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A highly sensitive high-performance liquid chromatography–mass spectrometry method for quantification of fludarabine triphosphate in leukemic cells

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Abstract

A high-performance liquid chromatography (HPLC)–mass spectrometry (MS) method has been developed for the analysis of 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-triphosphate (F-ara-ATP) from biological samples. Quantification is carried out by selected ion monitoring of the parent ion. Baseline separation of the monophosphate (F-ara-AMP) and diphosphate (F-ara-ADP) is achieved using the volatile ion-pairing reagent dimethylhexylamine. This method is selective and sensitive with an on-column detection limit of ∼50 fmol. It also permits simultaneous monitoring of endogenous adenosine phosphates. The utility of the assay has been demonstrated by the analysis of F-ara-ATP in human leukemic cells after incubation with 9-β-D-arabinosyl-2-fluoroadenine (F-ara-A) at clinically relevant concentrations.

Keywords: LC–MS; Fludarabine triphosphate; Leukemia

1. Introduction

9-β-D-Arabinofuranosyl-2-fluouroadenine[1] (F-ara-A) was first synthesized as an anticancer agent in the late 1960s [1–3]. It belongs to a class of anticancer nucleoside analogues in which the ribose moiety is replaced with arabinose and/or the nucleobase moiety (i.e. the purine or the pyrimidine) is halogenated. Other examples of such compounds include 2-chloro-adenosine, 2′-deoxycoformycin (pentostatin), 2-chloro-2′-deoxyadenosine (cladribine), 1-β-D-arabinosylcytosine (cytarabine, AraC), 1-β-D-arabinosyl-2-chlorocytosine and others. The cytotoxic effects of these compounds depend on intracellular conversion into their active triphosphate forms that interfere with DNA/RNA syntheses and repair [2,4].

F-ara-A is currently used in the treatment of indolent B-cell malignances such as chronic lymphocytic leukemia and follicular lymphoma [3]. It also exerts potent immunosuppressive activity by inhibiting cell proliferation and promoting apoptosis of lymphocytes [5]. Recently, F-ara-A has been introduced as a major immunosuppressive agent in reduced intensity myeloablative and non-myeloablative preparative regimens for hematopoietic stem cell transplantation (HSCT) [6,7]. Because of its low solubility, the drug is formulated as the 5′-monophosphate (F-ara-AMP) for administration and is rapidly dephosphorylated to F-ara-A in the circulating blood. F-ara-A is transported into cells mainly through an equilibrative nucleoside transporter [8]. Once inside the cells, it is successively phosphorylated to the mono-, di-, and tri-

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[1] "Fludarabine" has been used to describe both the non-phosphorylated and mono-phosphorylated nucleoside To minimize this nomenclature confusion, we choose to use the chemical names 9-β-D-arabinofuranosyl-2-fluouroadenine and F-ara-A monophosphate (F-ara-AMP) over the term "fludarabine".

