A Method for Quantitating the Intracellular Metabolism of AZT Amino Acid Phosphoramidate Pronucleotides by Capillary High-Performance Liquid Chromatography–Electrospray Ionization...

ARTICLE *in* MOLECULAR PHARMACEUTICS · JUNE 2005

Impact Factor: 4.38 · DOI: 10.1021/mp0500162 · Source: PubMed

4 AUTHORS, INCLUDING:

Jisook Kim
University of Tennessee at Chattanooga
13 PUBLICATIONS **76** CITATIONS
SEE PROFILE

Natalia Tretyakova
University of Minnesota Twin Cities
104 PUBLICATIONS **2,355** CITATIONS
SEE PROFILE

Carston R Wagner
University of Minnesota Twin Cities
86 PUBLICATIONS **1,539** CITATIONS
SEE PROFILE

molecular pharmaceutics

brief articles

A Method for Quantitating the Intracellular Metabolism of AZT Amino Acid Phosphoramidate Pronucleotides by Capillary High-Performance Liquid Chromatography−Electrospray Ionization Mass Spectrometry

Jisook Kim,[†] Soobong Park,[‡] Natalia Y. Tretyakova,[‡] and Carston R. Wagner*,[†]

Department of Medicinal Chemistry, College of Pharmacy, and the Cancer Research Center, University of Minnesota, Minneapolis, Minnesota 55455

Received March 24, 2005

Abstract: A methodology has been developed for the analysis of the intracellular metabolism of 3′-azido-3′-deoxythymidine (AZT) amino acid phosphoramidates utilizing reverse-phase high-performance liquid chromatography interfaced with negative ion electrospray ionization mass spectrometry (LC/ESI$^-$-MS). The presented work demonstrates the potential of capillary LC/MS and LC/MS/MS to identify and quantitate the cellular uptake and metabolism of nucleoside phosphoramidate. Significant intracellular amounts of D- and L-phenylalanine methyl ester or D- and L-tryptophan methyl ester AZT phosphoramidates were observed for human T-lymphoblastoid leukemia (CEM) cells incubated for 2 and 4 h with the prodrugs. AZT-MP was the primary metabolite observed for human T-lymphoblastoid leukemia (CEM) cells. In this paper, the details of using LC/MS to analyze AZT amino acid phosphoramidates in biological samples are discussed. LC/MS is an efficient method for analyzing multiple samples containing several analytes in a short period of time. The method also provides high selectivity and sensitivity, and requires minimal sample preparation. This approach should be broadly applicable for the analysis of the intracellular metabolism of nucleoside prodrugs and pronucleotides.

Keywords: Prodrugs; antiviral agent; AZT; phosphoramidate

Introduction

AZT has demonstrated potent inhibition against human immunodeficiency virus.[1−5] The mechanism of action requires conversion of AZT to the corresponding 5′-mono-, di-, and triphosphates by cellular kinases after cellular uptake. AZT triphosphate then competes with dTTP for incorporation in viral DNA, leading to inhibition of reverse transcriptase and DNA chain termination. Resistance to AZT treatment has been shown to depend on the downregulation of thymidine kinase-1, which is responsible for phosphorylating AZT to release AZT-MP, as well as the upregulation of exporters, such as the ATP-binding cassette (ABC) transporter, MRP4.[6−11] In order to circumvent this problem and deliver the monophosphate efficiently into the target cell,

* Corresponding author. Mailing address: Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55454. Tel: (612) 625-2614. Fax: (612) 624-0139. E-mail: wagne003@tc.umn.edu.

† Department of Medicinal Chemistry, College of Pharmacy.

‡ Cancer Research Center.

(1) Cheson, B. D.; Keating, M. J.; Plunkett, W. *Nucleoside analogs in cancer therapy*; Marcel Dekker: New York, 1990.

(2) Chabner, B. A.; Longo, D. L. *Cancer Chemotherapy and Biotherapy*; Lippincott-Raven: New York, 1996.

(3) De Clercq, E. Antiviral activity spectrum and target of action of different classes of nucleoside. *Nucleosides Nucleotides* **1994**, *13*, 1271.

(4) Herdewijn, P. Structural requirements for antiviral activity in nucleosides. *Drug Discovery Today* **1997**, *2*, 235.

(5) Ledneva, R. K.; Preobrazhenskaya, N. N.; Shinskii, N. G.; Shabarova, Z. A.; Prokofev, M. A. Analogs of uridine-5′-phosphoramides and thier hydrolysis by ribonucleoside-5′-phosphoramidase. *Dokl. Akad. Nauk SSSR* **1970**, *193*, 1308−1310.

10.1021/mp0500162 CCC: $30.25
Published on Web 05/04/2005

AZT amino acid phosphoramidates have been investigated as potential pronucleotides.[12−16] AZT amino acid phosphoramidates have reasonable cellular permeability and have shown potential as antiviral and/or anticancer agents with enhanced activity and reduced cytotoxicity.

Previous investigations carried out in our laboratory have shown that AZT amino acid phosphoramidates were able to generate phosphorylated AZT in the target cell.[17] This was confirmed by intracellular metabolism studies of AZT and AZT amino acid phosphoramidates utilizing a coupled radioimmunoassay−reverse phase HPLC method.[17] Recently, we have developed a method to investigate the intracellular metabolism of AZT amino acid phosphoramidates based on a ^{31}P NMR approach.[18] This technique allowed simultaneous monitoring of multiple analytes such as parent prodrugs and associated intracellular metabolites, with minimal sample preparation. However, due to the relatively large amount of tissue required for analysis with ^{31}P NMR, a more sensitive analytical method was desirable. Biological samples have been analyzed routinely with LC coupled to a UV detector, and this approach could be considered for studying pronucleotide metabolism.[19−21] However, this technique requires laborious sample purification in order to avoid potential background interferences from cellular debris, followed by derivatization.

LC/MS has proven to be a powerful technique for the structural identification and quantitative analysis of biological samples.[22−32] In this context, we have recently utilized LC/MS to investigate the intracellular metabolism of ^{18}O-

(6) Vrang, L.; Bazin, H.; Remaud, G.; Chattopadhyaya, J.; Oberg, B. Inhibition of the reverse transcriptase from HIV by 3′-azido-3′deoxythymidine triphosphate and its *threo* analogue. *Antiviral Res.* **1987**, *7*, 139−149.

(7) Mitsuya, H.; Jarett, R. F.; Matsukura, M.; Veronese, F. D. M.; De Vico, A. L.; et al. Long-term inhibition of human T-lymphotropic virus type III/lymphadenopathy-associated virus (human immunodeficiency virus) DNA synthesis and RNA expression in T cells protected by 2′,3′-dideoxynucleosides in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 2033−2037.

