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Analysis of intracellular didanosine triphosphate at sub-ppb level using LC-MS/MS

Xavier Cahours [a], Thanh Thu Tran [a], Nathalie Mesplet [a], Claudine Kieda [b], Philippe Morin [a], Luigi A. Agrofoglio [a,*]

[a] *Institut de Chimie Organique et Analytique, Associé au CNRS, Université d'Orléans, BP 6759 45067 Orleans, France*
[b] *Centre de Biophysique Moléculaire, UPR CNRS, CNRS 45067 Orleans, France*

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Abstract

An analytical procedure has been developed for the analysis of intracellular didanosine triphosphate (ddATP). An electrospray ionization tandem mass spectrometer (ESI-MS) was interfaced to liquid chromatography (LC) using a mobile phase CH_3OH/H_2O (25/75) containing 1% formic acid for the analysis of the 5′-triphosphate metabolite of the antiviral didanosine. In this procedure, ddATP was extracted from CEM-T4 cells, isolated using an exchange anion solid phase extraction procedure, enzymatically dephosphorylated and then analyzed by LC-MS/MS within a 1 min run time. The influence of several parameters (electrospray ionization interface, acidic modifiers of the mobile phase) has been studied. A calibration curve was generated and the linear regression analysis yielded a regression coefficient (r^2) greater than 0.999. Using LC-MS/MS detection in single reaction monitoring mode (SRM), the limit of quantitation of ddA in CEM-T4 cells was 0.02 ng ml^{-1}. Furthermore, this procedure could be used to perform simultaneous detection of five nucleoside reverse transcriptase inhibitors, such as AZT, 3TC, ddA, ddC and d4T and make LC-MS/MS a method of choice for Therapeutic Drug Monitoring (TDM) in a clinical environment.

Keywords: Didanosine; Didanosine triphosphate; HPLC; MS/MS; HIV; Electrospray; CEM-T4 cells

1. Introduction

Videx or Didanosine (ddI; 2′,3′-dideoxyinosine), an approved drug for the treatment of the human immunodeficiency virus (HIV) infection, is an analogue of the purine nucleoside inosine, which undergoes intracellular conversion to the active triphosphate metabolite, ddATP (2′,3′-dideoxyadenosine-5′-triphosphate) [1,2]. The active metabolite in Fig. 1 inhibits viral reverse transcriptase and acts as chain terminator of the proviral DNA.

Recent studies have demonstrated that the intracellular concentration of the triphosphate form of nucleoside reverse transcriptase inhibitors (NR

* Corresponding author. Tel.: +33-2-3849-4582; fax: +33-2-3841-7281.
E-mail address: luigi.agrofoglio@univ-orleans.fr (L.A. Agrofoglio).

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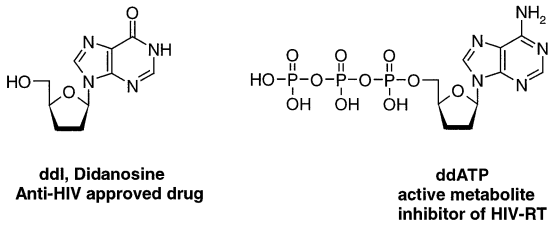

Fig. 1. Structural formulae of didanosine (2′,3′-dideoxyinosine) and its active triphosphate metabolite.