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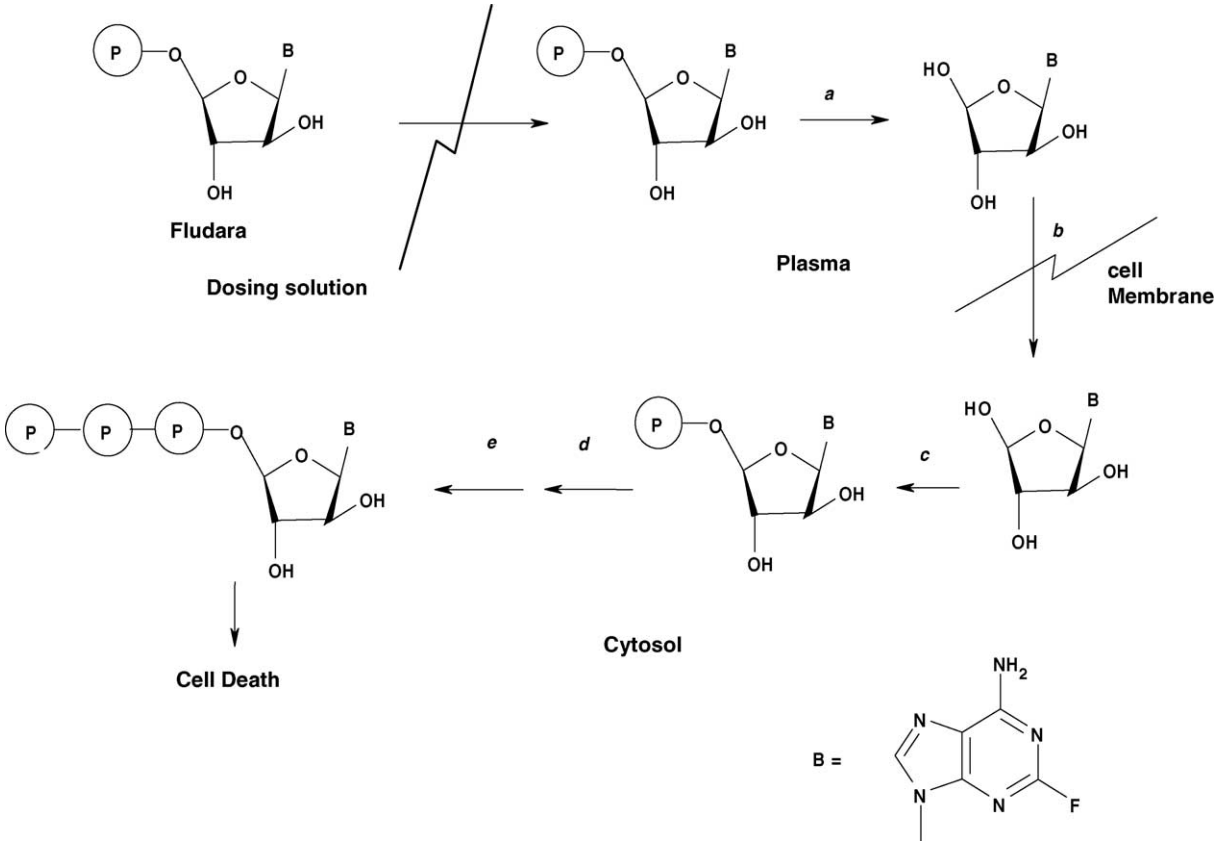

Fig. 1. The metabolic transformations of fludarabine from administration to cellular formation of F-ara-ATP. Enzymatic processes: (a) plasma 5′ nucleotidase; (b) equilibrative nucleoside transporter; (c) deoxycytidine kinase; (d) adenylate kinase and (e) nucleoside diphosphate kinase.

phosphates (F-ara-AMP, F-ara-ADP, and F-ara-ATP, respectively) by deoxycytidine kinase (dCK), adenylate kinase and nucleoside diphosphate kinase, respectively [3]. In addition, F-ara-AMP can be dephosphorylated to F-ara-A by intracellular 5′-nucleotidase. The cellular transport and activation of F-ara-A is summarized in Fig. 1.

F-ara-ATP is the only intracellular metabolite known to have pharmacological activity [2,3]. The final intracellular concentration of F-ara-ATP is determined by its cellular uptake and subsequent intracellular phosphorylation and dephosphorylation processes. Increasing evidence suggests that the parameter for predicting the clinical efficacy of a nucleoside analogue is not the plasma concentrations of the drug but its triphosphate metabolite in target cells [2,3]. Given the complexity of F-ara-ATP intracellular disposition pathway, quantification of intracellular F-ara-ATP in clinical samples (e.g. leukemic cells or lymphocytes) is critical for understanding its pharmacokinetic and pharmacodynamic relationships, which may provide useful information for dose adjustment and scheduling to improve clinical outcome of fludarabine treatment.

Historically, the development of analytical methods for F-ara-ATP has paralleled the procedures developed for natural nucleoside triphosphates. These methods depend on high-performance liquid chromatography (HPLC) using ion exchange [9–12] or ion pairing [13] chromatography with radiochemical or UV detection at 254 or 260 nm. Many of the synthetic nucleosides of interest are structural analogues of naturally occurring compounds and have similar UV characteristics. In addition, they exist in mono-, di-, and tri-phosphorylated forms. Thus, unequivocal quantification requires delicate, reproducible chromatographic separation and corresponding long run times. Furthermore, UV detection is relatively insensitive and the lower limit of detection generally ranges from 25 to 100 pmol on column. This often requires a large sample size, limiting its usefulness for quantifying clinical samples as the cell numbers that can be obtained from a single patient is restricted.

Mass spectrometry (MS)-based detection methods offer greater sensitivity. However, the chromatographic conditions used in the methods described above involve the use of non-volatile components in the mobile phase, which are not compatible with the use of MS methods. Recently, separations of phosphorylated drug metabolites have been developed which utilize protonated trialkyl amines as ion pairing reagents [14–16]. These volatile components are compatible with mass spectrometers, and thus, can provide increased selectivity and sensitivity. Such methods have been used successfully in the analysis of adenosine phosphates [14], phosphorylated metabolites of coronate and etiodronate, drugs used in the treatment of bone disorders [15], and carbovir

(Ziagen) used in the treatment of human immunodeficiency virus [16].

