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Simultaneous measurement of intracellular triphosphate metabolites of zidovudine, lamivudine and abacavir (carbovir) in human peripheral blood mononuclear cells by combined anion exchange solid phase extraction and LC–MS/MS

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We dedicate this article to the memory of John H. Rodman, our colleague, mentor and friend who passed away suddenly in April 2006. Through his hard work and dedication he made significant contributions to the field of HIV pharmacology.

Abstract

All nucleoside reverse transcriptase inhibitors (NRTI) must first be metabolized to their triphosphate forms in order to be active against HIV. Zidovudine (ZDV), abacavir (ABC) and lamivudine (3TC) have proven to be an efficacious combination. In order simultaneously to measure intracellular levels of the triphosphates (-TP) of ZDV, ABC (carbovir, CBV) and 3TC, either together or individually, we have developed a cartridge-LC–MS/MS method. The quantitation range was 2.5–250 pg/μl for 3TC-TP, 0.1–10.0 pg/μl for ZDV-TP and 0.05–5.00 pg/μl for CBV-TP. This corresponds to 0.1–11.0 pmol 3TC-TP per million cells, 4–375 fmol ZDV-TP per million cells and 2–200 fmol CBV-TP per million cells, extracted from 10 million cells. Patient samples demonstrated measured levels in the middle regions of our standard curves both at pre-dose and 4 h post-dose times.

Keywords: Human immunodeficiency virus; Zidovudine triphosphate; Carbovir triphosphate; Lamivudine triphosphate; Antiviral drug metabolism

1. Introduction

The nucleoside reverse transcriptase inhibitors (NRTIs) form the backbone of current antiretroviral treatment for HIV. The first drug approved for use, zidovudine (ZDV), is a member of this class of compounds. Lamivudine (3TC) and abacavir (ABC) are also NRTIs and are frequently given in combination with ZDV in a formulation marketed as TRIZIVIR®. All members of the NRTI class of antiretroviral agents, while differing in pharmacokinetics, toxicity and efficacy, require conversion to the triphosphate form in order to inhibit viral replication. Efficacy of this class of compounds depends on many factors including parent drug pharmacokinetics (absorbance, clearance, etc.), intracellular metabolism factors such as uptake/transport into the cell, multi-step phosphorylation and de-phosphorylation or other enzymatic processes and the activation status of the cells [1,2]. This complex series of interactions and associated variability are the reasons the relationship between plasma parent drug concentration and intracellular triphosphate concentration is difficult to predict. Since the triphosphate form is the active form of the NRTI class of compounds it is of interest to measure the triphosphate levels in the target cells, primarily peripheral blood mononuclear cells (PBMC). Previous studies have shown that intracellular concentrations of zidovudine triphosphate (ZDV-TP) correlate with anti-viral activity and immunological response to therapy [1].

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Intracellular ZDV-TP in human PBMC has been measured using several techniques. We and others have developed and implemented several methods. These include an enzymatic method but, while fairly rapid, this lacks the specificity of techniques that separate the phosphorylated metabolites [3,4]. HPLC–radioimmunoassay (HPLC–RIA) was one of the first methods used. It separates the intracellular phosphate metabolites but is time consuming because of the duration of the HPLC separation and the difficulty of multiplexing the separation method [5]. In order to circumvent the HPLC part of the assay we developed SPE anion exchange cartridge fractionation linked with RIA. This allowed several samples to be fractionated simultaneously [6]. Because the antibody kit for detection of ZDV is no longer available, we and others have turned to LC–MS/MS as a method of detection [7–9] since it provides good sensitivity. 3TC-TP has also been measured with cartridge-RIA, enzymatic methodology and cartridge-LC–MS with the same advantages and drawbacks as ZDV [7,10,11]. The active form of ABC, carbovir triphosphate (CBV-TP), is less well-characterized, but it too has been measured separately using enzymatic and LC–MS based assays [7,12,13]. Recently, a new LC-MS assay has been presented that allows ZDV, 3TC and ABC simultaneously in plasma along with several other nucleoside analogs but CBV derived from active triphosphate CBV-TP was not measured [8]. Other recent methods include direct measure of zidovudine and lamivudine triphosphates but the simultaneous measurement of ZDV-TP, 3TC-TP and CBV-TP together has not been done to date [15,16].

In this paper, we describe a new procedure, which represents an adaptation of separation techniques previously described by us, in which cartridge-RIA methods were used to measure ZDV-TP and 3TC-TP [6,10]. The new procedure relies on LC–MS/MS as the detection methodology. Using this methodology we can measure ZDV-TP, 3TC-TP and CBV-TP either together or individually in samples extracted from PBMC. In this study we validate the assay and demonstrate its potential to measure intracellular triphosphates as a result of TRIZIVIR® treatment.

