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Liquid chromatography–tandem mass spectrometric determination of tenofovir-diphosphate in human peripheral blood mononuclear cells

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Abstract

To facilitate the evaluation of drug safety, virologic activity, and pharmacokinetics, an anion exchange isolation of tenofovir-diphosphate (TFV-DP) from human peripheral blood mononuclear cells (hPBMCs), coupled with dephosphorylation, desaltation, and detection by LC–MS–MS was validated. hPBMCs were harvested from whole blood, lysed, and a suspension of intracellular tenofovir moieties was produced. TFV-DP was isolated from TFV-monophosphate (TFV-MP) and tenofovir (TFV), dephosphorylated with acid phosphatase to form TFV and then desalted and concentrated, making it possible for tandem mass spectral detection. An LC–MS–MS methodology was developed and validated for the determination of TFV concentrations, which directly correspond with the intra-hPBMC TFV-DP concentration. The assay was linear in the range of 50–10,000 fmol per sample. The lower limit of quantitation (LLOQ) of the method is 10 fmol per million cells with 5 million hPBMCs used. This paper outlines the development and validation of the determination of TFV-DP concentrations in femtomoles per million hPBMCs.

Keywords: Tenofovir-diphosphate; LC–MS–MS; Peripheral blood mononuclear cells

1. Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first antiretrovirals shown to be clinically effective against HIV infection and remain components of preferred treatment regimens to this day. Tenofovir disoproxil fumarate (TDF) is an increasingly important member of this family of antiretroviral drugs.

TDF, once administered, is converted by diester hydrolysis to tenofovir (TFV). Once taken up into the cell, cellular enzymes then phosphorylate TFV, creating the active diphosphorylated moiety. This species, tenofovir-diphosphate (TFV-DP), competes with deoxyadenosine 5′-triphosphate, inhibiting HIV-1 reverse transcriptase. After incorporation into the viral DNA, TFV-DP causes chain termination.

TDF is generally well-tolerated and effective for use in both treatment-naïve and experienced patients; the pharmacokinetic characteristics of the drug and its formulation allow one-tablet per day dosing. Additionally, TDF is FDA-approved in a combination product with NRTI emtricitabine (Truvada®). As a result, TDF has been increasingly prescribed since its approval by the FDA for use in the USA in 2001. This increase in use makes investigation into intracellular pharmacokinetics imperative in terms of the assessment of drug safety and appropriate dosing in diverse patient groups.

Because of the expense and analytical challenges of this type of assay, the intracellular pharmacokinetics of TFV, and other nucleos(t)ide reverse transcriptase inhibitors, are poorly characterized. TDF has been implicated in unexpected, yet clinically significant drug–drug interactions with other antiretroviral agents, including atazanavir, lopinavir/ritonavir, and didanosine [1–4]. The combination of TDF and didanosine has also been reported to paradoxically decrease CD4 lymphocyte counts in the face of adequate virologic suppression [5]. Additionally, tenofovir and its metabolites accumulate inside proximal renal tubules, and renal toxicities, including acute renal failure, Fanconi syndrome, and diabetes insipidus have been associated with TDF therapy [6]. Lastly, several triple NRTI antiretroviral drug

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regimens that included TDF have shown unpredictably high rates of virologic failure [7–10].

The mechanism(s) of these interactions, toxicities, and low efficacy rates remain largely unknown. Given that the pharmacologic activity of tenofovir and NRTIs depend on the intracellular profile of phosphate anabolites, studies of the intracellular concentrations of TFV-DP may uncover answers. At the time of our experiments, no validated LC–MS–MS method had been published to address the measurement of the intracellularly active TFV-DP moiety. Pruvost et al. and Hawkins et al. refer to direct LC–MS–MS ion-pairing methods in the context of clinical TFV-DP data, but provide too few details to reproduce the TFV-DP analytical method [11,12]. The following paper describes an indirect method for the quantitation of intracellular TFV-DP that describes more fully the inherent challenges and necessary validation steps associated with this type of intracellular LC–MS–MS quantitation. The development and validation closely follows our previously described method for zidovudine-triphosphate (ZDV-TP) detection [13]. Additionally, other previously described NRTI-TP indirect methods were reviewed for an initial developmental framework [14–17]. The LC–MS–MS methodology is an extension of our laboratory's previously validated and published TFV plasma detection method [18].

