Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 831 (2006) 248–257
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantitation of zidovudine triphosphate concentrations from human peripheral blood mononuclear cells by anion exchange solid phase extraction and liquid chromatography–tandem mass spectroscopy; an indirect quantitation methodology

Tracy King, Lane Bushman, Peter L. Anderson, Thomas Delahunty, Michelle Ray, Courtney V. Fletcher *

Antiviral Pharmacology Laboratory, Department of Clinical Pharmacy, School of Pharmacy, University of Colorado Health Sciences Center, Box C238, 4200 East 9th Avenue, Denver, CO 80262, USA

Received 7 September 2005; accepted 8 December 2005
Available online 10 January 2006

Abstract

To facilitate the assessment of drug safety and determination of phamacokinetics, an anion exchange isolation of zidovudine triphosphate (ZDV-TP) from human peripheral blood mononuclear cells (hPBMC), coupled with dephosphorylation, desaltation, and detection by liquid chromatography–tandem mass spectroscopy (LC–MS–MS) was validated. hPBMCs were harvested from whole blood, lysed, and a suspension of intracellular ZDV-TP was produced. ZDV-TP was isolated from ZDV, ZDV-monophosphate (ZDV-MP), and ZDV-diphosphate (ZDV-DP), which were all present in the cell lysate, by performing a salt gradient anion exchange SPE. Isolated ZDV-TP was dephosphorylated with acid phosphatase to its parent drug form, ZDV. ZDV was then desalted and concentrated for tandem mass spectral detection. An LC–MS–MS methodology was developed and validated for the determination of molar ZDV directly corresponding to the intra-hPBMC molar ZDV-TP concentration. ZDV-TP concentrations were determined in femtomoles per million hPBMCs (fmol/10^6 cells). The assay was able to determine ZDV-TP concentrations accurately and precisely within the range of 5–640 fmol/10^6 cells with 10 million cells per sample analyzed. Inter- and intra-day accuracy and precision data for back calculated standards and quality controls fell within 15% of nominal. The assay correlated well with a previous ELISA method developed and validated in our laboratory, and has been successfully used to quantitate ZDV-TP concentrations in patients being routinely monitored and treated with ZDV.

Keywords: LC–MS–MS; Zidovudine-triphosphate; hPBMC

1. Introduction

Nucleoside reverse transcriptase inhibitors (NRTI) were the first agents shown to be safe and effective for the treatment of patients infected with HIV, and they remain cornerstones of most treatment regimens today. These drugs are selectively phosphorylated to their active triphosphate moieties (NRTI-TP) within human peripheral blood mononuclear cells (hPBMC) [1]. It is the intracellular anabolite that competes with endogenous nucleotides for incorporation into the replicating HIV DNA, which results in termination of viral replication [2]. Zidovudine (ZDV), a thymidine NRTI, was the first anti-HIV drug approved by the FDA. It remains an important NRTI backbone component of many first-line HAART therapies used today [3]. The ability to detect and quantitate the NRTI-TP concentrations in hPBMCs will lead to a better understanding of the pharmacokinetic and pharmacodynamic characteristics of these agents. This understanding may potentially facilitate improved therapeutic approaches to inhibit viral replication, limit emergence of viral resistance, and reduce serious side effects. Generally, a few methods have been published for the determination of NRTI-TPs, some direct detection methodologies of the NRTI-TPs [4–6] and other indirect detection methodologies that isolate the NRTI-TP and dephosphorylate to the parent NRTI [7–10]. The

* Corresponding author. Tel.: +1 303 315 5229; fax: +1 303 315 1721.
E-mail address: courtney.fletcher@uchsc.edu (C.V. Fletcher).

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.12.033

direct methodologies utilize LC–MS–MS detection whereas the indirect dephosphorylation methodologies have utilized RIA, ELISA, and LC–MS–MS quantitation techniques. We previously validated an indirect methodology for the quantitation of ZDV-TP that utilized both RIA and ELISA detection methods [9]. These methods were accurate and precise, but the commercial unavailability of the tracer used in these methodologies has eliminated these modes of detection. Given the continued importance of ZDV in the treatment of HIV, we sought to develop a new method to detect ZDV-TP in PBMCs of patients. Herein, we present the development and validation of a novel ZDV-TP assay. We describe the issues that arose in these efforts and we present successful application of this new methodology to real-time patient samples.

2. Experimental

2.1. Chemicals and supplies

ZDV-TP and internal standard, isotopic ZDV (2 × N15 and 1 × C13), were obtained from Moravek Biochemicals (Brea, CA). ZDV was obtained from Sigma Chemical (St. Louis, MO). Methanol, ammonium acetate, and acetonitrile, all HPLC grade, were obtained from Fisher Scientific (Fairlawn, NJ). Glacial acetic acid, potassium chloride, sodium acetate, all A.C.S. certified, and acid phosphatase type XA from sweet potato were obtained from Sigma Chemical (St. Louis, MO). Deionized/distilled water was house purified on a Barnstead Mega-Pure System. Waters OASIS® HLB Extraction Cartridges, 3cc, and Waters Accell™ Plus QMA Cartridges, 3cc, were purchased from Waters Corporation (Milford, MA). Nitrogen gas (99.9% pure) and argon gas (99.999% pure) were supplied by General Air (Denver, CO). Citrate cell preparation tubes (CPT) were obtained from Fisher Scientific (Fairlawn, NJ).