2. Materials and methods

2.1. Materials

[^{13}C] ZDV, 3TC-TP, ZDV-TP, CBV-TP and [^{3}H]ZDV-TP were obtained from Moravek Biochemicals (Brea, CA). 3TC was provided by Glaxo SmithKline (Research Triangle, NC) and carbovir was kindly provided by Robert Vince (University of Minnesota, Minneapolis, MN). ZDV, Type XA acid phosphatase (EC 3.1.3.2) from sweet potatoes (catalog #P1435), GDP, CDP, and TDP were purchased from Sigma Chemical Co. (St. Louis, MO). Sep-Pak QMA anion exchange cartridges (catalog #WAT 020545) and OASIS HLB reverse-phase cartridges (catalog #WAT094226) were purchased from Waters (Milford, MA). Potassium chloride was purchased from JT Baker Co. (Phillipsburg, NJ). HPLC grade water and methanol were from Burdick and Jackson (Muskegon, MI). Glacial acetic acid and sodium chloride were purchased from Fisher Scientific (Fair Lawn, NJ) and ammonium acetate was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Ficoll (LSM) was purchased from Cellgro-Media Tech (Herndon, VA). Phosphate buffered salt solution (PBS), L-Glutamine, Penicillin/Streptomycin and non-essential amino acids (NEAA) were purchased from BioWhittaker (Cambrex) (Walkersville, MD). RPMI 1640 and fetal bovine serum were purchased from Invitrogen (Gibco) (Carlsbad, CA).

2.2. Instrumentation

Nucleotides were separated with a Supelco Visiprep 24™ DL with adjustable vacuum and disposable flow control valve liners to minimize contamination. The chromatographic system was an 1100 Series HPLC System from Agilent Technologies (Wilmington DE) consisting of a capillary pump with micro vacuum degasser, temperature-controlled micro well-plate sampler and column compartment. Chromatography was performed at 25 °C using a AQUASIL™ C18 column (2.1 mm × 50 mm, 3 μm particle size) from Thermo Electron Corporation (Bellefonte, PA) with a UNIGUARD™ AQUASIL™ C18 drop-in guard column (2.1 mm × 10 mm, 3 μm particle size). Mass spectrometric detection was performed using a Finnigan™ TSQ® Quantum™ triple quadrupole mass spectrometer from Thermo Electron Corporation (San Jose, CA), equipped with an electrospray ionization source.

2.3. Isolation and extraction of PBMC

PBMC were separated from blood obtained from healthy volunteer aphaeresis donors or HIV infected patients. In the case of the normal donors, the blood was diluted 1:2 with Phosphate buffered salt solution and layered over Ficoll. The PBMC were isolated by centrifugation for 20 min (700 × *g*) at room temperature. The cells were rinsed, the red cells removed by hypotonic lysis and then the PBMC were suspended in 50 ml PBS and an aliquot retained for determining cell counts and viability by trypan dye exclusion. The cells were centrifuged and extracted with 200 μl per 10 million cells of 70%:30% methanol:Milli-Q water for at least 15 min. PBMC extract corresponding to 10 million cells were isolated for each of the points in the studies described below. HIV infected patient samples were separated using heparinized blood cell preparation tubes from Becton Dickinson and the resultant PBMC extracted as described above except the tubes were initially spun at 1500 × *g*.

2.4. Intracellular triphosphate isolation

To separate CBV-TP, ZDV-TP and 3TC-TP from the mono and di-phosphates, QMA Sep Pak cartridges were used as previously described [6,10] but with the following modifications. The QMA cartridges were conditioned by rinsing with 10 ml of 500 mM KCl followed by 10 ml of 5 mM KCl. The samples were loaded onto the cartridges and unwanted metabolites removed by sequential washes with 12 ml of 50 mM KCl and 7 ml of 77.5 mM KCl. The triphosphate fraction was eluted with 3 ml of 500 mM KCl and collected for de-phosphorylation. The [^{13}C] ZDV internal standard was added at this point. The [^{13}C]

triphosphates of the analytes under study would have been the ideal internal standards but they were unavailable and cost prohibitive to have synthesized. [^{13}C] ZDV is the only nucleoside readily available and cost effective, and was added after the initial separation because it is not a triphosphate. The pH was lowered to 4.5 by the addition of ammonium acetate buffer, and phosphate groups were removed by addition of 1 unit of type XA sweet potato acid phosphatase per ml eluent and incubation at 37 °C for 30 min. Samples were loaded onto Waters OASIS HLB cartridges pre-conditioned with 3 ml MeOH and 3 ml H_2O, and washed with 3 ml of water to remove salt. The resultant nucleoside analogs of interest were eluted with 1 ml of methanol and dried at 43 °C under a stream of nitrogen for 30–40 min. Once dry, the residue was reconstituted with 100 μl of water and transferred to auto-sampler vials for quantitation by LC–MS/MS.