TIs) correlates directly with HIV viral load response [3]. Therefore, to fully assess the pharmacokinetic properties of a NRTI [4,5], it is necessary to quantitate the intracellular triphosphate (NRTI-TP) concentration. The analysis of intracellular NRTI-TPs has been performed mostly employing either LC/UV [6] or LC-RIA [7–9]. However, the main drawback is that the assay is cumbersome and time consuming, and consequently, unsuitable for clinical monitoring therapeutics in physiological fluids at low concentration levels. Thus, the development of modern, highly sensitive and selective chromatographic techniques such as LC-MS allows the identification and quantitation of studied nucleosides. To our best knowledge, only two articles report the intracellular analysis and quantification of anti-HIV nucleosides, AZT (3′-azido-3′-deoxythymidine) and FTC (β-L-(-)-2′-deoxy-5-fluoro-3′-thiacytidine) using LC-MS/MS [10,11]. However, none methods demonstrated the ability to concurrently measure two or more anti-HIV nucleosides, and none have been developed for the intracellular analysis of ddATP. Thus, as part of our on-going anti-HIV research, the present study focused on the development of the high potential of LC-MS/MS for the fast, specific and sensitive analysis of ddATP; furthermore, the proposed procedure could be used to perform simultaneous detection of five anti-HIV agents, i.e. AZT, 3TC, ddA, ddC and d4T. The LC-MS/MS methodology described here could provide data to support the pharmacodynamic studies of NRTI therapy as part of combination regimens. The standard of care for the HIV treatment is a three drugs combination of two nucleosides analogues plus a protease inhibitor. However, future regimens may well include three nucleosides (i.e trizivir-combination of AZT-3TC and ABV) or a 'two and two' combination (two nucleosides + two protease inhibitors). Therefore, the LC-MS/MS appears to be the most suitable technique due to its high sensitivity, selectivity and, particularly, its flexibility in the method development by comparison with radioimmunoassay.

2. Experimental

2.1. Chemicals

Type XA acid phosphatase and other chemicals were purchased from Sigma Chem. (St. Louis, MO, USA). The antiviral nucleosides have been synthesized following reported papers [12] or are gift from pharmaceutical companies. Formic acid and ammonia were of analytical grade and obtained from Sigma. The water used for the preparation of electrolytes was of HPLC quality obtained from Elgestat UHQ II system (Villeurbanne, France). All electrolytes and washing solutions were filtered before use through a polypropylene filter of 0.22 μm porosity (Prolabo, France). Anion exchange, Sep Pak QMA cartridges (100 mg × 1 ml) and reversed phase, Sep Pak C-18 (100 mg × 1 ml) were purchased from Waters Co. (Milford, Mass., USA).

2.2. Cell culture and incubation

CEM-T4 cells, obtained from the Centre de Biophysique Moléculaire (CNRS, Orléans), were cultured as reported earlier [8] in RPMI-1640 growth media (RPMI-1640 containing 10% heat inactivated fetal calf serum and 2 mM glutamine). Briefly, CEM-T4 cells used in this study were in log phase of growth when treated with drug or extracted for background determinations. The cells were pelleted by centrifugation and suspended in RPMI growth media plus non-essential amino acids and 10 U of penicillin/streptomycin. Then 6 h incubations with 10 μM ddI were per-

formed. Cell extracts were prepared from CEM-T4 cells in log phase. Ten CEM-T4 million cells were removed from culture at each time point and precipitated by centrifugation at $500 \times g$ for 7 min or $800 \times g$ for 10 min for CEMss cells and PBMCs. The media was removed by aspiration, and the cell pellet was treated with 400 ml of 70% CH_3OH buffered to pH 7.4. The samples were incubated for 15 min on ice and the debris removed by centrifugation. The supernate was transferred to a fresh tube and stored at -20 °C until analysis.

2.3. Intracellular triphosphate isolation

The separation conditions were as reported earlier [8,9] with following modifications: QMA cartridges (100 mg $\times$ 1 ml) were preconditioned with 5 ml of 1 M KCl followed by 5 ml of 5 mM KCl. The methanol extracts were first evaporated to dryness with nitrogen gas flow and reconstituted with 1 ml of Tris (pH 7.4, 1 M). The free, mono, and diphosphate nucleosides were eluted with 5 ml of 5 mM KCl followed by 15 ml of 75 mM KCl. Finally, the ddATP was eluted from the cartridge with 5 ml of 500 mM KCl and collected in a conical centrifuge tube. The eluted ddATP was adjusted to pH 6.0 by the addition of 150 μl of MES (2-[*N*-morpholino]ethanesulfonic acid) buffer. Five units (7 μl) of acid phosphatase were added and the mixture incubated for 30 min at 37 °C to remove the phosphate groups. After the hydrolysis of the phosphate groups, the samples were purified and desalted using a Waters SepPak C-18 cartridge. The C-18 cartridges were conditioned with 5 ml of methanol followed by 5 ml of water. The samples were loaded onto the cartridge and the protein and salt removed with a 15 ml water wash. The ddA was eluted with 3 ml of acetonitrile. The samples were then evaporated to dryness with a nitrogen gas flow system and stored at -20 °C until analysis. The recovery of the SPE extraction was studied using the corresponding radiolabeled analogues and reported [8,9]. The overall recovery (QMA and C18) was over 95%.