2.2. Instrumentation

The liquid chromatography system consisted of a Waters 2690 Separations Module (Waters Corporation, Milford, MA), equipped with an inline degasser, temperature controlled autosampler, and column oven. The analytical column was a Waters XTerra™ RP_{18} 3.5 μm, 2.1 mm × 150 mm (Waters Corporation, Milford, MA). The mobile phase consisted of aqueous 0.1% acetic acid and 10% acetonitrile at a flow rate of 0.2 mL/min. The mass analyzer was a TSQ Quantum Triple Quadrupole Mass Spectrometer equipped with an ESI source in the positive ionization mode utilizing the stainless steel spray needle (ThermoElectron, San Jose, CA). Data were collected using Xcalibur Software version 1.3 (ThermoElectron, San Jose, CA).

2.3. Standard and quality control preparation

ZDV working standards were prepared in water at concentrations ranging from 2.0 to 256 fmol/μL. Internal standard working solution was prepared in water at 8.55 ng/mL. The standards were stored at 4 °C and were stable for over 1 year. ZDV-TP quality controls were prepared in blank hPBMC cell lysate (10×10^6 cells/250 μL of lysate). The concentration of ZDV-TP controls were 100, 600, and 3000 fmol (ZDV-TP corresponding to 10, 60, and 300 fmol per million cells). The ZDV-TP quality controls were stored at −80 °C and were stable for over 1 year.

2.4. Determination of ZDV-TP

2.4.1. hPBMC collection and preparation (patient specimens and blank hPBMC processing)

hPBMCs were harvested within 60 min of blood collection. For each collection, approximately 25 mL of blood were collected in three cell preparation tubes (CPT). After gentle mixing, the tubes were centrifuged at 1500 × *g*, 20 min, 21 °C. After the top two layers were gently mixed, the remaining solution above the gel was decanted, the volume was recorded, and a small aliquot was used for cell counting with a hemacytometer. The solution was centrifuged at 400 × *g*, 10 min, 4 °C. All but about 1 mL of the supernatant was removed. The cells were resuspended and centrifuged at 400 × *g*, 10 min, 4 °C. The supernatant was completely removed and the cells were lysed with cold 0.5 mL 70% methanol/30% water. After pellet disruption and vortexing, the extraction was allowed to proceed for 15 min in an ice bath or at −20 to −80 °C refrigeration. The lysate solution was centrifuged at 13,000 × *g*, 1 min, 21 °C and the supernatant was transferred to a cryovial for storage at −80 °C until assayed.

2.4.2. ZDV-TP isolation (quality controls, validation samples, and patient specimens)

ZDV-TP was isolated from ZDV, ZDV-MP, and ZDV-DP in the cell lysis media by anion exchange SPE and a salt gradient. Cell lysate (equivalent to 10×10^6 cells) was applied to a Waters QMA SPE cartridge, previously prepared with ddH_2O (2 mL), 1 M KCL (1.5 mL), and 5 mM KCl (2 mL) solutions, and centrifuged at 100 × *g*, 2 min, 21 °C. Nine (2 mL) 74.5 mM KCl washes, followed by centrifugation at 100 × *g*, 2 min, 21 °C, were applied to the cartridge. The nine washes eluted ZDV, ZDV-MP, and ZDV-DP. The isolated ZDV-TP still remaining was eluted from the QMA cartridge with 1 M KCl (2 mL) centrifuged at 100 × *g*, 2 min, 21 °C into a collection tube.

2.4.3. Dephosphorylation

The isolated ZDV-TP solution (2 mL of 1 M KCl) was dephosphorylated to ZDV with the addition of acid phosphatase/sodium acetate working stock solution (0.1 mL) and incubated at 37 °C for 1 h. Stock acid phosphatase was diluted appropriately with 1 M sodium acetate solution, pH 5.0 into a working stock solution so that when 0.1 mL of the working stock was added to each sample, 0.4 units were applied. Working acid phosphatase solution was prepared prior to each extraction.

2.4.4. Standard curve preparation/desaltation and concentration of ZDV

ZDV standards at concentrations of 50–6400 fmol (25 μL of working ZDV standard) were prepared in 2.1 mL of 1 M

KCl/sodium acetate solution analogous to that used for QMA elution and dephosphorylation of the isolated ZDV-TP fractions. The dephosphorylated samples and standards were desalted and concentrated from the KCl solution making it possible for tandem mass spectral detection. Internal standard working stock (10 μL of 8.55 ng/mL) was added to all tubes. The samples were applied to prepared Waters Oasis HLB SPE cartridges (prepared by the addition of 2 mL of methanol, followed by 2 mL of ddH_2O) and centrifuged at 100 × *g*, 2 min, 21 °C. Three water (3 mL) washes, followed by centrifugation at 100 × *g*, 2 min, 21 °C, successfully desalted the solution. ZDV was eluted with methanol (1 mL). The sample was dried under nitrogen on a Zymark TurboVap at 37 °C for 30 min, reconstituted in mobile phase (100 μL) and injected onto the LC–MS–MS system (40 μL).