2.5. LC–MS/MS analysis

Samples were maintained at 4 °C and 40-μl aliquots were injected into the HPLC system for analysis. Mobile phase A consisted of water + 0.1% acetic acid, and mobile phase B was methanol + 0.1% acetic acid. Chromatography was performed at a flow rate of 200 μl/min, and separation was accomplished with an 8 min gradient from 5 to 85% B. After each separation the column was washed for 2 min with 100% B at 200 μl/min; then for 3 min with 5% B using a flow rate increasing linearly from 200 to 300 μl/min; and finally maintaining flow of 5% mobile phase B at 300 μl/min for a further 2 min. The flow rate was then returned to the initial set point of 200 μl/min and held for 2 min prior to the next injection. The total cycle time, injection-to-injection, was 17 min. The mass spectrometer was operated in the positive ion mode using selected reaction monitoring (SRM). The following SRM transitions were monitored: m/z 230 → 112 for lamivudine, m/z 248 → 152 for carbovir, m/z 268 → 127 for zidovudine, and m/z 271 → 130 for the internal standard (^{13}C) zidovudine. Ion source parameters were as follows: spray voltage 4000 V, nitrogen sheath gas pressure 40 (arbitrary units), nitrogen auxiliary gas pressure 20 (arbitrary units), capillary temperature 270 °C, capillary offset 35 V. The tube lens offset was set at the default value established by optimization using polytyrosine tuning and calibration solution (Thermo Electron, San Jose, CA). Collision induced dissociation was performed at 20 V collision energy with argon at 1.5 mTorr pressure as the collision gas. Data were acquired with scan width 0.2 m/z, scan time 0.1 s, and Q1 and Q3 resolution 0.7 (peak width FWHM). Instrument control, data acquisition, and processing were performed using Finnigan™ Xcalibur™ software version 1.4 SR1 (Thermo Electron Corporation, San Jose, CA).

2.6. Patient samples

Samples were collected in conjunction with PACTG protocol P1052. In this protocol samples were taken from persons receiving TRIZIVIR® therapy that withheld their morning dose until administration at the clinic. Two samples were taken from each patient, one before administration of drug (trough) and one 4 h post dose for determination of intracellular 3TC-TP, CBV-TP and ZDV-TP. The PBMC were isolated from these samples, extracted with 70% MeOH and assayed as described above. The protocol was approved by the Institutional Review Board at each participating institution.

3. Results

In order simultaneously to quantitate intracellular levels of ZDV-TP, 3TC-TP and CBV-TP, we investigated an analytical method that combines the strengths of anion SPE metabolite separation and LC–MS/MS detection. The methodology is summarized in Fig. 1.

3.1. Separation studies

The ability of the QMA cartridges to separate the triphosphates from the mono and diphosphates was tested by using the

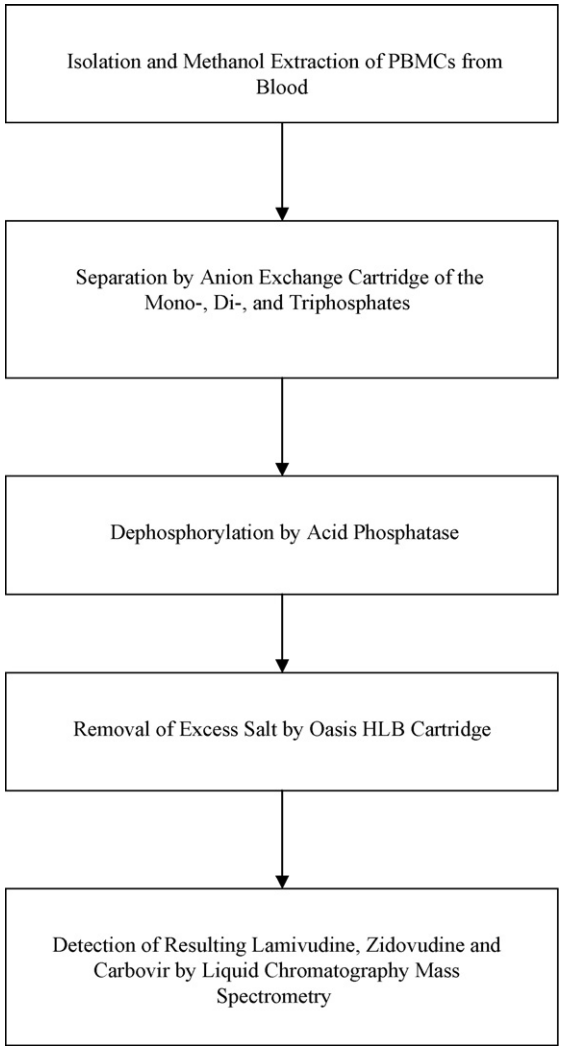

Fig. 1. Summary of the cartridge LC–MS/MS method for separation and detection of metabolites of abacavir, lamivudine and zidovudine.