2.4. LC-MS/MS

LC-MS experiments were performed on a Perkin-Elmer (Toronto, Canada) model LC-2000 binary pump and a Perkin-Elmer Sciex (Forster City, CA, USA) API 300 mass spectrometer in positive ionization mode. The orifice and focusing ring voltages were set at different values (25 and 250 V, respectively). Injections were done by Perkin-Elmer series 200 autosampler (Toronto, Canada) fitted with a 20 μl sample loop. For the study of the MS parameters, a Harvard Model 22 syringe pump (Harvard Apparatus, South Natick, MA, USA) was used to infuse the nucleoside solutions in the MS systems at a flow-rate of 5 μl min^{-1}. Mass spectra were acquired using a dwell time of 1 ms. A Macintosh computer was used for instrument control, data acquisition and MS-acquisition processing data were calculated with the LC2 Tune Software. The analytical column was a Purospher RP-18e column (30×21 mm ID, particle size 3 μm) from Merck (Darmstadt, Germany).

3. Results and discussion

The present study is, to our best knowledge, the first quantitation reported for the 5′-triphosphate of ddA and for the simultaneous analysis of AZT, ddC, ddA, d4T, and 3TC. The method developed was highly sensitive with an absolute limit of quantification of 0.1 ppb (0.1 ng ml^{-1}). Preliminary validation of this LC-MS/MS was established by performing analysis on a minimum of three replicates of ddA spiked with CEM-T4 cells for five separate assays; this process included the verification of analytical range, linearity, limit of quantification, accuracy for each analyte. Once the method will be applied to the analysis of anti-HIV nucleosides in biological fluids, the fully validation will be considered.

3.1. MS of ddA

The objective of this study was to develop a sensitive and selective procedure of intracellular ddA-TP analysis. In this method, ddATP was

Table 1
Main fragments for nucleosides observed by CAD

Nucleoside	MW	$(MH)^+$ ion (m/z)	Fragment ion in MS/MS (m/z)
Adenosine	267	268	136
Cytidine	243	244	112
Guanosine	283	284	152
Uridine	244	245	113
Thymidine	242	243	127
ddA	235	236	136

isolated from cells, extracted, and separated from other phosphorylated metabolites, then the triphosphate groups were removed with acid phosphatase. The efficacy of the enzyme hydrolysis was studied and reported earlier [8]. The analysis of the resulting ddA, which corresponds to the original ddA-TP, has been performed by LC-MS/MS. Table 1 reports for nucleosides, the different m/z ratio values of $[M + H]^+$ ions formed during the positive mode ionization and also the most intense fragment observed in Collisionally Actived Dissociation (CAD).

The m/z ratios corresponding to protonated nucleoside ($[M + H]^+$) show different values for each nucleoside; thus, any coelution of these compounds can be resolved in the event of different m/z ratios. Therefore, by Extracting Ion Current (XIC) at $m/z = 236$ it is possible to identify and quantitate by LC-MS the presence of ddA in a sample and in the presence of the other nucleoside analogues.

In order to increase the sensitivity of the method, MS/MS detection has been performed. Indeed, using CAD allows to increase signal/noise ratio and, consequently, offers better detection limits. CAD is carried out by accelerating selected ions (e.g.: $[M + H]^+$) into a region that has a higher density of gas molecules. The resulting collisions with the gas induce the dissociation of the protonated molecule. Since the fragments ions reflect the structure of $[M + H]^+$ ion, CAD offers structurally specific data. After CAD, only fragment ions derived from the analyte are selected, thus eliminating the CAD fragments from matrix contaminant. This two stage filtering process allows very selective data and improves the detection limits.