2.4.5. LC–MS–MS conditions

The chromatographic separation was performed on a Waters X-Terra, 2.1 mm × 150 mm, reversed phase column with a 3.5 μm particle size. The mobile phase consisted of a mixture of aqueous acetic acid (0.1%) and acetonitrile (10%) at a flow rate of 0.2 mL/min. The signal was achieved in positive ion mode with electro-spray ionization and SRM detection. The MS source and quadrupole conditions were as follows: spray voltage 1800 V, nitrogen sheath gas 30 and auxiliary gas 10 (arbitrary units), capillary temperature 310 °C, chrom filter peak width 30 s, collision gas pressure 1.0 mTorr, scan width 1.0 *m/z*, scan time 0.1 s, collision energy 10 V, quadrupole resolution was Q1:0.3 FWHM, Q3: 0.7 FWHM(H-SRM), [M + H] parent/product 268/127 *m/z* (ZDV) and 271/130 *m/z* (ZDV-IS). ZDV and its isotopic IS approximate retention time was 7.03 min. The assay was linear in the range of 50–6400 fmol ZDV/sample with a minimum quantifiable limit of 50.0 fmol/sample (equivalent to 5.0 fmol per million cells when 10 million cells were extracted).

2.5. Validation of hPBMC sample analysis

Validation samples at five different ZDV-TP concentrations were produced in 70% methanol:30% water blank hPBMC lysate (10×10^6 cells/250 μL of lysate), aliquoted, and stored at −80 °C for a full validation of this assay. The lower limit of quantitation (LLOQ) validation sample concentration was 50 fmol, the Low-1 validation sample concentration was 100 fmol, the Low-2 validation sample concentration was 300 fmol, the medium validation sample concentration was 1000 fmol, and the high validation sample concentration was 3000 fmol. The assay was validated for inter- and intra-day accuracy and precision (*n* = five validation samples on 5 different days), conditional stability, recovery, and matrix and interference effects.

2.5.1. Inter-day average back calculated calibration standards

Each validation run contained singlet calibration standards with theoretical concentrations of 6400, 3200, 1600, 800, 400, 200, 100, and 50 fmol/sample. All eight standard concentrations were run in singlet on 5 separate days.

2.5.2. Accuracy and precision

The accuracy and precision of this method were assessed by analyzing five validation sample levels in five replicates on 5 different assay days. The LLOQ validation sample acceptance criteria were ±20% for accuracy and precision. All other validation sample acceptance criteria were ±15% for accuracy and precision.

2.5.3. Conditional stability

The Low-2 and high validation samples were subjected to various conditions in order to test stability. First, freeze/thaw stability for three cycles was tested. Basically, this tested the warming of the 70% methanolic cell supernatant to room temperature for three cycles versus a traditional plasma freeze/thaw cycle since the supernatant does not freeze at storage conditions. The validation samples were "frozen" (−80 °C) for 24 h and "thawed" completely at room temperature. This freeze/thaw cycle was repeated three times. In addition, different samples were removed from storage and allowed to sit at room temperature for four hours to test for room temperature stability in the lysate media. A 4 h interval was checked to test for bench stability during extraction. The validation samples were then extracted and run in triplicate and compared with an extracted control triplicate set of samples frozen (−80 °C) and thawed immediately prior to the run. In addition to freeze/thaw cycles and room temperature stability, extracted sample stability was tested also. The same sets of Low-2 and high validation sample subjected to three freeze/thaw cycles and 4 h at room temperature were allowed to remain in the autosampler (4 °C) for 5 days. These samples were compared with a freshly extracted control set of high and Low-2 validation samples.

2.5.4. Recovery

Recovery for this procedure was comprised of both the front end anion exchange on the QMA and also the desaltation step using the Oasis HLB cartridge. Therefore, both isolation of the ZDV-TP and recovery of ZDV were assessed.

2.5.4.1. QMA recovery determination. QMA recovery was performed utilizing an ion pairing UV methodology to characterize the parent, MP, DP and TP elution in the washes. The column used was a Keystone BetaBasic-18, 250 × 3, 5 μm (Keystone Scientific, Inc., Bellefonte, PA) and the mobile phase was 85% 100 mM phosphate, 4 mM tetrabutylammoniumbromide, pH 6.0:15% acetonitrile at a flow rate of 0.5 mL/min. Detection was at 260 nm. In order to determine the correct number of washes to accurately remove ZDV, ZDV-MP, and ZDV-DP and retain the maximum amount of ZDV-TP on the QMA SPE cartridge, the following experiment was performed: known amounts of ZDV-TP, ZDV-DP, ZDV-MP, and ZDV were loaded onto a prepared QMA SPE cartridge. Each 74.5 mM KCl (2 mL) wash was eluted and saved for analysis on the LC system. The amount of each phosphate moiety in each elution was recorded and compared to 100% signal in the wash medium.

2.5.4.2. Desaltation/concentration recovery determination. ZDV recovery was compared between two different types of

SPE cartridges available in the laboratory: Oasis HLB 3cc and Oasis MAX 3cc. This recovery experiment was performed using 1000 and 10,000 fmol/sample of ZDV. Area response was measured in triplicate for unextracted/spiked sample, extracted first methanol elution (0.5 mL), and extracted second methanol elution (0.5 mL).

