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Simple and sensitive method for quantification of fludarabine triphosphate intracellular concentration in leukemic cells using isocratic liquid chromatography

Takahiro Yamauchi*, Takanori Ueda

First Department of Internal Medicine, Fukui Medical University, 23 Shimoaizuki, Matsuoka, Fukui 910-1193, Japan

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Abstract

A simple, isocratic HPLC method was newly developed for quantitating intracellular fludarabine triphosphate (F-ara-ATP). Samples (500 μl) were injected onto an anion-exchange column and eluted isocratically with phosphate-acetonitrile buffer (flow rate: 0.7 ml/min) at an ambient temperature. F-ara-ATP was quantitated according to its peak area at the absorbance of 261 nm. The standard curve was linear with minimal within-day and inter-day variability. The low and high quantification limits were 50 pmol and 20 nmol, respectively. The method was capable of measuring F-ara-ATP generated in cultured leukemic cells in vitro. Thus, our method will be useful because of its sensitivity and simplicity as well as applicability to biological materials.
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Keyword: Fludarabine phosphate

1. Introduction

Fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine-5′-monophosphate, F-ara-AMP) is a monophosphate form of a deaminase-resistant fluorinated analogue of arabinosyladenine [1]. The drug is currently used in the treatment of indolent B-cell malignancies such as follicular lymphoma and chronic lymphocytic leukemia [2]. It is effective both as an initial therapy and for diseases that have become refractory to other drugs [2–4]. Fludarabine is also promising in combination with cytarabine for treating acute myelogenous leukemia [5].

Upon administration, fludarabine is rapidly dephosphorylated to its active compound, 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A), in the plasma. After being taken up into leukemic cells, F-ara-A is again phosphorylated to fludarabine triphosphate (9-β-D-arabinofuranosyl-2-fluoroadenine-5′-triphosphate, F-ara-ATP), the intracellular active metabolite of fludarabine [1]. F-ara-ATP competes with dATP as a substrate for DNA polymerases and is incorporated into DNA [1,6,7]. The incorporation of the drug into DNA results in the inhibition of DNA synthesis, which is strongly associated with fludarabine-mediated cytotoxicity [1,6,7]. Since F-ara-A incorporation into DNA is also proportional to the intracellular F-ara-ATP concentration [1], F-ara-ATP is therefore the crucial metabolite of fludarabine as the mechanism of action. F-ara-ATP is also important for modulation of cytarabine therapy and inhibition of DNA repair [1,6–9].

In clinic, no correlation has been found between the therapeutic outcome and F-ara-ATP in treating chronic lymphocytic leukemia [10]. However, as demonstrated in the case of cytarabine, the parameter for predicting the clinical efficacy of a nucleoside analogue is not the plasma drug concentration but its triphosphate form in leukemic cells [11,12]. Therefore, taken together with the understanding of the mechanism of action, the pharmacokinetic evaluation of F-ara-ATP may provide crucial information on scheduling and dosing fludarabine.

The most widely used assay method was a gradient elution ion-exchange HPLC developed by Gandhi et al. [10,13,14]. This was sensitive and applicable to measuring F-ara-ATP in leukemic cells from patients receiving fludarabine. In general, however, a gradient mode requires a complicated

* Corresponding author. Tel.: +81-776-61-3111;
fax: +81-776-61-8109.
E-mail address: tyamauch@fmsrsa.fukui-med.ac.jp (T. Yamauchi).

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computer-based system to control different kinds of elution buffers, which might limit the use of the method only in hospitals highly equipped with laboratory sections. Moreover, a gradient elution sometimes shows a base-line drift, thereby leading to unexpected errors in measuring peak heights or areas of the target compound in crude, biological materials.

In the present study, we developed an isocratic HPLC method for measuring F-ara-ATP in leukemic cells. In contrast to the previous method, the isocratic mode is simple and inexpensive as it can be run using a single, mechanical pump. We have also demonstrated that our method was accurate, sensitive, and applicable to measuring biological samples.