3.2. Evaluation of the ESI interface parameters

In order to investigate the performance of LC-ESI-MS for the analysis of ddA, preliminary studies were carried out under both infusion and FIA conditions in full-scan mode. For all experiments, the position of the capillary versus ionspray needle and spatial positions were optimized to obtain a stable and highest signal and also to avoid source contamination. The different parameters, which have an impact on the performance of ESI are the ionspray voltage, the orifice and focusing ring voltages and the nebulizing and curtain gas, the effects of which on the MS response of ddA were investigated. A low orifice voltage (25 V) was found to ensure the best sensitivity. Other optimum conditions were established at the ring voltage of 250 V and the ionspray voltage of $+5$ kV. A slight decrease in curtain gas flow-rate allows to increase signal sensitivity. After optimization, the nebulizing gas (air) flow-rate was set at 1.13 l min^{-1}.

3.3. MS tandem parameters

The parameter determining the detection limit for a specific compound is the fragmentation efficiency. Quantitative LC-MS/MS analysis is usually performed using Single Reaction Monitoring (SRM) mode, which consists to monitor the intensity of a selected ion to one specific fragment ion. The first quadrupole (Q_1) was set to transmit protonated ion of ddA at $m/z = 236$. This precursor ion was fragmented by CAD with N_2 at 25 eV collision energy in the second quadrupole (Q_2) to generate some product ions. The main product ion of ddA at $m/z = 136$, corresponding to the loss of the sugar moiety, was monitored via the third quadrupole (Q_3). Optimization of the collision energy was performed in order to improve the intensity of a specific fragment ion. Fig. 2 depicts the relative fragment abundance of $[M + H]^+$ and $[MH^+$-sugar] ions of ddA versus the collision energy. It appears that an increase of the collision energy above 15 eV involves a decrease in the corresponding $[M + H]^+$ ion with the con-

comitant increase of fragment ion intensity. By increasing the collision energy, the fragment CAD ion abundance passes through a maximum (near 25 eV) for the ddA.

3.4. Influence of mobile phase additive on analyte detectability

Generally, for a given solute in MS, the abundance of $[M+H]^+$ ion formed depends on its proton affinity, on the nature of the solvent and on the presence of acidic or basic modifiers in the mobile phase [13]. For nucleosides, it has been already established that the sensitivity was improved by using volatile acids. The effects of two acidic modifiers (acetic and formic acids) upon the MS signal of ddA were investigated; we observed a total ion current of 1.7×10^5 counts by infusion of 5 mg l^{-1} ddA solution in CH_3OH/H_2O (25:75, v/v) containing 1% formic acid, 1.2×10^6 counts in CH_3OH/H_2O (25:75, v/v) containing 1% acetic acid and only 6.5×10^4 counts in CH_3OH/H_2O (25:75, v/v). The effect of formic acid concentration upon MS signal intensity was carried out by infusion of 5 mg ml^{-1} ddA solution in CH_3OH/H_2O (25:75, v/v) (Fig. 3). The best ionization efficiency was obtained at 3% formic acid added to the mobile phase. Otherwise, methanol has been substituted by acetonitrile or ethanol. It was found that these changes had only minor effect on signal intensity.

3.5. Preliminary validation of LC-MS/MS analysis

A LC-MS/MS method was developed in order to quantitate ddA in human cells and was shown to be fast, sensitive, accurate, precise and reproducible. This method was achieved by using a short LC column (2.1 cm) and a methanol content of 25% in the mobile phase, to shorten the analysis time. All experiments were carried out at room temperature and with a flow-rate of 100 µl min^{-1}; the part of the column effluent splitted towards