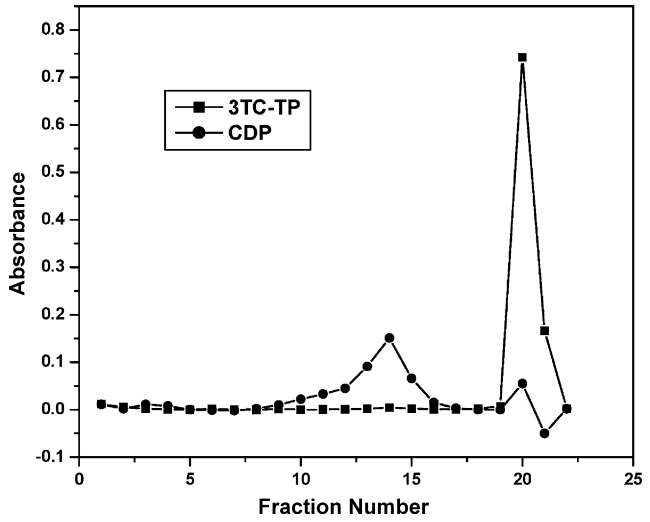

Fig. 2. Separation of 3TC-TP from its diphosphate analog, CDP, by anion exchange chromatography. Fractionation was performed using pre-conditioned QMA cartridges and the absorbance monitored at 272 and 254 nm using a spectrophotometer.

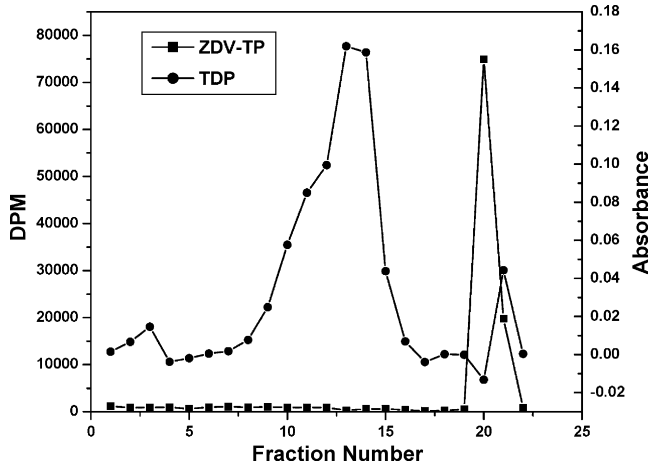

Fig. 4. Separation of TDP and [^{3}H]ZDV-TP from its diphosphate analog, TDP, by anion exchange chromatography. Fractionation was performed using pre-conditioned QMA cartridges. Elution of [^{3}H]ZDV-TP was monitored by scintillation counting, and elution of TDP was monitored by measuring the absorbance at 267 nm using a spectrophotometer.

appropriate analog triphosphates and surrogate diphosphates. These were thymidine diphosphate for ZDV, guanosine diphosphate for CBV and cytidine diphosphate for 3TC. The surrogates and their analogs were chosen because of the difficulty in obtaining the diphosphates and the closeness of the analogs and surrogates in HPLC profiles. Each of the triphosphates of interest was loaded onto a QMA cartridge and subjected to the separation gradient and 1 ml fractions were collected. Similarly, each of the appropriate diphosphate was loaded and 1 ml fractions were collected in the same manner. The resultant fractions were transferred to a quartz cuvette and read on a spectrophotometer. For ZDV-TP we used radiation as our monitoring method. Figs. 2–4 show that the triphosphates resolved well from endogenous diphosphates. Fig. 2 shows a 6 fraction separation from peak values of 3TC-TP and CDP. Less than 2% of the CDP was found in the triphosphate fraction and 95% of the 3TC-TP was recovered in the high salt fractions. Fig. 3 shows a 5 fraction separation between CBV-TP and GDP. Less than 4% of the GDP was found in the triphosphate fraction and 92% of the CBV-TP was recovered in the high salt fractions. Lastly, Fig. 4 shows a 6 fraction separation between ZDV-TP and TDP with less than 5% residual TDP in the triphosphate fraction, and 97% of the ZDV-TP was recovered.