(8) Furman, P. A.; St. Clair, M. H.; Weinhold, K.; Rideout, J. L.; Freeman, G. A.; et al. Phosphorylation of 3′-azido-3′deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8333−8337.

(9) De Clercq, E. New perspectives for the treatment of HIV infections. *Collect. Czech. Chem. Commun.* **1998**, *63*, 449−479.

(10) Cheng, Y. C.; Dutschman, G. E.; Bastow, K. F.; Sarngadharan, M. G.; Ting, R. Y. Human immunodeficiency virus reverse transcriptase: general properties and its interactions with nucleoside triphosphate analogs. *J. Biol. Chem.* **1987**, *262*, 2187−2189.

(11) Schuetz, J. D.; Connelly, M. C.; Sun, D.; Paibir, S. G.; Flynn, P. M.; et al. MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nat. Med.* **1999**, *5*, 1048−1051.

(12) Abraham, T. W.; Kalman, T. I.; McIntee, E. J.; Wagner, C. R. Synthesis and biological activity of aromatic amino acid phosphoramidates of 5-fluoro-2′-deoxyuridine and 1-β-arabinofuranosylcytosine: evidence of phosphoramidase activity. *J. Med. Chem.* **1996**, *39*, 4569−4575.

(13) Molema, G.; Jansen, R. W.; Pauwels, R. D. Targeting of antiviral drugs to T4-lymphocytes. Anti-HIV activity of neoglycoprotein-AZTMP conjugates in vitro. *Biochem. Pharmacol.* **1990**, *40*, 2603−2610.

(14) McIntee, E. J.; Remmel, R. P.; Schinazi, R. F.; Abraham, T. W.; Wagner, C. R. Probing the mechanism of action and decomposition of amino acid phosphomonoester amidates of antiviral nucleoside prodrugs. *J. Med. Chem.* **1997**, *40*, 3323−3331.

(15) Jones, A. S.; McGuigan, C.; Walker, R. T.; Balzarini, J.; De Clercq, E. Synthesis, properties, and biological activity of some nucleoside cyclic phosphoramidates. *J. Chem. Soc., Perkin Trans. 1* **1984**, 1471−1474.

(16) Wagner, C. R.; Chang, S.-l.; Griesgraber, G. W.; Song, H.; McIntee, E. J.; et al. Antiviral nucleoside drug delivery via amino acid phosphoramidates. *Nucleosides Nucleotides* **1999**, *18*, 913−919.

(17) Chang, S.-l.; Griesgraber, G. W.; Southern, P. J.; Wagner, C. R. Amino acid phosphoramidate monoesters of 3′-azido-3′-deoxythymidine: Relationship between antiviral potency and intracellular metabolism. *J. Med. Chem.* **2001**, *44*, 223−231.

(18) Kim, J.; Drontle, P. D.; Wagner, C. R. Monitoring the intracellular metabolism of nucleoside phosphoramidate pronucleotides by ^{31}P NMR. *Nucleosides, Nucleotides Nucleic Acids* **2004**, *23*, 483−493.

(19) Yoshida, S.; Urakami, K.; Kito, M.; Takeshima, S.; Hirose, S. High-performance liquid chromatography with chemiluminescence detection of serum levels of pre-column derivatized fluoropyrimidine compounds. *J. Chromatogr. A* **1990**, *530*, 57−64.

(20) Iwamoto, M.; Yoshida, S.; Hirose, S. High performance liquid chromatographic determination of pyrimidine nucleobases and 5-fluorouracil labeled with a fluorescent reagent. *Yakugaku Zasshi* **1984**, *104*, 1251−1256.

(21) Dass, C. *Principles and practice of biological mass spectrometry*; John Wiley & Sons: New York, 2001.

(22) Henner, G.; Becher, F.; Pruvost, A.; Goujard, C.; Grassi, J.; et al. Liquid chromatography-tandem mass spectrometry assays for intracellular deoxyribonucleotide triphosphate competitors of nucleoside antiretrovirals. *J. Chromatogr. B* **2003**, *789*, 273−281.

(23) Hua, Y.; Wainhaus, S. B.; Yang, Y.; Shen, L.; Xiong, Y.; et al. Comparison of negative and positive Ion electospray tandem mass spectrometry for the liquid chromatography tandem mass spectrometry analysis of oxidized deoxynucleosides. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 80−87.

(24) Lefebvre, I.; Perigaud, C.; Pompon, A.; Aubertin, A. M.; Girardet, J. L.; et al. Mononucleoside phosphotriester derivatives with *S*-acyl-2-thioethyl bioreversible phosphate-protecting groups: intracellular delivery of 3′-azido-2′,3′-dideoxythymidine 5′-monophosphate. *J. Med. Chem.* **1995**, *38*, 3941−3950.

(25) Lin, C.-c.; Yeh, L.-T.; Johnson, Y.; Lau, N. Specific, sensitive and accurate liquid chromatographic-tandem mass spectrometric method for the measurement of ribavirin in rat and monkey plasma. *J. Chromatogr. B* **2002**, *779*, 241−248.

(26) Lynch, T.; Eisenberg, G.; Kernan, M. LC/MS determination of the intracellular concentration of two novel aryl phosphoramidate prodrugs of PMPA and their metabolites in dog PBMC. *Nucleosides, Nucleotides Nucleic Acids* **2001**, *20*, 1415−1419.

(27) Rentsch, K. M. Sensitive and specific determination of eight antiretroviral agents in plasma by high-performance liquid chromatography−mass spectrometry. *J. Chromatogr. B* **2003**, *788*, 339−350.

(28) Roboz, J. *Mass spectrometry in cancer reserach*; CRC Press: Boca Raton, FL, 2002.

(29) Siuzdak, G. *Mass spectrometry for biotechnology*; Academic Press: San Diego, CA, 1996.

labeled AZT phosphoramidates.[33] The method proved to be highly sensitive and requires substantially less amounts of material than either NMR or HPLC with UV detection.

In this paper, we report the development of a capillary HPLC/ESI-MS/MS method for the efficient, specific, sensitive and rapid analysis of the intracellular metabolism of model pronucleotides of AZT.