2.5.5. Enzyme incubation time determination

In order to determine the minimum amount of incubation time necessary to produce the maximum amount of ZDV from ZDV-TP using acid phosphatase enzyme, the following experiment was performed. A solution of 24 μM ZDV-TP (100 μL), 400 mM KCL (375 μL), and a diluted enzyme solution (25 μL) was incubated at 37 °C. At time points 0, 15, 30, 45, 60, and 90 min, an aliquot (50 μL) of the solution was removed and 20 μL was injected onto an LC system specifically established for ZDV detection.

2.5.6. Comparison to previous ELISA methodology

The laboratory previously validated an indirect detection method for the determination of ZDV-TP concentrations in hPBMCs using the QMA isolation, dephosphorylation, and desalting technique as described above and applied an ELISA detection method. Thirty-five patient specimens that were previously analyzed by the ELISA method were analyzed by the method presented herein and compared. These patient specimens were stored in 70% methanol at −80 °C between analyses.

2.5.7. Clinical application

hPBMC samples from HIV-infected patients who were receiving a dose of 300 mg twice daily of ZDV were analyzed for ZDV-TP using the method described herein. The time elapsed from dosing time to blood draw varied from 0 to 8 h post dose.

3. Results

3.1. Inter-day average back calculated calibration standards

The ZDV to ZDV-IS ratio were plotted against concentration for each calibration curve. The experimental standard concentrations were back calculated using 1/concentration weighted linear regression curve. The resulting data are shown in Table 1. Standard back calculated values outside 15% from the nominal and 20% at the LLOQ were dropped and the standard curve was recalculated.

The inter-assay experimental mean for each calibration standard was compared against the theoretical concentration and percent deviation was calculated. The average percent deviation across all the concentrations was less than 6%. Precision statistics were represented by the standard deviation and the coefficient of variance (CV) data. Overall, the %CV for all concentrations was less than 6.1%. The slope, *y*-intercept, and coefficient of determination (R^2) values for each curve are also displayed in Table 1. The %CV for the five slopes was 0.0045 and all R^2 values were ≥0.9996. Overall, the standard curve responses for this assay were reproducible as well as significantly close to the theoretical values. Therefore, it was determined that the eight back-calculated concentrations of the standard curve could be used to accurately and precisely determine unknown sample concentrations used in this assay.

3.2. Accuracy and precision

Intra- and inter-assay accuracy and precision were tested for each ZDV-TP validation sample concentration (Table 2). Accuracy was the measure of deviation of the calculated concentration from the theoretical and precision was measured by calculating the coefficient of variance. Accuracy and precision acceptable limits were within ±20% at the LLOQ and within

Table 1
Inter-day average back calculated calibration standards

	Slope ($\times 10^3$)	Y-intercept	R^2
Run ID			
1	0.644	−0.0140	0.9996
2	0.618	−0.0153	0.9996
3	0.665	−0.0175	0.9996
4	0.648	−0.0055	0.9997
5	0.671	−0.0072	0.9996

AZT-TP	Cal 1-Std A	Cal 2-Std B	Cal 3-Std C	Cal 4-Std D	Cal 5-Std E	Cal 6-Std F	Cal 7-Std G	Cal 8-Std H
Theoretical concentration (fmol)	6400	3200	1600	800	400	200	100	50
Mean	6424	3200	1574	815	393	191	99	53
S.D.	74.9	45.7	31.5	16.8	15.2	8.9	6.0	2.1
%CV	1.17	1.43	2.00	2.06	3.88	4.66	6.09	4.01
%dev	0.37	0.00	−1.64	1.87	−1.83	−4.43	−1.22	5.96
n	5	5	5	5	5	4	5	5

Table 2
AZT-TP accuracy and precision

	LLOQ	Low VS-1	Low VS-2	Medium VS	High VS
Intra-assay statistics					
Run ID					
1					
Mean	49.8	104	302	1058	3016
S.D.	4.03	7.68	10.74	28.87	129.32
%CV	8.08	7.39	3.56	2.73	4.29
%dev	−0.34	3.95	0.57	5.79	0.53
n	5	5	5	5	5
2					
Mean	63.9	111	314	1055	3182
S.D.	3.06	4.88	16.94	35.56	60.24
%CV	4.79	4.39	5.40	3.37	1.89
%dev	27.78	11.12	4.56	5.53	6.08
n	5	5	5	5	5
3					
Mean	55.0	97.4	302	1014	3071
S.D.	3.57	6.44	4.96	23.17	82.07
%CV	6.50	6.61	1.65	2.29	2.67
%dev	9.97	−2.58	0.51	1.37	2.36
n	5	5	5	5	5
4					
Mean	43.7	85.9	308	1057	3051
S.D.	4.86	3.20	4.49	25.57	21.98
%CV	11.13	3.73	1.46	2.42	0.72
%dev	−12.70	−14.14	2.81	5.74	1.70
n	5	5	5	5	5
5					
Mean	61.7	104	294	961	2799
S.D.	10.28	4.95	16.01	15.91	93.33
%CV	16.64	4.75	5.44	1.66	3.33
%dev	23.48	4.12	−1.87	−3.90	−6.69
n	5	5	5	5	5
Inter-assay statistics					
Theoretical conc. (fmol)	50	100	300	1000	3000
Mean	54.82	100	304	1029	3024
S.D.	9.29	10.09	12.74	45.70	149.66
%CV	16.94	10.04	4.19	4.44	4.95
%dev	9.64	0.49	1.32	2.91	0.80
n	25	25	25	25	25