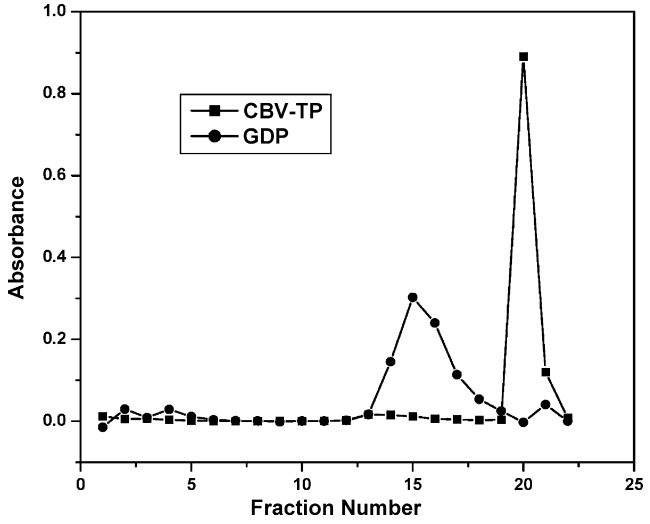

Fig. 3. Separation of CBV-TP from its diphosphate analog, GDP, by anion exchange chromatography. Fractionation was performed using pre-conditioned QMA cartridges and the absorbance monitored at 253 and 257 nm using a spectrophotometer.

3.2. Standard curve results

Prior to use the concentrated nucleoside analog stock solutions and their triphosphates were checked for purity by HPLC. The stock solution concentrations were checked by UV-spectrophotometry, when possible, and had to be within 10% of the calculated concentration.

PBMC extract was loaded onto the QMA cartridge and extracted as described in Section 2. The standard nucleoside solutions were then spiked into the 500 mM KCl fraction, desalted and reconstituted as also described in Section 2. The resulting solutions were subjected to LC–MS/MS for the construction of standard curves. The standard curves were linear over the range 2.5–250 pg/μl for lamivudine, 0.05–5.00 pg/μl for carbovir, and 0.100–10.0 pg/μl for zidovudine. Concentrations from cellular experiments and patients are normally expressed as fmol or pmol/10^6 cells. Given that we used 10 million cells to make our cell extracts and the cartridge purified dried samples derived from the 10 million cells were reconstituted in a final sample volume of 100 μl, the above pg/μl values correspond to 0.11–11 pmol/10^6 cells for lamivudine-TP, 2–200 fmol/10^6 cells for carbovir-TP, and 3.75–375 fmol/10^6 cells for zidovudine-TP. This is more than sensitive enough to measure levels in patient samples.

3.3. Limit of quantitation and assay variability studies

The intra-assay variability, inter-assay variability and limit of quantitation (LOQ) were determined in a 5-day series of experiments. PBMC extracts corresponding to 10 million cells from normal donors were applied to pre-conditioned QMA cartridges and the separation procedure described in Section 2 was performed. The triphosphate fraction consisting of 3 ml of solution containing 500 mM KCl was retained. Into this fraction was spiked nucleoside analogs and internal standard. Four different concentrations of each analyte were used, representing the limit of quantitation, and concentrations from the low, middle and high portions of the standard curves. The LOQ values were: 2.5 pg/μl for lamivudine, 0.05 pg/μl for carbovir, and 0.100 pg/μl for zidovudine. The final concentrations of lamivudine were 2.5, 7.5, 20 and 200 pg/μl; those of carbovir were 0.05, 0.15, 0.4 and 4 pg/μl; and those of zidovudine were 0.1, 0.3, 0.8 and 8.0 pg/μl. These samples were treated with ammonium acetate and acid phosphatase, desalted with Oasis HLB cartridges and measured by LC–MS/MS. Data for five replicates per day were acquired over a 5-day period.

The results are shown in Table 1. At the LOQ, daily %CV ranged from 3.17 to 10.45 for lamivudine, 4.38 to 17.97 for carbovir, and 6.28 to 35.47 for zidovudine. %E ranged from −14.85 to 13.49 for lamivudine, −1 to 19 for carbovir, and −4.5 to 23 for zidovudine. When all data points were used, variability at the LOQ was acceptable for all analytes with %E and %CV values less than 25. For a normal chemical UV assay the accepted %E and %CV values are less than 15% except at the LOQ where 20% is acceptable. However, wider ranges of 20% and 25% at the LOQ are acceptable for ELISA and other bioassays [17]. In our assay ZDV exhibited the most variability and was the only one with %E and %CV values over 20% at the LOQ. This was likely due to the fact that it had the least response ratio over background of the analytes tested. The average ratio of low standard to matrix blank was 99.61:1 for 3TC, 8.01:1 for CBV and 3.75:1 for ZDV in other words on average 3TC response of the low standard was about 100× background CBV about 8× background and ZDV about 4× background. The overall %CV for ZDV was 22% and the 2% difference between this and the 20% cutoff is unlikely to be biologically or clinically significant.