Materials and Methods

AZT was kindly donated by Toronto Research Chemicals Inc. (North York, Ontario, Canada). AZT-MP was purchased from Sigma. 4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), *N*,*N*-dimethylhexylamine (DMHA), and formic acid were purchased from Sigma-Aldrich. AZT tryptophan methyl ester phosphoramidate (ATO) and AZT phenylalanine methyl ester phosphoramidate (APO) were synthesized as previously described.[17,34] RPMI-1640 and fetal bovine serum (FBS) were purchased from Atlanta Biologicals, Inc. Trypan blue stain (0.4%) in saline (0.85%) was purchased from GIBCO (Grand Island, NY). Sterile stock solutions of penicillin G, streptomycin, human interleukin-2, and phytohemagglutinin were prepared with sterilizing filters. Solvents used for LC analyses are HPLC grade. All the solutions for instrumental analyses were filtered through a 0.45 μm membrane filter, and degassed prior to use.

HPLC Method. Chromatographic separation was achieved using a capillary Zorbax XDB-C18 column (150 mm $\times$ 0.5 mm, 5 μm, Agilent Technologies) eluted at a flow rate of 12 μL/min. An injection volume of 5 μL was used for standard samples as well as for cell extract samples. The mobile phase was composed of two solvents, A and B. Solvent A was 15 mM ammonium acetate, pH 6.65, and solvent B was methanol. Various gradient systems were employed for analyses, but the condition reported herein has the shortest running time. The representative gradient condition was as follows: 0−15% B in 5 min, 15−50% B in 25 min, 50−85% B in 30 min, and 85−87% B in 40 min. Column washing with 100% B was performed over 5 min, and the equilibration time before the next analysis was set at 10 min. The column temperature was maintained at 24 °C using a thermostated column compartment. To minimize salt contamination, HPLC effluent from the first 5−7 min of each run was diverted to waste.

Effect of Ion-Pairing Compounds on Retention Time and Signal. The effect of an ion-pairing agent was investigated by carrying out LC/MS experiments with standard samples containing adenosine phosphates, AZT, D4T, AZT-MP, and L-ATO at fixed concentrations for each analyte. The buffer was composed of DMHA and formic acid at pH 7.0. DMHA buffer concentrations were varied between 1 and 10 mM. The response was measured by comparing the peak area of L-ATO under various buffer concentrations.

Capillary HPLC/ESI-MS/MS Analysis. An Agilent 1100 capillary LC−ion trap MS system (Wilmington, DE) was employed for all analyses. The details of chromatographic separation were as discussed above. The mass spectrometer was operated in negative ion mode with nitrogen as a nebulizing and drying gas (15 psi, 5 L/min). The total eluent flow of 12 μL/min was directed to the ESI source. The HV capillary voltage was set to 4311 V. The drying gas temperature was set to 200 °C. The capillary exit voltage was −131 V. ESI source parameters and MS/MS parameters were optimized for maximum sensitivity during direct infusion of authentic standards. The MS experiment was divided into several segments to detect each analyte at a different retention time. Quantitation and identification of prodrugs as well as metabolites by HPLC/ESI-MS/MS was carried out with Chemstation software (Agilent).

Measurement of Intracellular Concentration of Metabolites and Phosphoramidates. Negative ion ESI and selective multiple reaction monitoring (MRM) mode was employed in all sample analyses of intracellular metabolites. The deprotonated molecular ions for each analyte ($[M - H]^-$ = m/z 546.5 for ATO, and 507.5 for APO) were trapped and fragmented simultaneously with an isolation width of 1 mass unit (mu).

Quantitation of AZT-MP was carried out as follows. The $[M - H]^-$ ions of AZT-MP (m/z 346) and the internal standard, D4T (m/z 223), were isolated and subjected to collision-induced dissociation (CID, fragmentation amplitude = 1). The product ions were detected within the scan range of m/z 100−400. The target ion abundance value was 30 000, and the maximum accumulation time was 300 ms. The instrument was tuned to maximize sensitivity while infusing an authentic standard solution of AZT-MP. AZT-MP was quantitated from fragment ions at m/z 125, 177, 303, and 346. A D4T internal standard was analyzed analogously on the basis of fragment ions at m/z 125, 150, 193, and 223. Calibration curves were constructed by injecting standard solutions containing known amounts of AZT-MP (2, 5, 10, and 20 pmol) and D4T (10 pmol), followed by analysis of

(30) St Claire, R. L. Positive ion electrospray ionization tandem mass spectrometry coupled to ion-pairing high-performance liquid chromatography with a phosphate buffer for the quantitative analysis of intracellular nucleotides. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1625−1634.

(31) Van den Driessche, B.; Lemiere, F.; Van Dongen, W.; Esmans, E. L. Alkylation of DNA by melphalan: investigation of capillary liquid chromatography-electrospray ionization tandem mass spectrometry in the study of the adducts at the nucleoside level. *J. Chromatogr. B* **2003**, *785*, 21−37.

(32) Witters, E.; Van Dongen, W.; Esmans, L. E.; Van Onckelen, A. H. Ion-pair liquid chromatography-electrospray mass spectrometry for the analysis of cyclic nucleotides. *J. Chromatogr. B* **1997**, *694*, 55−63.

(33) Kim, J.; Chou, T.-f.; Griesgraber, G. W.; Wagner, C. R. Direct measurement of nucleoside monophosphate delivery from a phosphoramidate pronucleotide by stable isotope labeling and LC−ESI$^-$-MS/MS. *Mol. Pharm.* **2004**, *1*, 102−111.

(34) Iyer, I. V.; Griesgraber, G. W.; Radmer, M. R.; McIntee, E. J.; Wagner, C. R. Synthesis, in vitro anti-breast cancer activity, and intracellular decomposition of amino acid methyl ester and alkyl amide phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (AZT). *J. Med. Chem.* **2000**, *43*, 2266−2274.

the HPLC/ESI-MS/MS peak area ratios. Then quantitative analyses were carried out on the basis of the ratio of the peak area in the selected ion chromatogram corresponding to AZT-MP to the peak area of D4T (relative response factors). The linearity of the relative response was checked using calibration graphs for AZT-MP using four standard solution samples.[21,28,29]

The procedure for quantitation of AZT was carried out as follows. The $[M - H]^-$ ions of AZT (*m*/*z* 265) and the internal standard, D4T (*m*/*z* 223), were isolated and subjected to collision-induced dissociation (CID, fragmentation amplitude = 1). The product ions were detected within the scan range of *m*/*z* 100−300. The target ion abundance value was 30 000, and the maximum accumulation time was 300 ms. AZT was quantitated from the fragment ion at *m*/*z* 223. D4T internal standard was analyzed analogously on the basis of fragment ions at *m*/*z* 125, 150, 193, and 223. Calibration curves were constructed by injecting standard solutions containing known amounts of AZT (2, 5, 10, and 20 pmol) and D4T (10 pmol), followed by analysis of the HPLC/ESI-MS/MS peak area ratios. Then quantitative analyses were carried out on the basis of the ratio of the peak area in the selected ion chromatogram corresponding to AZT to the peak area of D4T (relative response factors). The linearity of the relative response was checked using calibration graphs for AZT with four standard solution samples.