2. Experimental

2.1. Chemicals

Na_2HPO_4, acetonitrile (HPLC grade), phosphoric acid (85%), and perchloric acid (60%) were purchased from Nakalai (Kyoto, Japan). F-ara-A, ATP, CTP, UTP, GTP, and cytarabine were purchased from Sigma (St. Louis, MO, USA). F-ara-ATP (5 mg) was accurately measured and provided by Nihon Schering K.K. (Osaka, Japan). Water was home-purified by the combination of reversed osmosis and ion exchange on a multi-laboratory basis. All other chemicals were of analytical grade.

2.2. Preparation of standard F-ara-ATP in aqueous solution

An accurately measured 5 mg of F-ara-ATP was dissolved in water to make a stock solution (final concentration 10 mM). Serially diluted standards were prepared from this solution for drawing a standard curve. They were stored at –80 °C until analyzed.

2.3. HPLC apparatus and chromatographic conditions

The HPLC system consisted of a pump CCPM-II (TOSOH Corp., Tokyo, Japan), an autosampler AS-8020 (TOSOH Corp., Tokyo, Japan), an in-line degasser SD-8022 (TOSOH Corp., Tokyo, Japan), and a variable-wavelength detector UV-8020 (TOSOH Corp., Tokyo, Japan). The chromatography was controlled and analyzed using a personal computer equipped with the software LC-8020 (TOSOH Corp., Tokyo, Japan).

Samples were injected onto an anion-exchange column, TSK gel DEAE-2 SW (length × inside diameter 250 mm × 4.6 mm, particle size 5 µl, TOSOH Corp., Tokyo, Japan). The elution was performed isocratically with 0.06 M Na_2HPO_4 (pH 6.9)—20% acetonitrile at a constant flow rate of 0.7 ml/min and at ambient temperature. F-ara-ATP was monitored at 261 nm, the wavelength of the maximum UV absorption of the compound.

2.4. Standard curve and validation

To validate the method, aqueous solutions of standard F-ara-ATP at 10 different concentrations were made from the stock solution by serial dilution (Section 2.2). A 500-µl aliquot from each diluted standard was injected onto the HPLC to give a final amount of F-ara-ATP ranging between 20 pmol and 20 nmol (20, 50, 100, 200, and 500 pmol; 1, 2, 5, 10, and 20 nmol). Measurements of these standard solutions in triplicate were performed on three separate days to determine the within-day and the inter-day variation. Data were combined and plotted to determine the linearity between the amounts of F-ara-ATP and the corresponding peak areas. The standard curve was fitted by the weighed least-squares linear regression analysis method using the equation, $y = ax + b$, where a represented the slope and b represented the intercept of the standard curve at $x = 0$.

2.5. Determination of unknown samples

F-ara-ATP of unknown samples was identified by its retention time by comparison with that of the standard F-ara-ATP solution. The amount of F-ara-ATP was calculated by extrapolating the peak area count into the equation of the standard curve.

2.6. Cell culture and treatment

To validate applicability of the method to biological materials, the human leukemia HL 60 cells were cultured in RPMI1640 (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA) in 5% CO_2 humidified atmosphere at 37 °C. The cells in a logarithmic growth phase (1×10^6 cells/ml, 20 ml) were incubated with F-ara-A, an active compound of fludarabine, at various concentrations for the indicated time periods at 37 °C. The cells treated or untreated were then washed twice into fresh media. The cell pellet was collected by centrifugation (400 × g, 10 min, 4 °C) in a micro-test tube, and 200 µl of 0.3 M cold perchloric acid was added to the pellet. The mixture was vortexed for 10 s, and allowed to stand for 15 min at 4 °C. The acidic supernatant was isolated by centrifugation of the sample (15,600 × g, 20 s, 4 °C), and then mixed with 100 µl of 0.5N potassium hydroxide for neutralization. After another centrifugation (15,600 × g, 20 s, 4 °C), the neutralized supernatant was obtained as an acid soluble fraction (ASF), a nucleotide pool [12,15]. The sample loop for the HPLC had a 500-µl space, and the dead volume for the aspiration of the sample by the autosampler was 170 µl. By adding 500 and 170 µl and some room, the volume of each ASF sample was adjusted to 700 µl by the addition of water, and the 500-µl aliquot was applied to the chromatographic analysis. The intracellular concentration of F-ara-ATP was expressed as pmol/1×10^7 cells. The samples were stored at −80 °C when they were not used immediately.