The statistical results for the inter- and intra-day precision and accuracy of the assay over the full range of the standard curves are shown in Table 2. Daily %CV values varied from 1.46 to 9.72 for lamivudine, 1.31 to 22.13 for carbovir and 2.16 to 10.5 for zidovudine. Daily %error varied from −8.44 to 10.29 for lamivudine, −5.02 to 10.78 for carbovir and −1.44 to 11.89 for zidovudine. When all data points across all days for each concentration were taken into consideration (inter-assay variation), the assay performed very well with %CV and %E values less than 10 at the indicated concentrations.

3.4. Triphosphate study

To further validate the assay, triphosphates of 3TC, CBV and ZDV were purchased. Concentrations of 3TC-TP, CBV-TP and ZDV-TP that corresponded to LOQ and QC values used for the variability studies were added to PBMC extracts from normal donors corresponding to 10 million cells. The resulting samples were subjected to QMA cartridge, de-phosphorylation, HLB cartridge and LC–MS/MS analysis as before. Samples were analyzed in duplicate over 5 days to conserve cell extract and analog triphosphates and to provide sufficient data for analysis. This resulted in a total of 10 data points for each concentration and the resultant statistical information is shown in Table 3. They exhibit overall %CV and %E values less than 25% at the LOQ for all three triphosphates and less than 20% for all the rest of the concentrations studied. These values indicate acceptable precision and accuracy over the range of the assay, although there is a slight negative bias with 3TC.

Potential interference from endogenous compounds was investigated by analyzing blank PBMCs. No significant interference from endogenous substances was observed at

Table 1
LOQ values for lamivudine, carbovir and zidovudine

Target (pg/μl)	Lamivudine 2.5	Carbovir 0.05	Zidovudine 0.1
Day 1			
Median	2.641	0.052	0.095
Mean	2.624	0.051	0.096
SD	0.108	0.003	0.017
%CV	4.100	6.075	18.146
%E	4.953	2.000	−4.500
Day 2			
Median	2.346	0.053	0.126
Mean	2.413	0.053	0.123
SD	0.252	0.004	0.019
%CV	10.453	6.773	15.579
%E	−3.467	6.333	23.000
Day 3			
Median	2.825	0.060	0.109
Mean	2.837	0.060	0.108
SD	0.090	0.005	0.012
%CV	3.174	8.941	10.924
%E	13.487	19.000	7.500
Day 4			
Median	2.108	0.049	0.102
Mean	2.129	0.050	0.102
SD	0.163	0.002	0.006
%CV	7.668	4.380	6.285
%E	−14.847	−1.000	1.833
Day 5			
Median	2.295	0.049	0.121
Mean	2.328	0.051	0.121
SD	0.083	0.009	0.043
%CV	3.550	17.973	35.466
%E	−6.873	2.333	21.000
Overall			
Median	2.418	0.052	0.105
Mean	2.466	0.053	0.110
SD	0.286	0.006	0.024
%CV	11.598	11.498	21.978
%E	−1.360	6.000	10.000