Parent prodrugs (L-/D-ATO and L-/D-APO) were quantitated similarly to AZT and AZT-MP. The $[M - H]^-$ ions of L-/D-ATO (*m*/*z* 546), L-/D-APO (*m*/*z* 507), and the internal standard, L-DPO (*m*/*z* 464), were isolated and subjected to collision-induced dissociation (CID, fragmentation amplitude = 0.85). The product ions were detected within the scan range of *m*/*z* 100−700. The target ion abundance value was 25 000, and the maximum accumulation time was 300 ms. ATO was determined from fragment ions at *m*/*z* 265, 377, 503, and 546. APO was quantitated from fragment ions at *m*/*z* 226, 278, 338, 464, and 507. L-DPO internal standard was analyzed analogously on the basis of fragment ions at *m*/*z* 226, 278, 306, 338, and 464. Calibration curves were constructed by injecting standard solutions containing known amounts of L-/D-ATO (1, 5, 25, 50 pmol) or L-/D-APO (1, 5, 25, 50 pmol) and L-DPO (20 pmol), followed by analysis of the HPLC/ESI-MS/MS peak area ratios. Then, quantitative analyses were carried out on the basis of the ratio of the area of the peak in the selected ion chromatogram corresponding to prodrug to the peak area of L-DPO. The linearity of the relative response was checked by calibration curves generated with four standard prodrug solutions.

Sample Preparation for Recovery Tests. The percentage recovery of AZT phosphoramidates (L-ATO as a representative), AZT-MP, and AZT during the assay was determined. Two sets of experiments were conducted: one with cell extract and the other with a 60% methanol−40% 15 mM ammonium acetate buffer (pH 6.65). The procedure is described as follows: 5 million CEM cells were suspended in a tissue culture flask with a density of 10^6 cells/mL in fresh growth media and incubated for various time periods. The cells were counted using the trypan-blue dye exclusion method and were centrifuged to form a cell pellet (1500 rpm, 10 min, 0 °C). Then the supernatant was removed, and cells were treated with 0.5 mL ice-cold 60% methanol−40% 15 mM ammonium acetate buffer (pH 6.65). To this cellular residue was added a known concentration of L-ATO (0.02 μmol). The residue was frozen at −20 °C overnight and dried in vacuo. To the dried cell extract was added 100 μL of 20 mM HEPES (pH 7.2). After addition of 20 pmol of the internal standard L-DPO, samples were diluted 100- or 1000-fold and submitted for LC/MS analysis. For recovery test without cell extract, 0.02 μmol of L-ATO was dissolved in 0.5 mL of ice-cold 60% methanol−40% 15 mM ammonium acetate buffer (pH 6.65). Then, the remained procedure was carried out in the same way as described above. The experimental procedure for measuring percentage recovery of AZT-MP (at 5 nmol) and AZT (at 5 nmol) was carried out in the same way as described above.

Cell Culture. CCRF-CEM cells (human T-lymphoblastoid leukemia cell lines that were purchased from American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, penicillin G (Fisher) (100 units/mL), streptomycin (Sigma) (10 μg/mL), and human interleukin-2 (IL-2) (Boehringer Mannheim, IN) (10 units/mL).

Incubation of Cells with L-/D-ATO and L-/D-APO. Cells were suspended in a tissue culture flask with a density of 10^6 cells/mL in fresh growth media. For each experiment, 5 million cells were incubated with a known concentration of AZT phosphoramidate in 10% CO_2/90% air incubator at 37 °C. At various time intervals, cells were counted using the trypan-blue dye exclusion method and were centrifuged to form a cell pellet (1500 rpm, 10 min, 0 °C). Then the supernatant was removed and the cell pellet was treated with ice-cold 60% methanol−40% 15 mM ammonium acetate buffer (pH 6.65). The residue above was set at −20 °C overnight and then dried in vacuo. One hundred microliters of 20 mM HEPES (pH 7.2) was added to the dried cell extract. The sample above was then diluted, and internal standards (D4T and L-DPO) were added before submission to LC/MS analysis. To prevent contamination of the LC column and MS detector by salts and cellular debris, an online switching method was used.

Results and Discussion

Ion-Pairing Agents. In developing an LC-based assay, an efficient separation profile of each analyte while ionic strength is minimized is important in order to generate the maximum MS signal for a molecule of interest. The use of ion-pairing agents for tuning the retention time of ionic analytes is well-known.[30,32,35,36] In initial studies, we em-

(35) Monkkonen, H.; Moilanen, P.; Monkkonen, J.; Frith, C. J.; Rogers, J. M.; et al. Analysis of an adenine nucleotide-containing metabolite of clodronate using ion pair high-performance liquid chromatography-electrospray ionisation mass spectrometry. *J. Chromatogr. B* **2000**, *738*, 395−403.

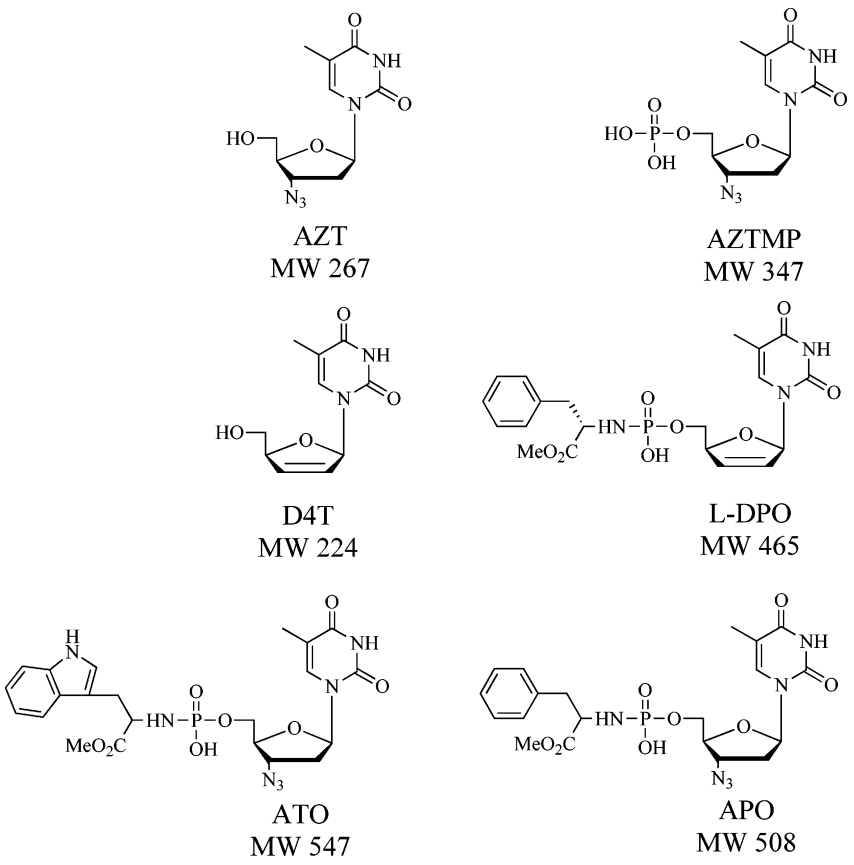

Figure 1. Structures of analytes.