2.7. Preparation of standard F-ara-ATP mixed with ASF

To further confirm the utility to biological samples, another set of F-ara-ATP standards was made using an ASF. Known amounts of standard F-ara-ATP were dissolved an ASF extracted from untreated HL 60 cells, and the sample volume was adjusted to 700 μl by the addition of water. The 500-μl aliquots were applied to the HPLC to give final amounts of F-ara-ATP that were equivalent to those made in water. Then, the variation was assessed in the measurement of F-ara-ATP in the ASF. The solutions were stored at −80 °C until analyzed.

2.8. Calculations and statistical analyses

The standard curve was obtained by the weighed least-squares linear regression analysis method using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). The values of coefficient of variation (CV) and standard deviation for F-ara-ATP measurements were obtained using Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA).

3. Results and discussion

3.1. Standard curve and validation

To determine the standard curve, 10 concentration levels of standard F-ara-ATP in aqueous solutions (Section 2.2) in triplicate were applied to the present HPLC scheme on three separate days. A linear relation was obtained between the amounts of F-ara-ATP (50–20,000 pmol) and the corresponding peak areas using all the data sets ($r^2 = 0.97$, $P < 0.0001$). The parameters for the curve were; intercept $= -0.09 \pm 0.05$; slope $= 0.59 \pm 0.01$. For validation, the within-day and the inter-day variability were determined at different amounts of F-ara-ATP (Table 1). The variations were minimal, as all the values of % coefficients of variation were less than 10%. The low and high limits of quantification were 50 and 20,000 pmol (20 nmol), respectively. The limit was defined as the lowest or the highest concentration that gave the % coefficient of variation <10%. The low limit was as sensitive as the previous HPLC method (25 pmol) [10,13,14]. Thus, these results suggested that the present HPLC assay was accurate and sensitive for quantitating F-ara-ATP.

Table 1
Within-day and inter-day variation for F-ara-ATP in aqueous solution

F-ara-ATP (pmol)	Variation (mean, S.D., % CV)	
	Within-day (day 1)	Inter-day
50	31.3	30.0
	2.3	1.0
	7.3	3.3
200	132.3	131.3
	5.1	6.5
	3.9	4.9
500	323.7	305.7
	9.5	6.4
	2.9	2.1
2000	1004.3	1071.7
	45.5	96.8
	4.5	9.0
5000	2861.0	2893.0
	74.4	169.5
	2.6	5.8
20000	12385.0	12572
	52.0	1065
	0.41	7.8

Various amounts of standard F-ara-ATP in aqueous solution in triplicate were determined on three separate days. The within-day variation was the value for day 1. The value of the inter-day variation was calculated from the mean value of the peak area count on each separate day.

3.2. Separation of F-ara-ATP in biological samples

To assess the separation of F-ara-ATP, the mixture of standard nucleotides (CTP, UTP, ATP, GTP, and F-ara-ATP) was injected onto the HPLC. The peak of F-ara-ATP was clearly isolated from the other nucleotides (Fig. 1A). The retention time of the F-ara-ATP peak was 90 min. The retention times of standard deoxyribonucleotides (dATP, dCTP, dTTP, dGTP) were similar to those of the corresponding ribonucleotides (data not shown).

To evaluate if the present HPLC condition was applicable to the measurement of F-ara-ATP in biological samples, nucleotide pools extracted from leukemic cells were tested. Fig. 1B represented a blank chromatogram of an ASF extracted from untreated human leukemia HL 60 cells. Endogenous nucleoside triphosphates (CTP, UTP, ATP, and GTP) were clearly separated in the same way (Fig. 1B), as had been demonstrated using standard markers (Fig. 1A). The chromatogram did not show any peaks between ATP and GTP, which might otherwise have interfered with the peak of F-ara-ATP. When the ASF was co-eluted with standard F-ara-ATP, the peak of F-ara-ATP was clearly isolated from the other endogenous peaks of the leukemic nucleotides (Fig. 1C), suggesting the applicability of the method to biological samples.