Table 2
Variability of the LC–MS assay in PBMC extracts

Target (pg/μl)	Lamivudine			Carbovir			Zidovudine		
	7.5	20	200	0.15	0.4	4	0.3	0.8	8
Day 1									
Median	7.522	19.908	195.694	0.163	0.411	4.284	0.328	0.850	8.200
Mean	7.464	19.868	194.110	0.166	0.405	4.293	0.336	0.852	8.211
SD	0.369	1.497	10.486	0.018	0.015	0.056	0.035	0.041	0.177
%CV	4.950	7.535	5.402	11.054	3.599	1.310	10.495	4.862	2.160
%E	−0.487	−0.662	−2.945	10.778	1.250	7.325	11.889	6.525	2.631
Day 2									
Median	7.892	20.628	198.675	0.149	0.395	4.036	0.319	0.833	8.259
Mean	7.856	20.546	199.262	0.153	0.401	4.049	0.315	0.835	8.293
SD	0.176	0.430	2.910	0.015	0.015	0.068	0.024	0.068	0.261
%CV	2.243	2.091	1.461	9.901	3.735	1.680	7.622	8.120	3.143
%E	4.751	2.730	−0.369	1.889	0.292	1.229	4.944	4.417	3.660
Day 3									
Median	7.835	19.276	187.215	0.164	0.412	4.245	0.322	0.842	8.208
Mean	7.701	18.993	183.120	0.162	0.412	3.799	0.331	0.837	8.208
SD	0.528	1.846	13.267	0.007	0.014	0.841	0.029	0.028	0.263
%CV	6.858	9.719	7.245	4.113	3.442	22.132	8.682	3.308	3.206
%E	2.673	−5.037	−8.440	8.000	2.958	−5.021	10.222	4.625	2.604
Day 4									
Median	7.778	21.919	214.814	0.151	0.380	3.892	0.319	0.786	8.043
Mean	7.756	21.854	213.725	0.154	0.392	3.855	0.305	0.789	7.961
SD	0.328	0.570	7.343	0.010	0.031	0.093	0.025	0.031	0.226
%CV	4.235	2.608	3.436	6.784	7.847	2.411	8.172	3.892	2.844
%E	3.413	9.270	6.863	2.444	−1.917	−3.629	1.556	−1.438	−0.492
Day 5									
Median	8.244	22.295	217.991	0.148	0.404	4.120	0.317	0.841	8.046
Mean	8.029	22.058	217.781	0.146	0.405	4.125	0.314	0.840	8.084
SD	0.507	0.809	7.244	0.007	0.022	0.086	0.027	0.033	0.176
%CV	6.316	3.668	3.326	4.903	5.352	2.089	8.515	3.906	2.177
%E	7.049	10.292	8.891	−2.444	1.292	3.113	4.667	4.979	1.050
Overall									
Median	7.841	20.827	202.395	0.152	0.401	4.118	0.320	0.840	8.130
Mean	7.761	20.664	202.237	0.156	0.403	4.024	0.320	0.830	8.151
SD	0.419	1.607	15.055	0.014	0.020	0.399	0.029	0.045	0.239
%CV	5.401	7.776	7.444	8.690	4.982	9.927	8.957	5.467	2.935
%E	3.480	3.320	1.120	4.000	0.750	0.600	6.670	3.750	1.890

the retention times of the analytes and internal standard. Any matrix effects on ionization were assessed by comparing the response obtained from solutions of analytes or internal standards in water with the same compounds spiked into blank PBMC at the same concentration. The results indicated that there was no detectable ion suppression.

Carry over was less than 0.005% with 3TC, less than 0.03% with CBV and less than 0.1% with ZDV as determined by placing blanks after the high standards.

Table 3
Validation of triphosphate quantitation by exogenous addition to PBMC extracts

Metabolite	3TC-TP				CBV-TP				ZDV-TP			
Target (pg/μl)	2.5	7.5	20	200	0.05	0.15	0.4	4	0.1	0.3	0.8	8
Median	2.566	7.463	20.345	186.463	0.059	0.169	0.468	4.662	0.118	0.310	0.809	7.761
Mean	2.661	7.671	20.748	191.661	0.060	0.172	0.470	4.626	0.114	0.312	0.810	7.872
SD	0.257	0.768	2.298	28.082	0.005	0.014	0.025	0.379	0.018	0.026	0.012	0.389
%CV	9.65	10.01	11.08	14.65	8.82	8.41	5.26	8.19	15.49	8.19	1.53	4.94
%Error	6.43	2.28	3.74	−4.17	19.07	14.91	17.41	15.65	13.80	4.11	1.30	−1.60
Min	2.242	6.568	17.512	157.351	0.054	0.150	0.432	4.098	0.083	0.281	0.785	7.293
Max	3.045	9.364	23.895	242.129	0.068	0.202	0.520	5.221	0.141	0.359	0.835	8.583

Table 4
Results from patients receiving TRIZIVIR® therapy

Patient	Time (hours)	3TC-TP (pmol/10^6 cells)	CBV-TP (fmol/10^6 cells)	ZDV-TP (fmol/10^6 cells)
1	0	5.72	31.0	19.1
	4	7.43	67.5	56.1
2	0	1.9	26.7	27.4
	4	8.32	70.8	77.4
3	0	3.88	29.7	22.0
	4	5.95	60.0	51.6