ployed *N*,*N*-dimethylhexylamine (DMHA) to increase the retention times of AZT-MP and the phosphoramidates (Figure 1). The efficiency of this approach depends on the pH of the buffer solution and on the alkyl chain length of the ion-pairing agent. A UV chromatogram of the standard sample with an eluant buffer containing 5 mM DMHA is shown in Figure 2. In general, due to their polar nature, nucleotides (especially di- and triphosphates) tend to elute with the solvent front. However, the retention times of ribonucleotides (AMP, ADP, and ATP) increased with added DMHA.

To examine the effect of DMHA concentration on the ionization, DMHA at 1, 5, and 10 mM was used as a buffer and the signal response of L-ATO, while the DMHA concentration was varied, was monitored. The data suggested that the peak area of L-ATO was highly dependent on DMHA concentration (data not shown). A balance between signal suppression and chromatographic efficiency could only be obtained by optimizing the DMHA concentration. Nevertheless, efficient separation of the adenosine phosphates (mono-, di-, and triphosphates), a likely key contaminant in any intracellular-based assay, was difficult to achieve with DMHA.[13,18] In contrast, when ammonium acetate was employed as an ion pairing agent, AZT, AZT-MP, and the phosphoramidates exhibited an acceptable retention profile, while contaminating adenosine phosphates eluted at the solvent front. Thus, ammonium acetate was employed for the study, and this approach resulted in efficient isolation of the peaks of interest from the major potential intracellular ribonucleotides (AMP, ADP, and ATP) (Figure 3A).

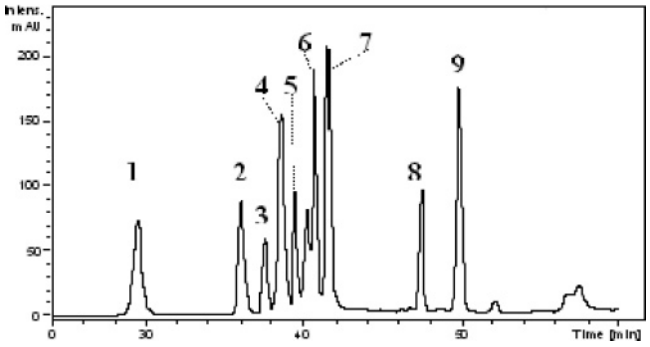

Figure 2. UV chromatogram of the standard sample containing IS1 (**1**), AMP **(2)**, IS2 (**3**), AZT (**4**), ADP (**5**), AZT-MP (**6**), ATP (**7**), IS3 (**8**), and L-ATO (**9**). Solvent A: 5 mM DMHA, pH 7.0. Solvent B: methanol. Gradient: 0−15% MeOH in 25 min, 15−50% MeOH in 40 min, 50−80% MeOH in 60 min at a flow rate of 12 μL/min. IS1: internal standard candidate for metabolites. IS2: internal standard candidate for metabolites. IS3: internal standard candidate for AZT amino acid phosphoramidate.

(36) Auriola, S.; Frith, J.; Rogers, J. M.; Koivuniemi, A.; Monkkonen, J. Identification of adenine nucleotide-containing metabolites of bisphosphonate drugs using ion-pair liquid chromatography-electrospray mass spectrometry. *J. Chromatogr. B* **1997**, *704*, 187−195.