To further confirm the clinical utility, the present method was applied to the measurement of F-ara-ATP generated in leukemic cells in vitro. An ASF was extracted from HL 60 cells after treatment with F-ara-A, and then injected onto the HPLC. Even at the minimal concentration of the drug at the shortest incubation time period, the production of F-ara-ATP in leukemic cells was clearly demonstrated in the chromatogram (Fig. 1D). Thus, these results suggested that the appropriate F-ara-ATP separation was obtained in the biological materials under the present HPLC condition.

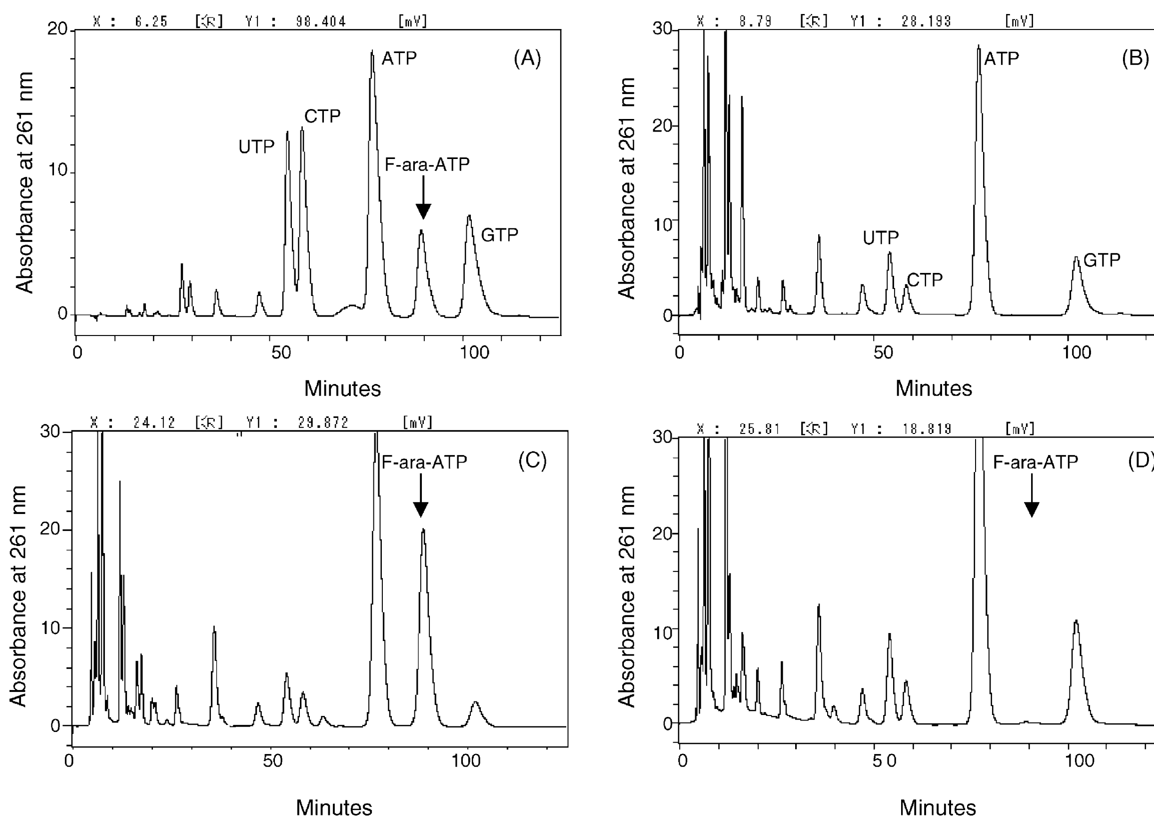

Fig. 1. HPLC profiles of nucleotides. (A) Standard nucleotides (ATP, CTP, UTP, GTP, and F-ara-ATP) dissolved in water were applied to the present HPLC scheme; (B) an acid soluble fraction was extracted from untreated human leukemic HL 60 cells (2×10^7 cells), and the volume of the extract was adjusted to 700 μl by the addition of water. A 500-μl aliquot was then applied to the HPLC; (C) the above acid soluble fraction was co-eluted with standard F-ara-ATP in the same HPLC run; (D) an acid soluble fraction was extracted from HL 60 cells after 1-h incubation with 1 μM F-ara-A (the minimal dose and incubation time period detectable). The volume of the extract was adjusted to 700 μl by the addition of water, from which a 500-μl aliquot was applied to the HPLC.