±15% at all other levels tested. The greatest mean inter-assay percent deviation for ZDV-TP was 9.64% for the LLOQ validation sample concentration. All non-LLOQ validation sample mean inter-assay percent deviations were less than 3%. The highest mean inter-assay %CV for ZDV-TP was 16.94% for the LLOQ validation sample. All non-LLOQ validation sample mean inter-assay %CVs were less than 10.1%. The greatest mean intra-assay percent deviation was 27.78% for the LLOQ validation sample on Run 2. All non-LLOQ validation sample mean intra-assay percent deviations were less than 14.2%. The highest intra-assay %CV was 16.64% for the LLOQ validation sample on Run 5. All non-LLOQ validation sample CV values were less than 7.4%. As expected, it was observed that the 50 fmol validation sample showed the highest % deviation and %CV values compared with the other validation sample percentages. The 50 fmol validation sample exceeded the 20% acceptance criterion for accuracy on one of the five validation runs. Four of the five validation runs were within the 20% criterion and was thus defined as the LLOQ for the assay. Table 2 demonstrates that the assay was both accurate and precise between runs and within individual runs. The LLOQ response for ZDV-TP was at least five times greater than the blank response for all validation runs. In addition, the isotopic ZDV internal standard did not give a response in the ZDV detection window.

3.3. Conditional stability

Conditional stability results are presented in Table 3. Mean, standard deviation, %CV, and % deviation were calculated for each triplicate Low-2 and high validation sample. From the mean values of these triplicates, a percent difference (treated

Table 3
Conditional stability

	Low VS	High VS	Low VS: %diff treated vs. control	High VS: %diff treated vs. control
3 F/T cycles				
Treated				
Theoretical concentration	300	3000	2.5	3.2
Mean	316	3148		
S.D.	13.1	84.3		
%CV	4.2	2.7		
%dev	5.4	4.9		
n	3	3		
Controls				
Theoretical concentration	300	3000		
Mean	308	3051		
S.D.	4.5	22.0		
%CV	1.5	0.7		
%dev	2.8	1.7		
n	5	5		
4 h room temperature				
Treated				
Theoretical concentration	300	3000	1.1	1.0
Mean	312	3081		
S.D.	4.5	40.2		
%CV	1.4	1.3		
%dev	3.9	2.7		
n	3	3		
Controls				
Theoretical concentration	300	3000		
Mean	308	3051		
S.D.	4.5	22.0		
%CV	1.5	0.7		
%dev	2.8	1.7		
n	5	5		
5 day autosampler				
Treated				
Theoretical concentration	300	3000	2.2	6.3
Mean	301	2975		
S.D.	9.6	41.0		
%CV	3.2	1.4		
%dev	0.3	−0.8		
n	6	6		
Controls				
Theoretical concentration	300	3000		
Mean	294	2799		
S.D.	16.0	93.3		
%CV	5.4	3.3		
%dev	−1.9	−6.7		
n	5	5		

versus control) was calculated for the Low-2 and high levels. The greatest percent difference between the treated and control samples was 6.3% for that of high validation sample treated to 5 days in the autosampler. This experiment showed that the Low-2 and high validation samples were stable under the tested temperature and time conditions both prior to extraction and post extraction. Stability was shown for three freeze/thaw cycles, room temperature for 4 h, and extracted sample stability for up to 5 days in the autosampler, which was maintained at 4 °C.

3.4. Recovery

3.4.1. QMA recovery determination

It was necessary to wash the QMA cartridge with 18 mL of 74.5 mM KCl solution. This was accomplished by performing nine (2 mL) washes with 74.5 mM KCl. SPE preparation, washes, and elutions were performed in a Jouan centrifuge programmed at 100 × *g* for 2 min at 21 °C. Using the centrifuge we were able to process up to 72 samples at a time and subject each SPE to the same relative force/flow rates during the extraction

procedure. We determined after nine (2 mL) KCl washes 99.3% triphosphate remained with only 0.6% diphosphate remaining on the cartridge. After 10 (2 mL) KCl washes, 95% triphosphate remained and 0.2% diphosphate remained. The assay was validated with nine KCl washes instead of 10 so that the loss of ZDV-TP would be minimized, with less than 1% ZDV-DP still remaining on the QMA cartridge for elution.

3.4.2. Desaltation/concentration recovery determination

The means of the two methanol elution areas were added together and compared with the mean of the unextracted/spiked sample areas to show total percent recovery. The MAX 1000 and 10,000 fmol sample percent recovery equaled 74.5 and 79.6%, respectively. The HLB 1000 and 10,000 fmol sample percent recovery equaled 82.9 and 90.3%, respectively. The recovery percent difference between the two cartridges was −10.19% for the 1000 fmol sample and −11.86% for the 10,000 fmol sample. This experiment showed the HLB cartridge performed with a higher overall recovery of ZDV than the MAX. Therefore, the HLB cartridge was the cartridge used in the protocol. Recoveries between two (0.5 mL) methanol elutions and one (1 mL) methanol elution were identical. For time saving purposes, a one (1 mL) elution was used in validation.

3.5. Enzyme incubation time determination

The ZDV area and height responses were recorded versus time and maximum ZDV production was determined to be after the 30 min time point. The production of ZDV did not change significantly from the 30 min time point through the 90 min time point. Additionally, incubation was allowed to continue overnight and the response was not different from the 30 min time point. We also compared ZDV response for a 30 min incubation between separate equimolar ZDV and ZDV-TP samples. The results showed that ZDV-TP was completely converted to ZDV in the 30 min incubation.