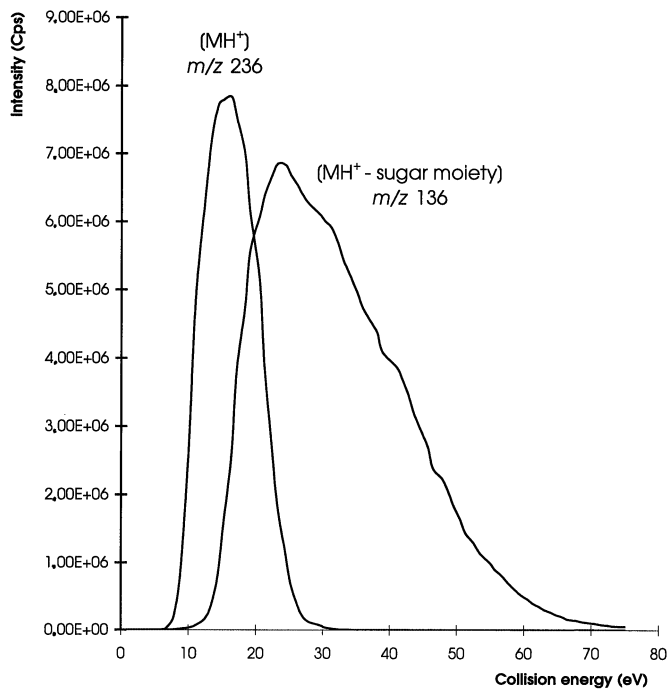

Fig. 2. Variation of relative fragment abundance of ddA versus the collision energy in MS/MS (SRM mode). Liquid chromatography and mass spectrometry conditions are as described in Section 2.

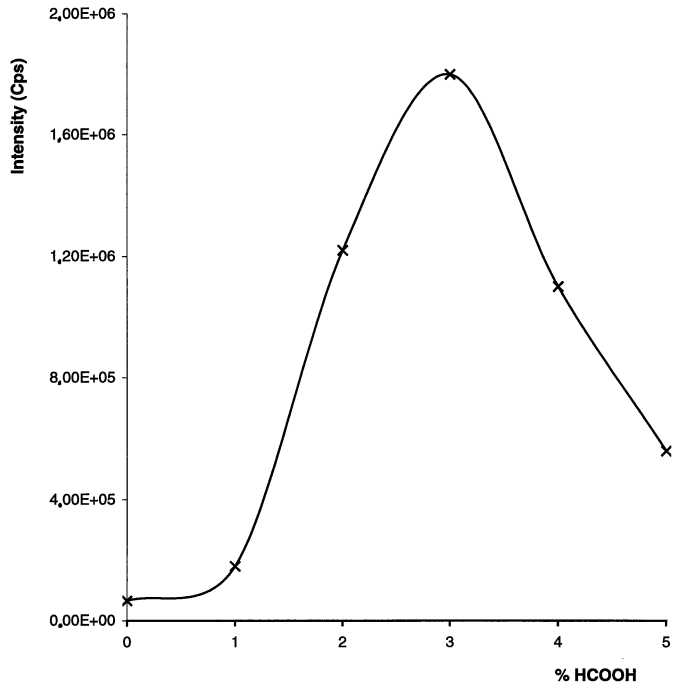

Fig. 3. Variation of the total ion current of ddA versus amount of formic acid added to CH_3OH/H_2O (25/75) mobile phase. Liquid chromatography and mass spectrometry conditions are as described in Section 2.

the ion source (split 1/20) was 5 μl min^{-1}. To assess the precision and accuracy of the method, five replicates of the ddA samples were analyzed in each of three assay runs.

3.5.1. Stability of analytical standard solutions

Stock solutions of each nucleoside (10 mg l^{-1}) in CH_3OH/H_2O (25:75, v/v) were freshly prepared and kept at controlled temperature (20 °C) or stored at −10 °C when not in use. Samples of these solutions were analyzed by the reversed-phase HPLC method at appropriate times. Standard solutions of nucleosides were stable and no degradation of the products was observed during the analysis.

3.5.2. Specificity and selectivity

Specificity was assessed by utilizing a triple quadrupole SMR detection technique, whereby analyte specific ions were selected in both the Q1 and Q3 regions of the mass spectrometer. To verify the selectivity, unspiked CEM-T4 were extracted and analyzed for possible interference from endogenous nucleotides/nucleosides. Fig. 4 shows chromatograms (A) of blank CEM-T4 cell extracts and (B) CEM-T4 cell extract spiked with 1 ng ml^{-1} of ddA. No endogenous interfering peaks were observed in the chromatograms from these samples. Thus, the detection of ddA was carried out by MS/MS using SRM mode with a dwell time of 600 ms and turned out to be selective and specific.