3.3. Validation of the F-ara-ATP measurement in biological samples

To validate the measurement in biological samples, the method was applied to measuring standard F-ara-ATP in an ASF. Various amounts of standard F-ara-ATP dissolved in the ASF of untreated HL 60 cells (Section 2.7) were injected onto the HPLC to give the final F-ara-ATP contents equivalent to those made in water. Measurements of these F-ara-ATP-ASF solutions in triplicate were performed on three separate days to see the variability. The within-day and inter-day variability for F-ara-ATP quantitation was minimal, which were indicated as the % coefficient of variation less than 10% (Table 2).

Table 2
Within-day and inter-day variation for F-ara-ATP in acid soluble fraction

F-ara-ATP (pmol)	%CV	
	Within-day	Inter-day
200	9.0	9.8
500	6.5	6.0
2000	4.3	5.5
5000	3.2	5.1

Various amounts of F-ara-ATP were mixed with an acid soluble fraction extracted from untreated HL 60 cells. These samples in triplicate were quantitated on three separate days. The within-day variation was the value for day 1. The value of the inter-day variation was calculated from the mean value of the peak area count on each separate day.

The corresponding peak areas were compared between the F-ara-ATP-ASF solution and the F-ara-ATP-water solution. Various amounts of standard F-ara-ATP in the ASF or in aqueous solution were applied to the present HPLC method. Upon injection, both solutions gave final F-ara-ATP contents of 50, and 200 pmol; 1, and 5 nmol. The peak area counts were similar to each other in both solutions, and a close correlation was found ($r^2 = 0.99$, $P < 0.0001$, slope $= 0.95 \pm 0.006$, y-intercept $= 0$, x-intercept $= 0$). The result suggested that F-ara-ATP was quite stable in the ASF and that the measurement of F-ara ATP was not disturbed by the endogenous peaks arising from the ASF. Therefore, the present HPLC method would be applicable to the quantification of F-ara-ATP in biological samples.

3.4. F-ara-ATP generation in leukemic cells in vitro

Using the present HPLC method, the production of F-ara-ATP in leukemic cells was quantitated. When HL 60 cells were incubated with different concentrations for

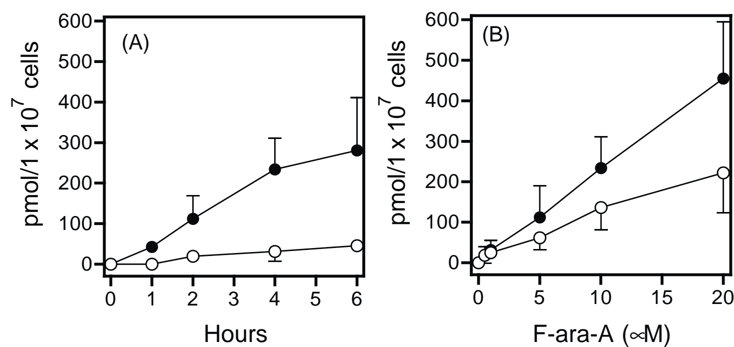

Fig. 2. Dose- and time-dependent increase in F-ara-ATP production in leukemic cells. Human leukemia HL 60 cells (1×10^6 cells/ml, 20 ml) were incubated with 1 μM (○) or 10 μM (●) F-ara-A for indicated time periods (0, 1, 2, 4, 6 h) (A). Alternatively, the cells were incubated for 2 h (○) or 4 h (●) with the indicated concentrations (0, 0.5, 1, 5, 10, 20 μM) of F-ara-A (B). After washing the cells into fresh media, acid soluble fractions were extracted from the samples, and the volume of the extracts was adjusted to 700 μl by the addition of water. A 500-μl aliquot from each sample was then applied to the HPLC. The amount of F-ara-ATP was calculated by extrapolating the peak area count into the equation of the standard curve.

the indicated time periods, the production of intracellular F-ara-ATP became greater in time- and concentration-dependent manners (Fig. 2A and B). The F-ara-ATP concentrations in HL 60 cells measured here might be within the range of the expected F-ara-ATP concentrations (approximately 6–52 μM, which corresponds to 60–520 pmol/1 $\times$ 10^7 cells) in primary leukemic cells from patients receiving fludarabine (25–30 mg/m^2 per day) [10,16]. Thus, the present method was capable of measuring F-ara-ATP generated in the leukemic cells in vitro, which strongly suggested its clinical applicability.