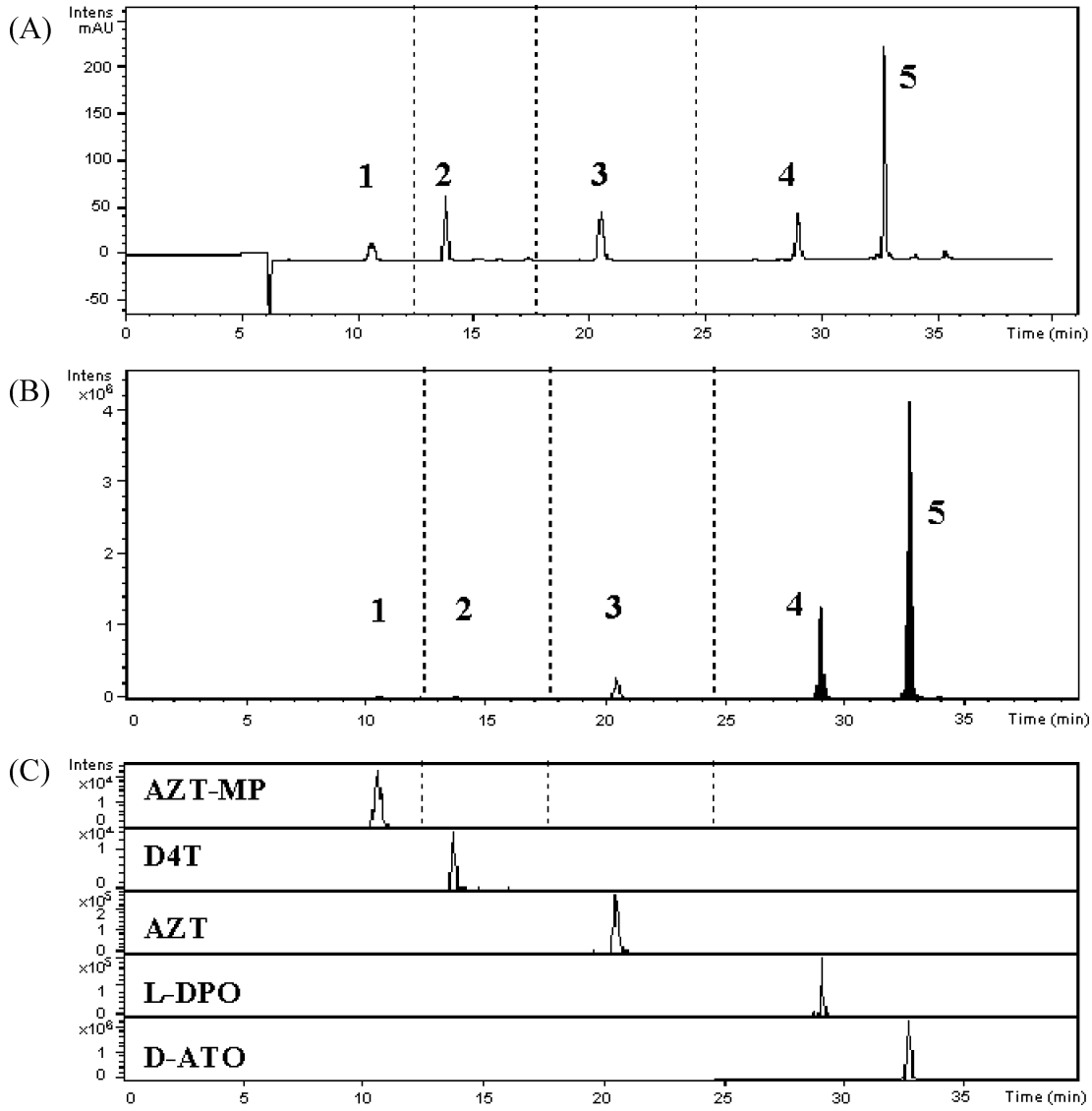

Figure 3. (A) Representative UV chromatogram of a standard sample containing authentic AZT-MP (**1**), D4T (**2**), AZT (**3**), L-DPO (**4**), D-ATO (**5**), AMP, ADP, and ATP (adenosine phosphates eluted with the solvent front). (B) Total ion chromatogram. (C) Extracted ion chromatogram at MRM mode. Solvent A: 15 mM ammonium acetate, pH 6.65. Solvent B: methanol. Gradient: 0−15% MeOH in 5 min, 15−50% MeOH in 25 min, 50−85% MeOH in 30 min, 85−87% MeOH in 40 min, at a flow rate of 12 μL/min.

Analysis of MS. The utility of determining the amounts of intracellular phosphoramidates and their metabolites with positive and negative ion electrospray ionization mode was investigated. MS spectra from ESI-MS experiments carried out in the positive ionization mode with standard samples exhibited unacceptably low sensitivity with a greater degree of adduct formation. In addition, the phosphate species were found to be unstable under acidic conditions required for positive ion mode detection. Spectra obtained in the negative ionization mode, however, generated interpretable simple spectra with greater sensitivity, revealing only the molecular ion $[M - H]^-$.[32,37]

In the LC/MS study carried out with authentic standards, phosphoramidate pronucleotides had significantly higher sensitivity to MS detection than AZT-MP. As shown in Figure 3A, the peak height observed for AZT-MP at 10 pmol on the UV chromatogram is comparable to that of D-ATO at 25 pmol, while the total ion chromatogram of both species at the same concentration revealed that a greater signal intensity was observed for D-ATO than AZT-MP (Figure 3B). Similar behavior was observed for the other phosphoramidates. In order to enhance the signal sensitivity, extraction of the selected ion was carried out at multiple reaction monitoring (MRM) mode (Figure 3C).[21,26,29] MRM mode has been shown to be effective for enhancing ion detection, especially for low-intensity species.

(37) Esmans, E. L.; Broes, D.; Hoes, I.; Lemire, F.; Vanhoutte, K. Liquid chromatography−mass spectrometry in nucleoside, nucleotide and modified nucleotide characterization. *J. Chromatogr. A* **1998**, *794*, 109−127.

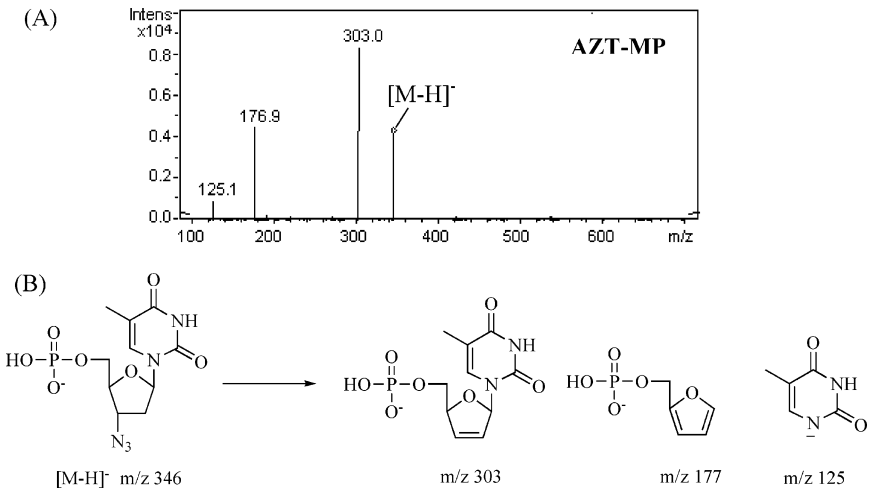

Figure 4. (A) ESI⁻-MS/MS spectrum of authentic AZT-MP at *m*/*z* 346. (B) Proposed fragmentation pathway of the ion at *m*/*z* 346.

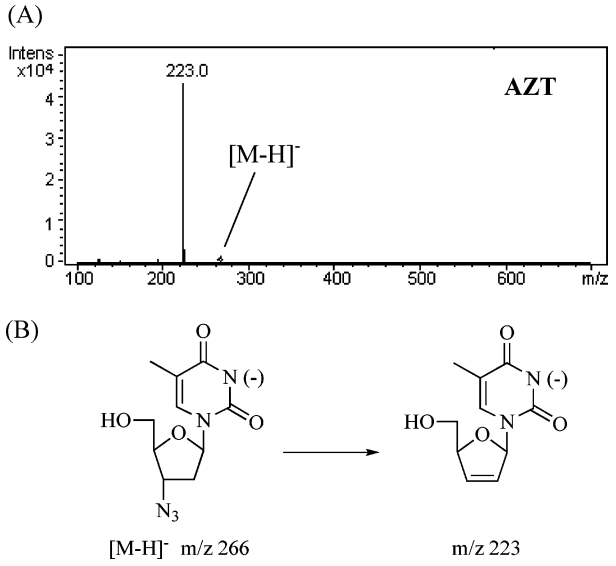

Figure 5. (A) ESI⁻-MS/MS spectrum of authentic AZT at *m*/*z* 266. (B) Proposed fragmentation pathway of the ion at *m*/*z* 266.