3.6. Comparison to previous ELISA methodology

We analyzed 35 samples with the current LC–MS–MS methodology that had been previously analyzed with the ELISA method. We found a good correlation between the two methods

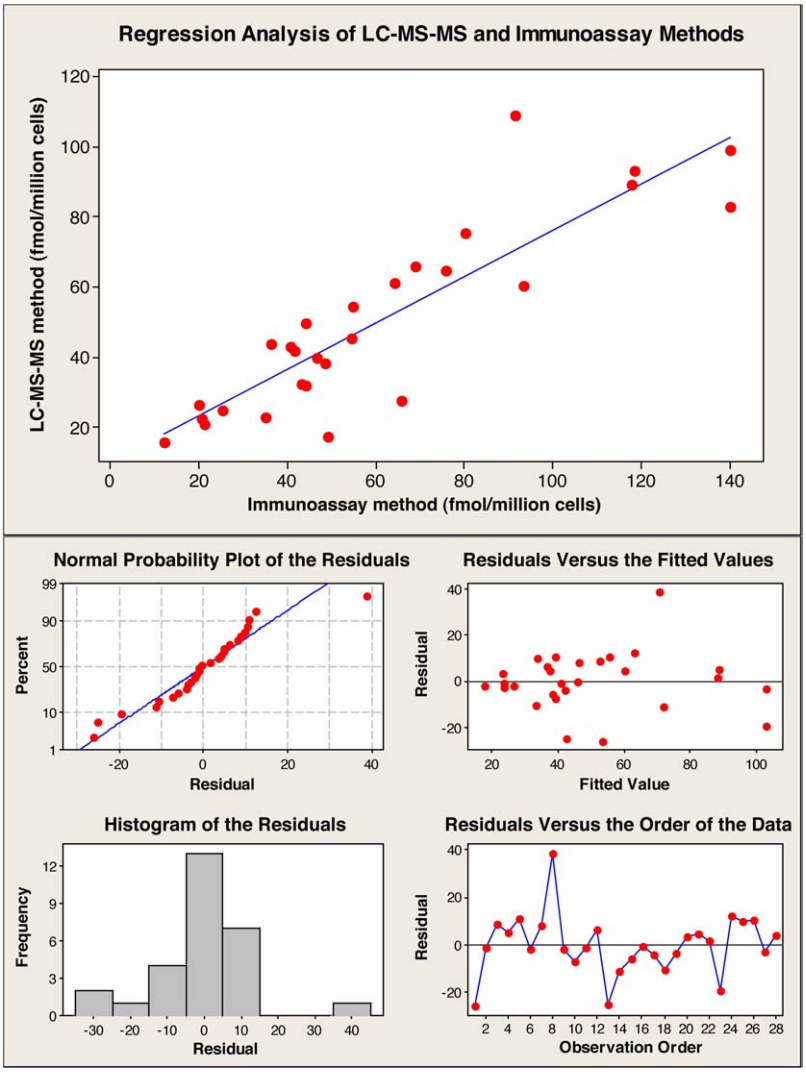

Fig. 1. Comparison data of previous RIA method to current LC–MS–MS Method.