In this study, we describe a method for the analysis of F-ara-ATP in which separation is achieved using a reverse phase column with a volatile tertiary amine, dimethyhexylamine (DMHA), as an ion-pairing reagent. Detection is achieved using atmospheric pressure ionization–mass spectrometry in the negative electrospray mode (API–ES$^-$). In contrast to previous methods, the liquid chromatography–mass spectrometry (LC–MS) method provides much greater selectivity and sensitivity for quantification of intracellular F-ara-ATP with an on-column detection limit of ∼50 fmol. In addition, it also permits simultaneous monitoring of other F-ara-A metabolites and endogenous adenosine phosphates. The application of this method in analysis of F-ara-ATP was demonstrated in human Jurkat leukemia cells.

2. Experimental

2.1. Chemicals

MeOH (Optima), perchloric acid, potassium bicarbonate, acetic acid and ammonium hydroxide were from Fisher (Atlanta, GA). Adenosine mono-, di-, and tri-phosphates, 2′-chloroadenosine triphosphate (Cl-ATP), and DMHA were from Sigma (St. Louis, MO). The sodium salt of F-ara-AMP was the kind gift of the National Cancer Institute. All other chemicals were of reagent grade or better.

2.2. Synthesis of F-ara-ATP

F-ara-ATP was prepared from F-ara-AMP according to the method of Novotny [17]. Briefly, the sodium salt of F-ara-AMP was converted first to the pyridinium salt and then to the tributyl ammonium salt using cation exchange gel. After drying thoroughly, the active intermediate was formed using 1,1-carbonyldiimidazole in dimethylformamide (DMF) and F-ara-ATP formed upon the addition of tributylammonium pyrophosphate. The product was isolated using semi-preparative HPLC equipped with a Develosil RP-Aqueous C-30 column and an aqueous mobile phase (10 mM formic acid). The resulting product showed no impurities by LC–UV. The concentration of the F-ara-ATP was calculated using the F-ara-AMP as a standard assuming the same UV extinction coefficient for both compounds.

2.3. Preparation of standard solutions

To prepare F-ara-ATP standard solutions, a stock solution of F-ara-ATP (80 μM) was diluted 1:40 with cold 1.0 M perchloric acid solution. Serial dilutions were prepared from this solution ranging in concentration from 0.031 to 2.0 μM. Aliquots (40 μL) were transferred to 0.5 mL microfuge tubes and stored at −80 °C. The stock solution of Cl-ATP was prepared by the addition of 1.0 mg tetrasodium Cl-ATP to 10.5 mL water forming a 150 μM solution. One hundred microlitres of aliquots were stored at −80 °C until use.

2.4. HPLC

HPLC separation was performed on a HP-1100 liquid chromatography system equipped with a thermostated autosampler (Agilent Technologies, Palo Alto, CA, USA). Separation was achieved using a Prodigy 5u C-8 100 mm × 2.0 mm column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.025% (v/v) DMHA, 20 mM acetic acid adjusted to pH 7.5 with ammonium hydroxide/MeOH. A gradient system was used starting at 5.0% MeOH for 0.5 min. The MeOH was increased to 40% at 4.0 min and then to 70% at 6.0 min. After returning to initial conditions at 7.0 min, the column was re-equilibrated for 5.0 min prior to the next injection. The injector was maintained at 4 °C and injection volumes were 10 μL. The solvent flow was maintained at 0.3 mL/min throughout and the column temperature was not controlled.