Once the selected ion was extracted, the peak area of each analyte was integrated and compared with the peak area of an internal standard. D4T showed an ion abundance/mole ratio similar to that of AZT-MP and AZT, which were lower than the values observed for the phosphoramidates. L-DPO exhibited an ion abundance/mole ratio similar to that of the parent phosphoramidates. Therefore, D4T was chosen as the internal standard for AZT-MP and AZT, and L-DPO for the parent phosphoramidate prodrugs.

The full-scan negative ion mode ESI-MS spectrum of the standard sample containing the authentic AZT-MP, D4T, AZT, L-DPO, and parent prodrugs (ATO or APO) revealed a peak for each deprotonated molecule $[M - H]^-$ at *m*/*z* 346, 223, 265, 464, and 546 or 507, respectively. The identity of the five ions was confirmed by an ion trap instrument at the MS/MS mode. MS/MS spectra of standards were compared with those of the analytes derived from cell

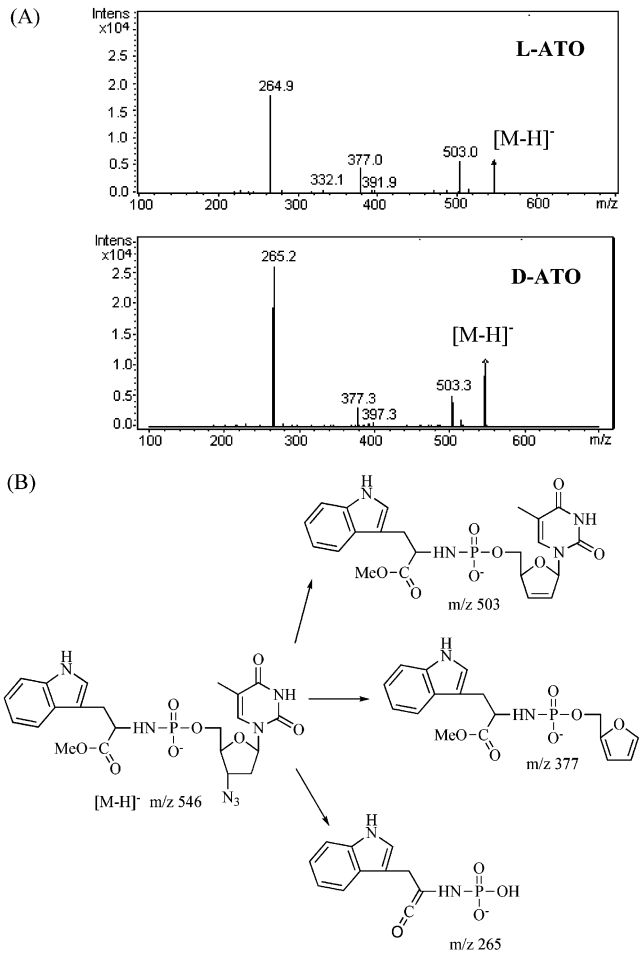

Figure 6. (A) ESI⁻-MS/MS spectrum of authentic L-/D-ATO at *m*/*z* 546. (B) Proposed fragmentation pathway of the ion at *m*/*z* 546.

samples, and quantitation based on the fragment ions generated from MS/MS of molecular ions of the associated analyte.

The ESI-MS/MS spectrum of authentic AZT-MP revealed a peak for the deprotonated molecule $[M - H]^-$ at *m*/*z* 346.

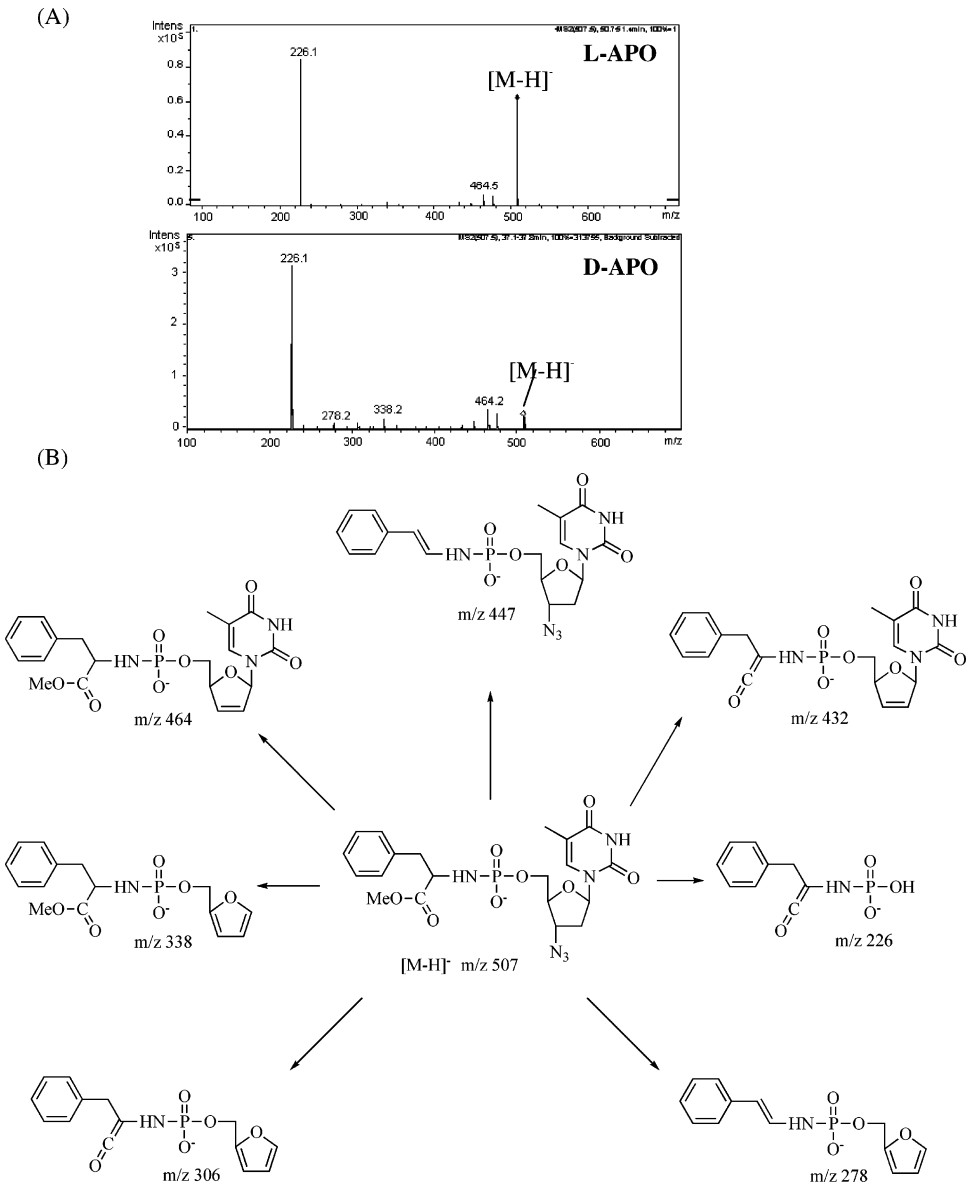

Figure 7. (A) ESI⁻-MS/MS spectrum of authentic L-/D-APO at *m*/*z* 507. (B) Proposed fragmentation pathway of the ion at *m*/*z* 507.