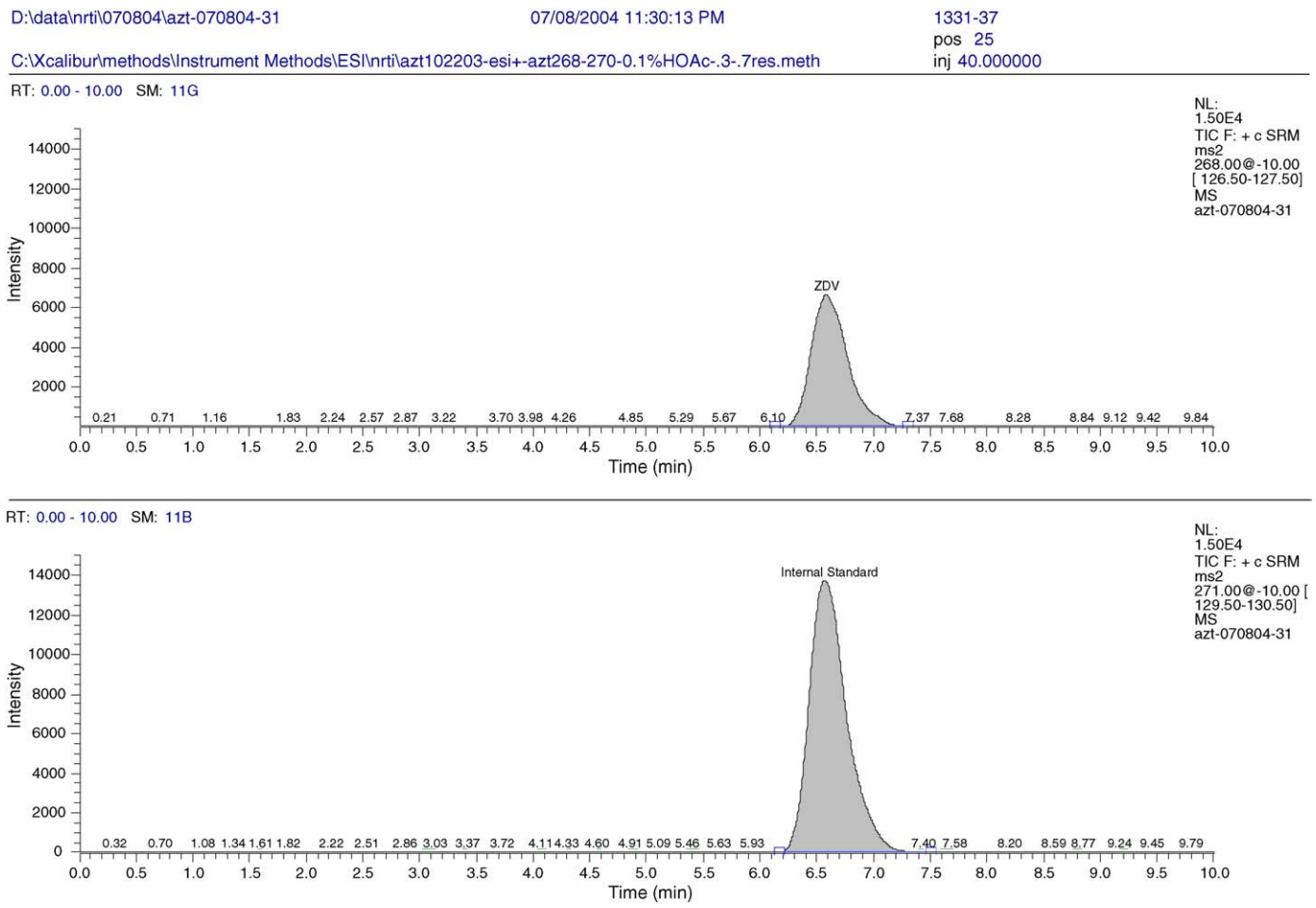

Fig. 2. Typical patient chromatogram of ZDV-TP (76.7 fmol/million hPBMC).

(Fig. 1). Furthermore, the samples being analyzed were stored at −80 °C in the 70:30 methanol:water lysate for over 5 years. In addition to the good correlation, the data presented also provide evidence of long-term stability of ZDV-TP in the lysate solution used when stored at −80 °C.

3.7. Clinical application

The current methodology has been applied to real-time patient specimens. A typical chromatograph can be seen in Fig. 2. The samples were drawn in CPTs between 0 and 8 h post ZDV dose. A median ZDV-TP concentration of 22.5 fmol/million cells was determined among 55 patient samples analyzed.

4. Discussion

It was our intention to develop and validate a direct quantitation method for ZDV-TP from hPBMCs. Such a method would be potentially faster and perhaps more precise with the elimination of several steps used in the indirect detection methodologies. However, developmental attempts for determining ZDV-TP concentrations directly from patient hPBMCs were unsuccessful. We found that published direct methodologies could not be replicated in our laboratory because of supply and methodology issues. The supply obstacles included the unavailability of a ZDV-TP specific SPE cartridge used by Becher et al. and no access to MALDI instrumentation as used by Van Kampen et al. [11].

There were also multiple methodological obstacles that limited our ability to replicate published methods. First, ATP levels were very high in the hPBMC lysate. Volatile ion pairing agents were able to separate ATP from ZDV-TP, but the large amount of ATP on the column caused a continuous column bleed, indicative of ATP column overloading, which interfered with detection of ZDV-TP in the patient cellular extracts. Normally, this would not be an issue for tandem mass spectrometry detection (notwithstanding matrix effects), but in the case of ZDV-TP detection, ATP and dGTP (endogenous nucleotides found in the hPBMC lysate) have the same precursor ion (506 *m*/*z*) and the same resulting major product ion (159 *m*/*z*) in the ESI negative ion mode. Unique minor ZDV-TP precursors exist but the resulting signals are too low for quantitation of ZDV-TP in the femtomolar range. Attempts to eliminate or reduce the ATP content with chemical treatment (periodate) were successful in that they did eliminate much of, or all of the ATP while not affecting the ZDV-TP, but sensitivity remained a factor probably because the ion pairing content suppressed ZDV-TP signal.

Generally, the use of volatile ion pairing agents was a methodological obstacle. The agents caused ion suppression in the ESI source and they were nearly impossible to wash from the system. We have seen a bleed of some of these ion pairing agents well over 1 year after their use on the analytical system. We believe this led to carryover issues of the TP analytes seen over time. We suspect the ion pairing agents adhere to the system and create binding sites for the TP analogues. This caused carryover issues that hindered our development of this methodology.