2.5. Mass spectrometry

All mass spectral experiments were performed on an Agilent MSD single quadrapole instrument (Agilent Technologies, Palo Alto, CA) using API–ES in negative ion mode. The gas temperature (nitrogen) was maintained at 350 °C at a flow of 12 l/min. The nebulizing pressure was 35 psi g; the Capillary voltage was 4500 V and the fragmentor 110 V. Initial scans were run from 140 to 1000 on chromatographically resolved standards. For quantitative purposes, the MS was run in the SIM mode. Ions monitored were *m*/*z* 524, 444, and 364 for the $(M-H)^-$ ions of F-ara-A tri-, di-, and mono-phosphates; *m*/*z* 506, 426, and 346 for $(M-H)^-$ of ions ATP, ADP and AMP; and *m*/*z* 540 for the $(M-H)^-$ ion of Cl-ATP. The MS conditions were as described above: the fragmentor and capillary voltage were optimized under analytical conditions with Chemstations FIA software. During analyses, eluent before 4.0 and after 7.5 min was diverted to waste. Typical retention times are AMP – 4.3 min, ADP – 5.8 min, ATP – 6.2 min, F-ara-AMP – 5.8 min, F-ara-ADP – 6.3 min, and F-ara-ATP – 6.5 min. The internal standard, Cl-ATP, elutes at 6.7 min. It should be noted that we had no authentic sample of F-ara-ADP. The identity was assigned based on the *m*/*z* value (444) of the formed ion, the chromatographic characteristics as it eluted between F-ara-AMP and F-ara-ATP, and was only observed in cells which had been incubated with F-ara-A.

2.6. Test for linearity and limits of detection

The absolute limit of detection was determined using diminishing concentrations of F-ara-ATP solutions diluted with mobile phase from 200 to 10 nM. Ten microlitres of each solution was repeatedly injected and the limit of detection was set as the concentration having an average integrated peak

area with a CV less than 20% over five injections. Test standard curves were run to 10 μM F-ara-ATP to confirm linearity within the range of these concentrations. It was possible that the large concentrations of ATP observed in cellular extracts affected the response of F-ara-ATP. Samples of F-ara-ATP containing 0 or 150 uM ATP were analysed by standard techniques to test for ion suppression. Standard curves were routinely run from 0.062 to 1.0 μM. Linearity considerations were based upon R^2 values of unweighted data.

2.7. Cell culture and sample preparation

Jurkat cells were obtained from American Type Cell Collection (ATCC) and cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine in 5% CO_2 humidified atmosphere at 37 °C. Cells in logarithmic growth phase were incubated with F-ara-A at concentrations ranging from 2.5 to 20 μM for 5 h at 37 °C. Unless otherwise noted, 2×10^6 cells were used per incubation. At the end of the incubation period, the cells were transferred to a microfuge tube and centrifuged at 4000 rpm for 5 min at 4 °C. The cell pellets were then washed once with 1.0 mL cold PBS. The pelleted cells were then lysed and protein precipitated by the addition of 40 μL cold 1.0 M $HClO_4$. The samples were vortexed and stored at −80 °C until analysis.

Immediately prior to analysis, the samples and a set of standards were thawed on ice, vortexed briefly, and 4.0 μL of 150 μM IS added. The samples were centrifuged at 20000 × *g* for 5 min at 4 °C and an aliquot (40 μL) of the supernatant transferred to a 0.5 mL microfuge tube. The samples were then neutralized by the addition of 40 μL 1.0 M $KHCO_3$, mixed and allowed to sit on ice for 15 min to complete precipitation of $KClO_4$. The resulting suspension (pH 7.0–8.0) was centrifuged for 5 min at 4 °C, 50 μL of the supernatant transferred to an injection vial and 10 uL applied to the column for analysis. Chromatograms were processed using ChemStations software; quantitative analysis was based on peak area ratio data.

2.8. Variability, stability and specificity of biological samples

2.8.1. Variability

Intraday variability was evaluated by: (1) running and comparing preparations of multiple standard curves on one day and (2) running and comparing replicates of cell incubates at several F-ara-A concentrations. Control samples were prepared from large pools (10^8) of cells incubated with 20 or 2.5 μM of F-ara-A. After all of the samples were processed, 40 μL aliquots of the acidic, protein-free supernatant were stored at −80 °C. Two samples of each incubation concentration were run with each sample set. The F-ara-ATP concentrations were determined empirically and variations and trend analysis were performed over time.