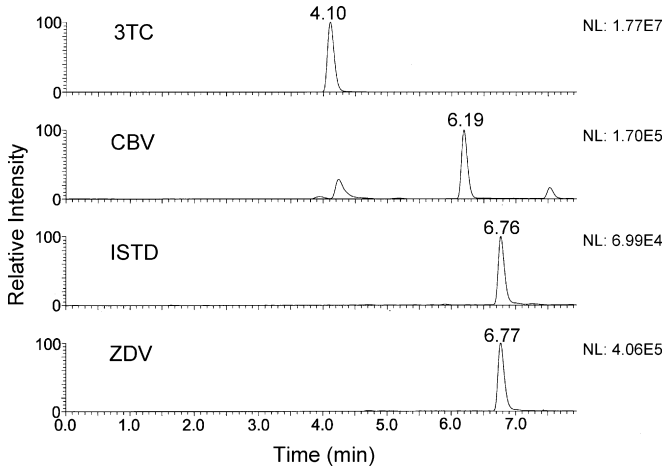

Fig. 5. Mass spectral data for quantitation of nucleoside analogs from a patient sample at 4 h post dose. Single ion mass chromatograms for 3TC, CBV and ZDV, and internal standard (^{13}C)ZDV. Base peak intensities are indicated by normalized (NL) signal strength values indicated. Retention times for principal components are indicated above each peak.

3.5. Patient samples

Samples from patients receiving TRIZIVIR® were then tested. The results are shown in Table 4 and are similar to those seen in previous studies [7,11,14]. The values, presented in pmol and fmol/10^6 cells in Table 4, are near the middle of the three standard curves described above. Fig. 5 shows chromatograms for transitions corresponding to each of the three NRTIs and demonstrates co-elution of [^{13}C] ZDV and ZDV.

4. Discussion

Combination therapy is the current standard of care for HIV-infected persons. TRIZIVIR® is a combination of abacavir, zidovudine and lamivudine, and is currently formulated in a single dose to ease the pill burden on patients receiving therapy. The regimen also spares protease inhibitors for possible later use in the patient's regimen if the virus develops resistance to nucleoside analogs. Alternatively, TRIZIVIR® can be used if the patients develop a toxic reaction to protease inhibitors.

All three of the NRTIs in TRIZIVIR® must be phosphorylated to be active, suggesting that measuring the levels of these triphosphates may be of value in predicting clinical outcome. In this study we have adapted the cartridge purification method used in our earlier RIA methods [6,10], and replaced RIA detection with LC–MS/MS. We have developed the procedure to enable simultaneous measurement of 3TC TP, CBV-TP and ZDV-TP. While at present we are interested exclusively in the triphosphate forms of NRTIs, the same methodology can be adapted to measure the mono- and di-phosphate intermediates as we have done previously with the ZDV and 3TC in cartridge RIA assays.

The present assay had LOQ values of 2.5, 0.05 and 0.1 pg/μl for 3TC-TP, CBV-TP and ZDV-TP, respectively. For a sample size of 10 million cells, these values correspond to 0.11 pmol/10^6 cells, 2 fmol/10^6 cells and 3.75 fmol/10^6 cells. If only 5 million cells are available then the assay LOQ becomes higher: 0.22 pmol/10^6 cells 3TC-TP, 4 fmol/10^6 cells CBV-TP, and 7.5 fmol/10^6 ZDV-TP. In either case these values are at least as sensitive as 3TC values from our previous cartridge RIA assay and more sensitive for ZDV with concentrations of 20 fmol/10^6 cells for ZDV and 0.21 pmol/10^6 cells for 3TC [6,10]. We compared our assay for CBV-TP to previous measurements [5,7,11] and we were able to determine the levels of CBV-TP in a subset of samples from patients receiving TRIZIVIR® therapy (Table 4). The results combining the 0 h and 4 h post-dose for CBV-TP varied from 26.7 to 70.8 fmol/10^6 and ZDV-TP levels varied from 19.1 to 77.4 fmol/10^6 cells. King et al. observed contamination by external ZDV from the environment due to extensive plasma ZDV determinations in their laboratory. We encountered a similar problem, and took special measures to prevent cross-contamination between samples and contamination of samples from equipment harboring residual nucleoside analog. These measures included use of disposable liners for the valve assembly in the Supelco Visiprep 24^{TM} DL manifold system used for cartridge purification, a dedicated area of the laboratory and dedicated glassware that was thoroughly washed after each experiment to minimize any cross-contamination. Minimal carry over was seen with any of the analytes as determined by placing blanks after the high standards.

Further modification of the assay can likely result in the ability to measure the other intracellular metabolites of these compounds, including the MP, DP and intracellular NRTIs, and would provide even more information on the metabolism of these compounds.

We have developed and validated an assay to measure intracellular triphosphate levels of the active forms of ABC, 3TC and ZDV, and have demonstrated its use for determining intracellular levels of active metabolites for regimens containing ABC, 3TC or ZDV alone or in combination in patient-derived samples.

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