3.5. Separation of cytarabine triphosphate from F-ara-ATP

One of the strategies to overcome the resistance of leukemic cells is the combination use of fludarabine with cytarabine. When HL 60 cells were treated with F-ara-A

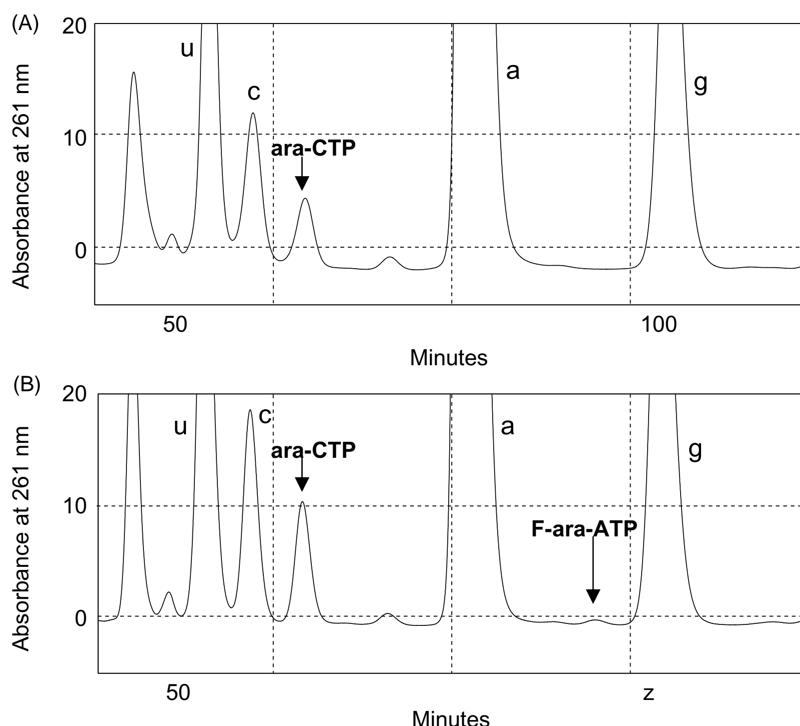

Fig. 3. Separation of cytarabine triphosphate from F-ara-ATP. HL 60 cells (1×10^6 cells/ml, 20 ml) were incubated with (B) or without (A) 10 μM F-ara-A for 2 h, followed by washing, resuspension in fresh media, and a subsequent incubation with 10 μM cytarabine for 3 h. After washing the cells into fresh media, acid soluble fractions were extracted from the samples, and the volume of the extracts was adjusted to 700 μl by the addition of water. A 500-μl aliquot from each sample was then applied to the HPLC. (a) ATP, (g) GTP, (c) CTP, and (u) UTP, and (ara-CTP) cytarabine triphosphate.

combined with ara-C, the present HPLC analysis was capable of separating cytarabine triphosphate (retention time 64 min) from F-ara-ATP in the nucleotide pool (Fig. 3). The results suggested the possibility of simultaneous measurement of the corresponding triphosphates of these two compounds in the same run.

4. Conclusions

Fludarabine is currently effective not only for indolent hematological malignancies but also for acute leukemia. The pharmacokinetic evaluation of F-ara-ATP may provide crucial information on scheduling and dosing fludarabine [17]. The quantitation method should be simple with the use of less expensive apparatus so that clinicians might utilize the pharmacokinetic data for treating patients on a daily basis in hospitals. The whole procedure of the present method was similar to that of the previous methods, since this approach might be the best for monitoring phosphate metabolites of nucleoside analogues in leukemic cells. In addition, the isocratic HPLC method described here can be controlled not only using a computerized system but also using a conventional, mechanical single pump. Moreover, an isocratic elution may allow an accurate measurement of small amounts of F-ara-ATP in crude biological extracts because it does not induce a base-line drift. Thus, the present method will be of great advantage because of its sensitivity and simplicity as well as its clinical applicability.

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