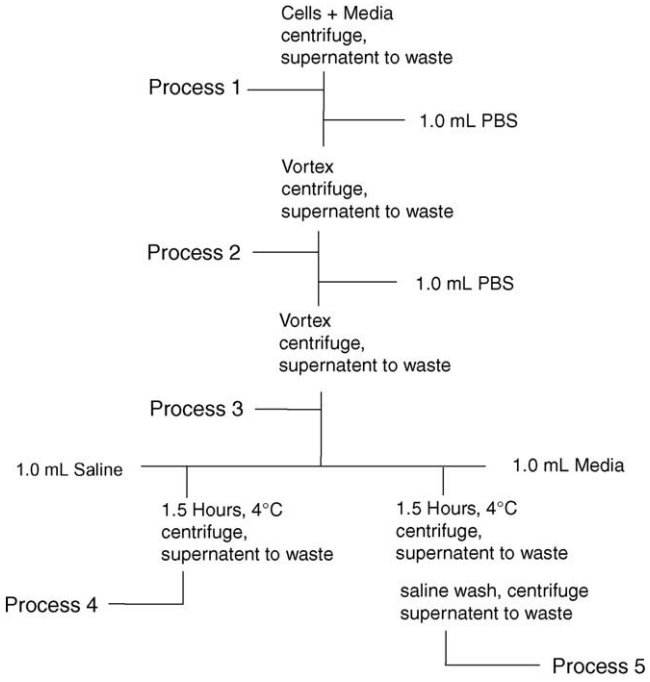

Fig. 2. Different sample preparation procedures to test intracellular F-ara-ATP stability prior analysis.

2.8.2. F-ara-ATP stability during sample preparation

To test the stability of intracellular F-ara-ATP during sample preparation, samples were prepared with different washes and delay times according to the scheme shown in Fig. 2. After F-ara-A treatment, cells in Process 1 were harvested directly from the media by centrifugation without washing and the cells lysed and protein precipitated by the addition of PCA. This basic procedure was considered to represent 100% recovery to which modifications including an increased number of washes or prolonged exposure to PBS or media were compared. In all cases, the final cell preparations were treated with PCA and stored at −80 °C until analysis.

2.8.3. Specificity

The specificity of the assay was determined by comparing cells incubated with or without F-ara-A. Other nucleoside triphosphates were injected to ensure there was no interference from endogenous compounds.

3. Results and discussion

3.1. Mass spectrometry

Mass spectra of ATP, F-ara-ATP, and Cl-ATP are shown in Fig. 3. The compounds showed a base signal consistent with $(M-H)^-$ at *m/z* 506 for ATP, 524 for F-ara-ATP, and 540 and 542 for the ^{35}Cl-ATP and ^{37}Cl-ATP respectively. In addition, at higher ionization (fragmentor) voltages, some breakdown of F-ara-ATP was observed. Signals at *m/z* 444 and 364

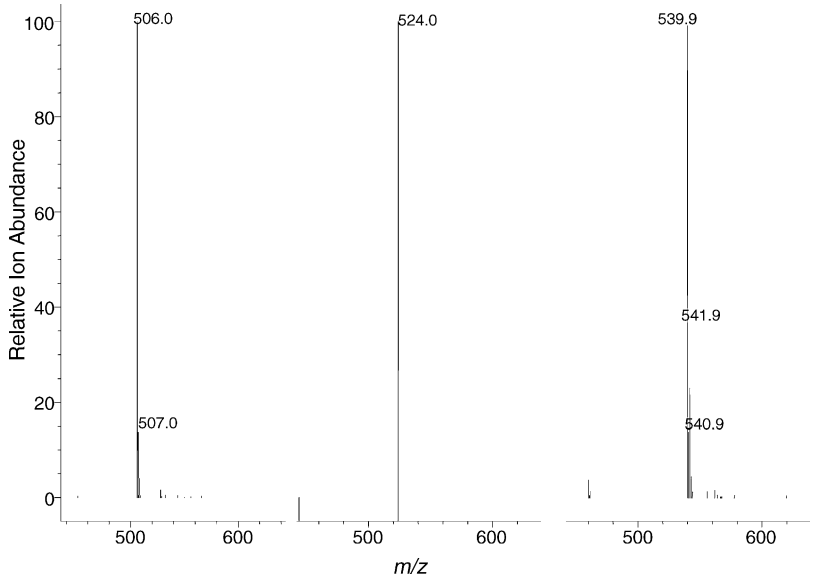

Fig. 3. Negative ion electrospray scans of chromatographically separated adenosine and modified adenosine phosphate standards. From left to right: ATP, F-ara-ATP, and Cl-ATP all show the signal corresponding to $(M-H)^-$ ion.

are consistent with $(M-H\text{-}PO_3)^-$ and $(M-H\text{-}P_2O_6)^-$ fragmentation, representing F-ara-ADP and F-ara-AMP formed during ionization. This pattern is analogous to that of ATP where observed signals at *m/z* 426 and 346 are consistent with the loss of one and two PO_3 groups from the molecular anion forming ADP and AMP, respectively. In these scans of chromatographed samples, there is no sign of signals for the $(M+Na\text{-}2H)^-$ species which do appear when the sample is introduced via direct injection.

