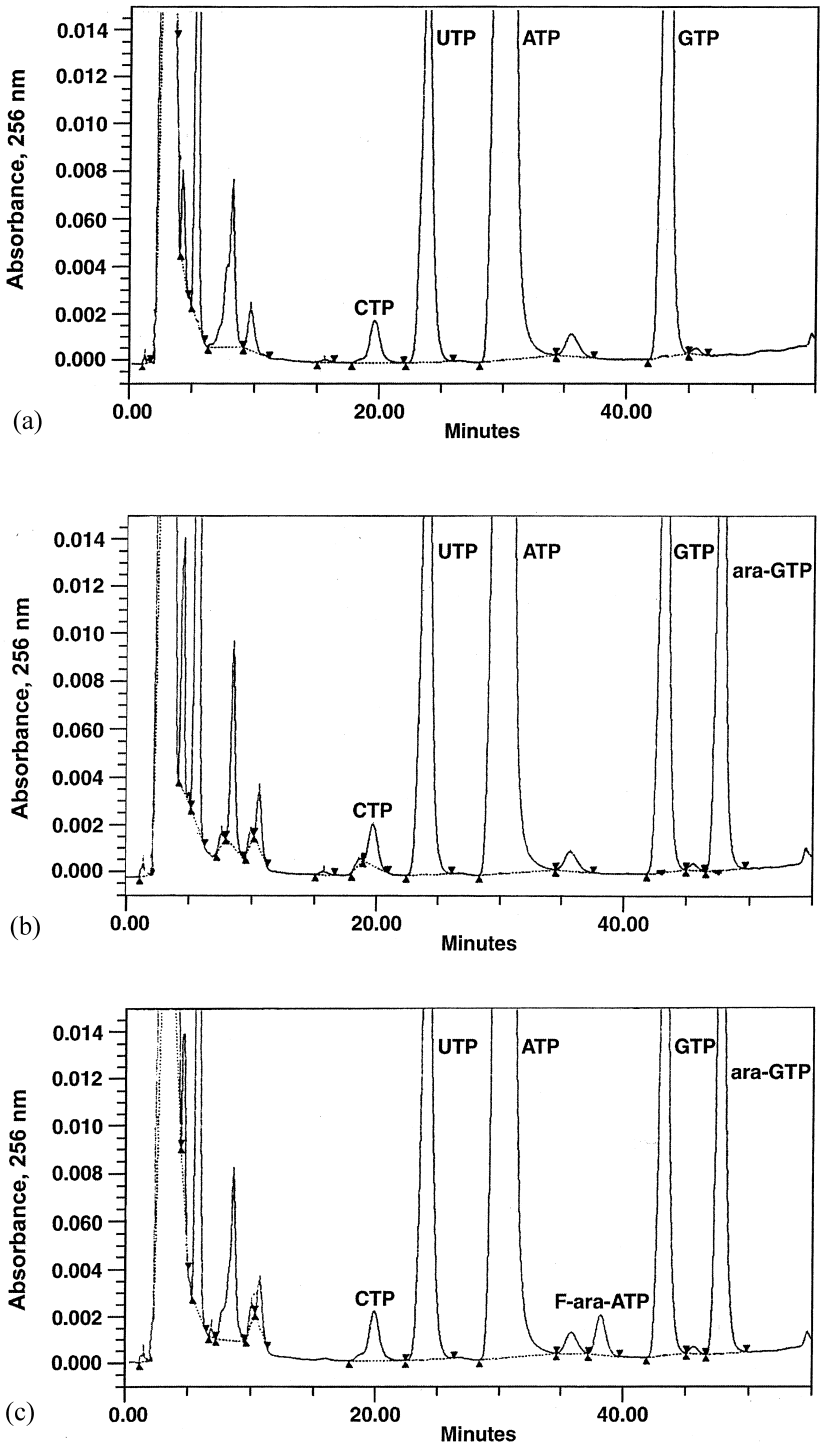

Fig. 4. Representative chromatogram of cellular extract from a patient with B-cell chronic lymphocytic leukemia. (A) before the start of therapy, (B) at the end of a 2-h infusion of 1.2 g/m^2 of nelarabine, and (C) at the end of infusion of a 30-min infusion of 30 mg/m^2 of fludarabine followed 4 h later with a 2-h infusion of 1.2 g/m^2 of nelarabine. Each sample was prepared and the nucleotides were quantitated as described in the Experimental section.

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