3.5.3. Linearity

The linearity of the calibration curve for ddA was assessed using five standard solutions from 0.05 to 5 ng ml^{-1}. The curve was forced to cross the origin; least-squares linear regression was performed and it gave the following equation: $y = 2.8 \times 10^6 x$, with a regression coefficient (r^2) equal to 0.9997, and a 0.57% relative standard deviation (R.S.D.) on the slope.

3.5.4. Accuracy

Standard solutions ($n = 5$, from 0.05 to 5 ng ml^{-1}) were injected in triplicate to assess the

accuracy of the assay, which was expressed as the percentage of the theoretical concentration. The results of recovery studies are shown in Table 2.

3.5.5. Quantification and detection limits

Using MS/MS detection in the SRM mode, an injected ddA concentration of 0.1 ng ml^{-1} in CEM-T4 cell extract gave a signal-to-noise ratio of 50. Calculated LOQ (S/N 10) and LOD (S/N 3) values are equal to 0.02 and 0.006 ng ml^{-1}, respectively.

3.5.6. Repeatability

The intraday system repeatability was evaluated at two different levels of ddA, e.g. 0.1 and 10 ng ml^{-1}, by injecting seven times each standard solution. The R.S.D. for areas of ddA were 1.40 and 0.47%, respectively.

3.6. Simultaneous analysis of five NRTIs

The described procedure can be used for the simultaneous determination of five nucleoside reverse transcriptase inhibitors currently used for

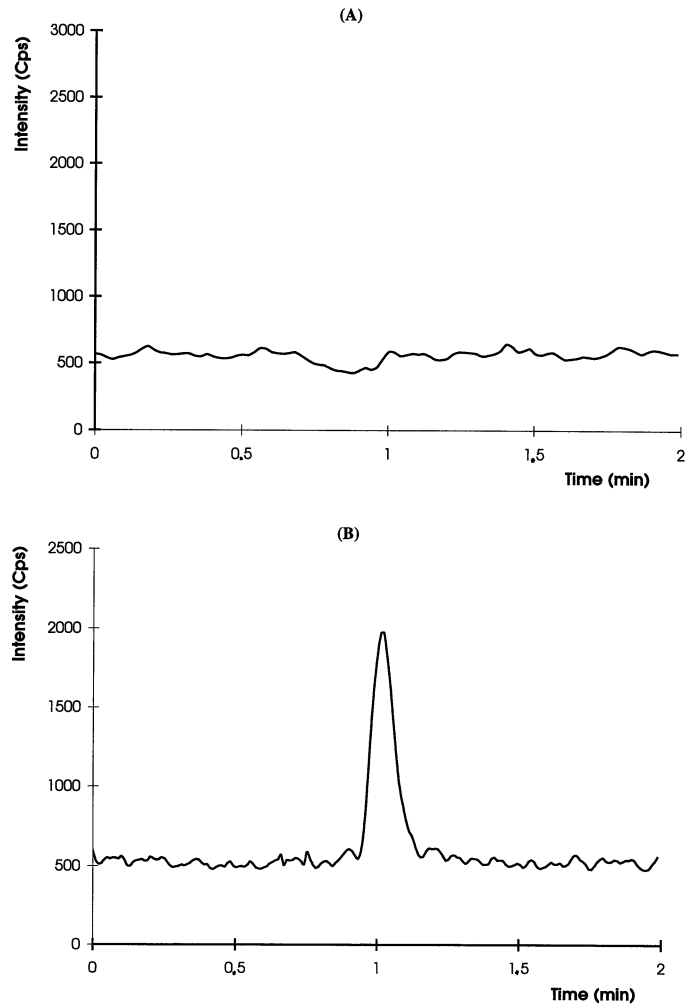

Fig. 4. SRM chromatograms derived from the analysis of (A) blank CEM-T4 cell extracts, containing 10×10^6 CEM-T4 and (B) 10×10^6 CEM-T4 cell extract spiked with ddA (1 ng ml^{-1}). Liquid chromatography and mass spectrometry conditions are as described in Section 2.