3.2. Chromatography

The chromatography developed for F-ara-mono-, di-, and tri-phosphates ions corresponding signals from a cell extract after 5 h incubation with or without 2.5 μM F-ara-A is shown in Fig. 4. The analytes, F-ara-ATP, F-ara-ADP, and F-ara-AMP, were observed in cell samples incubated with F-ara-A. The baseline separation of the F-ara-A phosphates allows quantification of all the intermediates of interest. The assay also provides separation of ATP, ADP, and AMP. More rapid separations are possible if F-ara-AMP and F-ara-ADP levels are not of interest. However, complete separation of the F-ara-A phosphates is necessary to distinguish biologically formed analytes from signals resulting from in-cone fragmentation. The response of F-ara-ADP observed resulting from such fragmentation is routinely 9% of the F-ara-ATP peak area; that of F-ara-AMP is less than 1%. Although recent work [18] has suggested that residual PCA in samples can interfere with the separation of nucleotide phosphates, we have circumvented the problem by neutralizing the sample with potassium bicarbonate. The resulting sample has low ion strength at pH ∼7.5, ideal for subsequent chromatography.

3.3. Linearity and reproducibility

The standard curves routinely run ranging from 1.0 to 0.065 μM show R^2 values consistently >0.99, suggesting excellent linearity. The data in Table 1 summarize the precision and accuracy of the F-ara-ATP assay. The variation in all cases was less than 15% and found F-ara-ATP content within 5% of the nominal concentrations. The reproducibility of the procedure in a biological study which included incubation and sample work up (washing and deproteinization) is demonstrated in Fig. 5, which illustrates a linear relationship between F-ara-ATP formation and the F-ara-A concentration in the incubation media. Incubations were run with F-ara-A concentrations ranging from 1.25 to 20 μM with an *N* of four for each condition. The formation rates ranged from 7.5 to 125 pmol/10^6 cells/5 h. The coefficient of variation was less than 15% in all cases, demonstrating good precision over a wide range of incubation conditions.

3.4. Limits of detection

Limits of detection were calculated empirically using serial dilutions injected. The standard deviations at each concentration were calculated. Based on a coefficient of variation

Table 1
Precision and accuracy of F-ara-ATP assay

Nominal [F-ara-ATP](μM)	*N*	Found [F-ara-ATP]	Standard deviation	CV %
1	5	1.00	0.03	2.9
0.5	5	0.498	0.015	3.1
0.25	5	0.254	0.029	11.6
0.125	5	0.126	0.015	12.7
0.062	5	0.0603	0.0077	12.7