For these reasons we decided to turn our efforts to replacing the ELISA detection method with LC–MS–MS detection and to continue utilizing an indirect methodology previously validated and used in our laboratory. This method, while more labor intensive for sample preparation than the direct methodologies, allows for the quantitation of ZDV-TP at the necessary concentrations found in hPBMCs of patients. The method also allows for a built-in quality assurance (QA) of the ZDV-TP stocks used and the extraction process by using the parent ZDV as the standard and the ZDV-TP as the QC. Importantly, we have found significant deviations from the purity and potency reported on the certificate of analysis for these triphosphate stock solutions. Most of these deficiencies will be detected with indirect assays, as this methodology uses parent NRTI standards. A large error could potentially go undetected in direct assays where the triphosphate stock is used for standards and quality controls. We have found that impurities of the triphosphate analogs include the parent, MP, and DP entities likely from instability or impurities from synthesis. The largest purity discrepancy found was a 76% pure stock that was listed as 93% pure on the certificate of analysis. We have found potency discrepancies as low as 70% of that listed and as high as twofold more concentrated than listed. We suggest that the purity determinations be made using ion pairing UV determinations similar to our QMA recovery experiment above. For potency determination we dephosphorylate to the parent NRTI and utilize the parent NRTI standards to determine the equimolar concentration of the triphosphate. There are other options for the potency determination such as absorbance and molar extinction coefficients, but these are probably utilized by the manufacturer from where witnessed discrepancies have been determined. Because stated purity and potency of triphosphate reference standards may be inaccurate, we recommend all reference standards be characterized prior to the reporting of any analytical results.

Lastly, a source of concern with highly sensitive LC–MS–MS assays is environmental contamination. During the methods development process we found an “apparent interference” coming from the front-end SPE processes. As mentioned above, ZDV-TP has a direct interference with ATP and dGTP in the hPBMC lysate when analyzed directly with the mass spectrometer in ESI negative ion mode using the 159 $[M-H]^-$ product ion. It was first thought that this same interference may be carrying into the indirect analysis method detecting ZDV, but theoretically this did not make sense since ZDV has independent daughter ions from both adenosine and deoxyguanosine. Furthermore, we could not chromatographically separate this interference from ZDV while separation from the endogenous entities was being performed. Finally, we found that the interference was in fact an environmental contamination of ZDV from the extensive NRTI plasma work previously performed in the laboratory. The laboratory has been measuring ZDV concentrations in plasma for several years and the potential for the environmental contamination was a hidden issue. Remedies for the contamination included isolation of the work area and instruments used for the analysis (pipets, test tube racks, pipet tips, etc.). In addition, all supplies and instruments were washed with copious amounts of tap water and rinsed with distilled water between assays (ZDV is highly water soluble).

5. Conclusions

We present in this paper a method to quantitate ZDV-TP concentrations from hPBMCs by LC–MS–MS. The method is both accurate and precise and can be applied to real-time patient specimens. This methodology is an indirect determination of ZDV-TP that allows for MS–MS determination by eliminating the interferences seen with ATP and dGTP in direct MS–MS determinations with standard supplies available to most MS–MS capable laboratories. While the QMA isolation of ZDV-TP is time-consuming, it is extremely specific and provides for a highly sensitive methodology. Additionally, the indirect method has the added benefit to quality assure that the ZDV-TP concentrations of the stock solution are at the correct and determined purity and potency. The current assay has been shown to correlate well with a previously established immunoassay method utilizing the same indirect extraction process. Finally, this methodology has been successfully applied to the analysis of patient specimens in real-time for support of pharmacological and clinical studies.

Acknowledgement

This research is supported by grant RO1 AI33835-12 from the National Institute of Allergy and Infectious Diseases to Courtney V. Fletcher and also supported by a junior faculty grant to Peter L. Anderson from the Colorado Center for AIDS Research (UO1 AI054907). Additionally, this research was supported by General Clinical Research Center Grant RR000051.

References

[1] T.N. Kakuda, Clin. Ther. 22 (2000) 685.
[2] P.A. Furman, J.A. Fyfe, M.H. St Clair, K. Weinhold, J.L. Rideout, G.A. Freeman, Proc. Natl. Acad. Sci. USA 83 (1986) 8333.
[3] Panel on Clinical Practices for Treatment of HIV Infection, Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents [updated 6 October 2005], available from: http://www.aidsinfo.nih.gov/guidelines/adult/AA_100605.pdf.
[4] F. Becher, A. Pruvost, C. Goujard, C. Guerreiro, J.F. Delfraissy, J. Grassi, H. Benech, Rapid Commun. Mass Spectrom. 16 (2002) 555.
[5] F. Becher, A. Pruvost, J. Gale, P. Couerbe, C. Goujard, V. Boutet, E. Ezan, J. Grassi, H. Benech, J. Mass Spectrom. 38 (2003) 879.
[6] F. Becher, R. Landman, S. Mboup, C.N.T. Kane, A. Canestri, F. Liegeois, M. Vray, M. Prevot, G. Leleu, H. Benech, AIDS 18 (2004) 181.

[7] B. Robbins, J. Rodman, C. McDonald, R.V. Srinivas, P.M. Flynn, A. Fridland, Antimicrob. Agents Chemother. 1 (1994) 115.
[8] E. Font, O. Rosario, J. Santana, H. Garcia, J. Sommadossi, J. Rodriquez, Antimicrob. Agents Chemother. 12 (1999) 2964.
[9] C. Fletcher, S. Kawle, T. Kakuda, P. Anderson, D. Weller, L. Bushman, R. Brundage, R. Remmel, AIDS 14 (2000) 2137.
[10] J.F. Rodriguez, J.L. Rodriguez, J. Santana, H. Garcia, O. Rosario, Antimicrob. Agents Chemother. 11 (2000) 3097.
[11] J.J. Van Kampen, P.L. Fraaij, V. Hira, A.M. Van Rossum, N.G. Hartwig, R. de Groot, T.M. Luider, Biochem. Biophys. Res Commun. 315 (1) (2004) 151.