The proposed fragmentation pathway for AZT-MP is shown in Figure 4A. The fragment ions detected in the ESI-MS/MS spectrum of authentic AZT-MP were found at *m*/*z* 125, 177, and 303 (Figure 4A). The major ion, *m*/*z* 303, is likely to be formed through dehydroazidonation of the molecular ion as shown in Figure 4B. Formation of AZT via dephosphorylation of AZT-MP was not observed. The fragment ion, *m*/*z* 177, arises from loss of thymine, since the fragment ion corresponding to thymine (*m*/*z* 125) is observed. For the internal standard D4T, fragment ions at *m*/*z* 125, 150, and 193 were attained by MS/MS of the parent ion at *m*/*z* 223 (see Supporting Information). D4T was also generated from AZT by dehydroazidonation of the molecular ion (Figure 5). For the internal standard, L-DPO, the loss of thymine resulted in a major fragment ion at *m*/*z* 338 (see Supporting Information). Figure 6A represents the negative ion ESI-MS/MS spectrum for D-/L-ATO. Dehydroazidonation again resulted in a fragment ion at *m*/*z* 503, dethymination produced a fragment ion at *m*/*z* 377, and the cleavage of the P−O bond with loss of a methoxy group resulted in a fragment ion at *m*/*z* 265 (Figure 6B). For L-/D-APO, fragment ions similar to those for the internal standard L-DPO were observed (see Supporting Information). In addition, dehydroazidonation resulted in a fragment ion at *m*/*z* 464, and dethymination produced a fragment ion at *m*/*z* 338 (Figure 7). In contrast to the relatively similar fragmentation pattern observed for D- and L-ATO, more fragmentation was monitored for D-APO than for L-APO. As expected, the stereochemistry of the amino acid did not affect the fragmentation pattern of the phosphoramidates, although minor differences in molecular ion peak intensities were observed.

Determination of the Intracellular Amounts of Phosphoramidates and Their Metabolites. Prior to investigating

Table 1. Recovery Percentage of L-ATO, AZT-MP, and AZT during Workup Procedure with Cell Lysate ($n = 3$)

	mol treated	% recovery ± std[a]
L-ATO	0.02	103 ± 33
AZT-MP	0.005	146 ± 24
AZT	0.005	74 ± 6

[a] Standard deviation ($n = 3$).

Table 2. Intracellular Concentration (nmol/5 million cells) of AZT Phosphoramidate, AZT-MP, and AZT

	L-ATO	D-ATO	L-APO	D-APO
		(A) 2 h[a]		
prodrug	106 ± 16	188 ± 18	77 ± 9	82 ± 14
AZT-MP	8.0 ± 0.8	10.0 ± 1.3	5.0 ± 1.4	3.0 ± 0.3
AZT	0.30 ± 0.05	2.0 ± 0.4	2.0 ± 0.4	0.30 ± 0.08
		(B) 4 h[a]		
prodrug	143 ± 23	154 ± 25	97 ± 9	102 ± 10
AZT-MP	11.0 ± 0.2	9.0 ± 0.2	8.0 ± 3.4	3.0 ± 0.6
AZT	0.50 ± 0.12	1.0 ± 0.1	3.0 ± 1.2	0.50 ± 0.16

[a] Five million CEM cells were incubated with each phosphoramidate at 2.5 mM for (A) 2 h and (B) 4 h ($n = 3$).

the intracellular metabolism of the phosphoramidate prodrugs, the recovery of L-ATO, AZT-MP, and AZT with or without CEM cell lysate was determined. As shown in Table 1, approximately 100% of the L-ATO and AZT-MP was recovered, while 74% of the added AZT was recovered. In order to determine the intracellular levels of phosphoramidates and their metabolites, CEM cells were incubated with D-/L-ATO or D-/L-APO for 2 and 4 h, respectively, and then subjected to analysis. L- and D-ATO accumulated in CEM cells to a greater extent than L- and D-APO (Table 2). The intracellular levels of phosphoramidates, typically, reached steady state by 2 h. AZT-MP was observable for both sets of phosphoramidates and accumulated to levels corresponding to approximately 4−8% of the amounts of intracellular phosphoramidate. Low concentrations of AZT were observed for each phosphoramidate, with the highest percentage found for incubations with L-APO and the least with L-ATO.

Conclusions

A reverse-phase liquid chromatography/mass spectrometry method has been successfully used for the identification and quantitation of intracellular phosphoramidates and their metabolites. Structural identification of these analytes in CEM cell extracts was confirmed by capillary HPLC/MS/MS experiments utilizing MRM mode. Although we have developed this protocol for AZT phosphoramidate pronucleotides, it is likely that the intracellular behavior of other phosphoramidates could be characterized by the same methodology with minor modifications.

Abbreviations Used

AZT, 3′-azido-3′-deoxythymidine; AZT-MP, 3′-azido-3′-deoxythymidine monophosphate; ATO, AZT tryptophan methyl ester phosphoramidate; APO, AZT phenylalanine methyl ester phosphoramidate; ESI, electrospray ionization; LC/MS, liquid chromatography−mass spectrometry; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; DMHA, *N*,*N*-dimethylhexylamine; TEA, triethylamine; MRM, multiple reaction monitoring mode; D4T, 2′,3′-didehydro-3′-deoxythymidine; DPO, D4T phenylalanine methyl ester phosphoramidate; mu, mass unit; CID, collision-induced dissociation.

Acknowledgment. The authors are thankful to Dr. Balfour's group in the ACTU unit in the Department of Laboratory Medicine and Pathology at the University of Minnesota for providing PBMCs. This research is supported by NIH-NCI (CA 89615).

Supporting Information Available: ESI-MS/MS spectra of authentic D4T at *m*/*z* 223 and of L-DPO at *m*/*z* 464 and proposed fragmentation pathways of ions at m/z 223 and 464, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

MP0500162