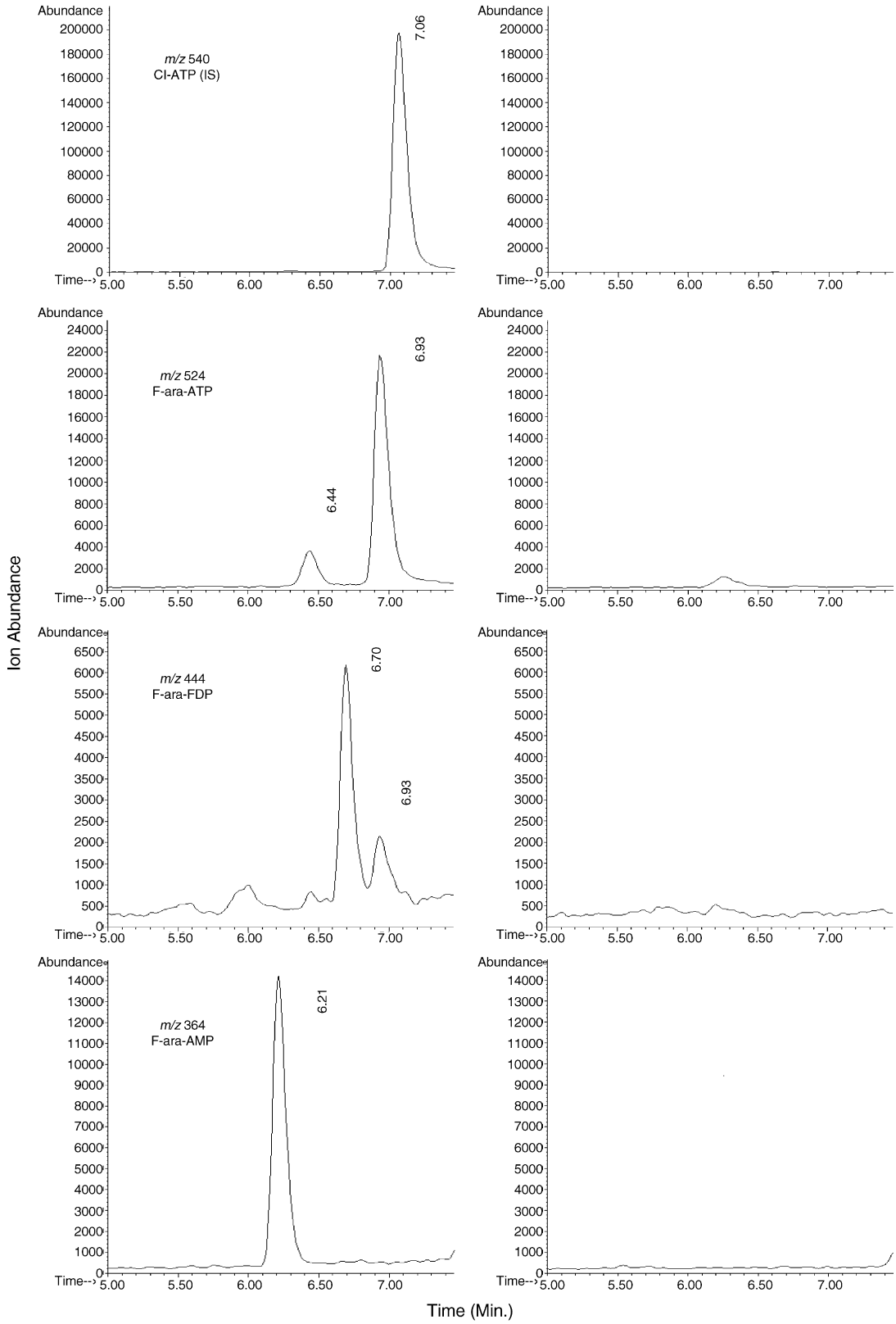

Fig. 4. Chromatograms from human Jurkat leukeamic cells incubated with (left panels) or without (right panels) 5 μM F-ara-A. The analytes, Cl-ATP (I.S., 15 uM) F-ara-ATP (0.7 μM), F-ara-ADP (concentration unknown), and F-ara-AMP (0.62 μM) correspond SIM chromatograms *m/z* 540, 524, 444, and 364, respectively.

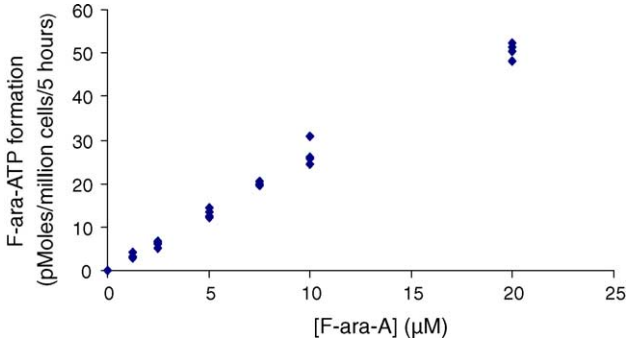

Fig. 5. Effect of varying F-ara-A concentration on F-ara-ATP formation in human Jurkat leukemic cells.

of 20%, the limit of quantification was found to be 50 fmol on column. Assuming a 100% recovery, this would be equivalent to samples of an original concentration of 30 nM. We routinely run standard curves to 62 nM to avoid any problems that might arise from fluctuations in instrument response. A typical chromatogram of F-ara-ATP at 70 nM from cell extract is shown in Fig. 6 superimposed on appropriate blank. The peak at 6.2 min is commonly observed in cell extracts but does not interfere with F-ara-ATP quantification. The response was linear up to 10 μM concentrations of F-ara-ATP.

Potential ion suppression due to cellular constituents in the sample was investigated in the presence of ATP at physiologically relevant concentration. There was no loss of sensitivity of F-ara-ATP in the presence of 150 μM ATP, a concentration that we normally see in Jurkat cell lysates (data not shown),