Table 2
Results of recovery studies for samples in injection medium

Concentration (ng ml^{-1})	Recovery (%)	Average recovery ($\% \pm t.s/\sqrt{n}$)
0.05	98.31	99.21 ± 0.90
	99.83	
	99.50	
0.1	98.42	97.34 ± 1.23
	96.24	
	97.37	
0.5	94.63	95.31 ± 1.83
	97.15	
	94.13	
1	105.79	102.50 ± 4.26
	103.32	
	98.39	
5	97.16	99.95 ± 2.73
	101.44	
	101.24	

the HIV therapy. CEM-T4 cell extracts were spiked with standard solution of AZT (3′-azidothymidine), d4T (2′,3′-didehydro-3′-deoxythymidine), ddC (2′,3′-dideoxycytidine), 3TC (3′-thiacytidine) and ddA. Fig. 5 shows SRM chromatogram for the simultaneous determination of those five NRTIs (10 ppm per each) in the same sample. This approach is of great interest to quantitate the metabolites of drugs used currently into the Highly Active AntiRetoviral Therapy (HAART).

4. Conclusion

An LC-MS/MS method using a solid phase extraction step has been developed for the determination of ddATP in CEM-T4 cells. A calibration curve was generated and the linear regression analysis yielded a regression coefficient (r^2) greater than 0.999. This method afforded a limit of quantitation of 0.02 ng ml^{-1} for ddA, with a chromatographic run time under 1 min. This has to be compared with general LC-RIA techniques that are cumbersome and time consuming (over 8 h). A cross-validation study with established procedures (SPE-RIA or LC-RIA) methodology would be of interest, nevertheless to date, no assay for ddA or ddATP is available. The main

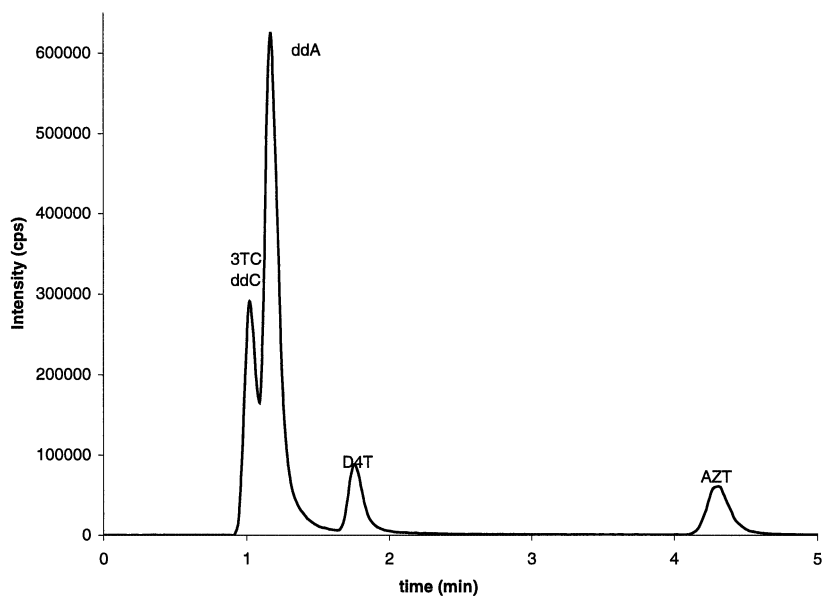

Fig. 5. Chromatograms for simultaneous analysis of a solution of ddC, d4T, 3TC, ddA, and AZT in CEM-T4 cell extract. Concentration: 10 ppm per each; precursor $(M + H)^+$ and fragment ions $(MH\text{-sugar})^+$: for ddC: 212– > 136; for 3TC: 230– > 112; for ddA: 236– > 136; for AZT: 268– > 136.

advantage of the method we described here is the simultaneous determination of several nucleoside reverse transciptase inhibitors used in the present HIV polytherapy. We have currently investigated the direct quantitation of ddATP by LC-MS/MS from a pool of endogenous nucleotides.

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