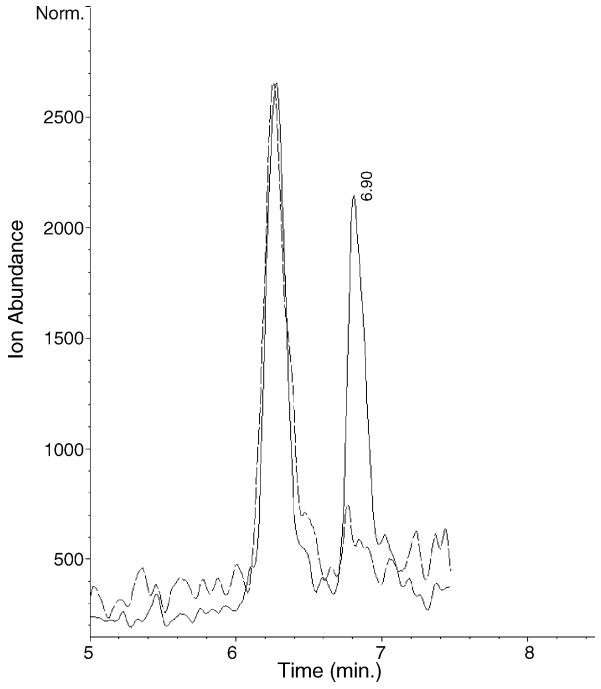

Fig. 6. Jurkat cell extract after incubation with F-ara-A showing F-ara-ATP at LLOQ (0.07 μM, solid lines) superimposed on blank extract (dashed lines).

indicating that ion suppression due to cellular constituents is probably not a concern in this assay. Efforts were made to quantify the degree to which DMHA and buffer concentration affected the MS response. However, these variables affected the retention times of the analyte and the results were confounded by the effect of varying organic fractions on ionization efficiency. The suppression could not be quantified by direct injection procedures partially because the $[M + Na\text{-}H_2]^-$ species varied with DMHA and buffer concentrations. Qualitatively, the effects of tertiary amines in negative ion MS are minimal compared to the drastic effects encountered in the positive mode.

3.5. F-ara-ATP stability

After incubation of cells with F-ara-A, the intracellularly formed F-ara-ATP may be subject to biological degradation during the period of sample preparation for LC–MS analysis. To test the effects of washing time, washing medium and temperature on the stability of intracellular F-ara-ATP, cells were prepared in several different procedures (Fig. 2) and intracellular F-ara-ATP was assayed. The analyte is stable showing virtually no loss even after multiple washes with saline. There was approximately 25% loss when kept in saline at 4 °C for 1.5 h. When maintained in media at 4 °C for 1.5 h, there was no change in recovered F-ara-ATP. These data suggest that with rapid work-up using cold solutions, the loss of F-ara-ATP due to either chemical or enzymatic breakdown is minimal during sample preparation. After samples had been deproteinized and neutralized, repeated injection over 12 h showed no detectable changes in the peak area ratios of F-ara-ATP, ATP, F-ara-ADP, or ADP (data not shown), suggesting that these metabolites are chemically stable for the period required for the analysis.

4. Conclusions

Fludarabine has become and will probably remain an effective agent in cancer therapy, either as an anticancer drug used in the treatment of B-cell malignances or as a major immunosuppressive agent in reduced intensity or non-myeloablative preparative HSCT. Because the pharmacological effect of F-ara-A depends on its intracellular conversion to the active metabolite F-ara-ATP, direct measurement of the intracellular concentration of this metabolite in target cells could be very useful in predicting clinical outcomes. In the past, analyses of F-ara-ATP in biological samples mainly rely on HPLC-based methods using ion exchange [9–12] or ion pairing [13] chromatography with radiochemical or UV detections. These methods have several limiting factors. The low specificity of these methods requires complex chromatography to completely resolve F-ara-adenosine phosphates from naturally occurring nucleotides. Sample turnover times in excess of 1 h are not uncommon. This results in practical limitations on the number of vari-

ables explored and/or the *N* value of any one condition. The method presented, with a turnover time of fifteen minutes, allows greater flexibility and capacity in experimental design. In addition, it takes advantage of the lower background noise characteristic of negative ESI. Moreover, the greater sensitivity of MS vis-à-vis UV allows researchers to use fewer cells and lower, clinically relevant fludarabine concentrations. As the present method is 250–1000 times more sensitive than conventional UV-based assays based on applied analyte, it has a great potential for being developed into a standard therapeutic drug monitoring procedure to directly measure F-ara-ATP intracellular concentration in clinical samples (e.g. leukemia cells or lymphocytes). Pharmacokinetic evaluation of the relationship of plasma F-ara-A concentrations versus intracellular F-ara-ATP formation may provide crucial information on scheduling and dosing fludarabine to improve its therapeutic efficacy. In addition, since many other clinically used nucleoside analogs, such as cytarabine, gemcitabine, and cladribine, undergo similar cellular activation pathways, our method has the potential to be adapted to quantify the active tri-phosphate form of these drugs.

Acknowledgements

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