2. Experimental

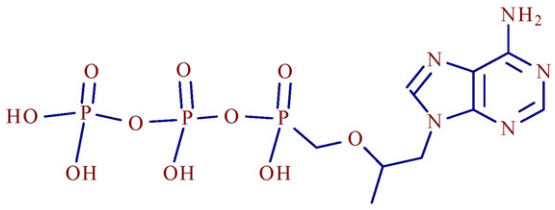
2.1. Chemicals and reagents

TFV-DP, TFV-MP, TFV, and stable labeled isotopic TFV, used as internal standard, were all obtained from Moravek Biochemicals (Brea, CA, USA) (Fig. 1). Methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Fairlawn, NJ, USA). Glacial acetic acid, sodium acetate (ACS grade), potassium chloride (USP/EP grade), trifluoroacetic acid (TFA), and acid phosphatase type XA from sweet potato were obtained from Sigma Chemical (St. Louis, MO, USA). Deionized/distilled water (ddH_2O) was produced in-house using a Barnstead Mega-Pure system. Nitrogen (99.9% pure) and argon (99.999%) gases were supplied by General Air (Denver, CO, USA). BD Vacutainer® sodium citrate cell preparation tubes (CPT™) were obtained from Fisher Scientific (Fairlawn, NJ, USA).

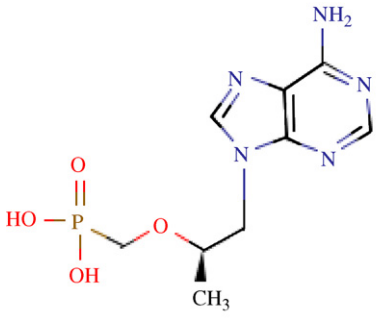
2.2. Equipment

The two solid phase extraction (SPE) cartridges used were Waters Accell™ Plus QMA Cartridge, 3 cm^3, and Waters OASIS® HLB Extraction Cartridge, 3 cm^3 (Waters Corporation, Milford, MA, USA). The analytical column was a Synergi 4μ Polar RP, 80A, 2.0 mm × 150 mm (Phenomenex, Torrance, CA, USA). The HPLC system consisted of Waters 2690 Alliance pump with autoinjector, inline degasser, and column heater (Waters Corporation). A TSQ Quantum mass spectrometer equipped with an electrospray ionization (ESI) source, utilizing a stainless steel spray needle in positive ion mode (ThermoElectron, San Jose, CA, USA) was used for detection. The Waters 2690 Alliance and the TSQ Quantum were both configured to Xcalibur™ version 1.3 software. Additional laboratory equipment included: Mettler Toledo analytical balance (Mettler Toledo, Switzerland), Vortex Genie 2 vortex mixer (Fisher Scientific, Fairlawn, NJ, USA), Jouan GR422 centrifuge (Jouan, Winchester, VA, USA), Zymark Turbo Vap LV nitrogen evaporator (Zymark Corp., Hopkinton, MA, USA), Aqua Bath 18000 water bath (LabLine Instruments, Inc., Melrose Park, IL, USA), and Beckman Φ11 pH meter (Beckman, Fullerton, CA, USA).

TFV - DP



TFV



Isotopic TFV Internal Standard [5-^{13}C of ring]

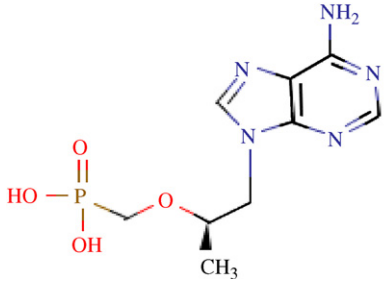


Fig. 1. Molecular structures of TFV-DP, TFV, and isotopic TFV internal standard.

2.3. Standard, and quality control/validation sample preparation

Isotopic TFV IS (1 mg/mL; 3.42×10^6 fmol/μL) stock solution was prepared from reference standard by dissolving 1 mg solid with 1 mL ddH_2O. This stock was diluted with ddH_2O to yield a final 0.01 mg/mL (3.42×10^4 fmol/μL) preparation stock. Both preparation stock solutions were stored at −80 °C and prepared as necessary. A working internal standard solution was prepared by diluting 0.01 mg/mL (3.42×10^4 fmol/μL) preparation stock solution with ddH_2O to yield a final 0.03 μg/mL (102.7 fmol/μL) solution. This solution was stored at 4 °C and prepared as necessary. TFV preparation stock 1

(3.27×10^4 fmol/μL) and stock 2 (1.09×10^4 fmol/μL) were prepared from reference standard by dissolving appropriately with 50:50 methanol:ddH_2O. These solutions were stored at −80 °C and prepared as necessary. Purity and salt content were taken into consideration when preparing. TFV standard curve preparation working solutions in ddH_2O (2.5–500 fmol/μL) were prepared by appropriate dilution of preparation stocks. The TFV standard curve working solutions were stored at 4 °C in 10 mL aliquots. These standards have been in use for over 1 year without observed degradation. TFV-DP validation sample/quality control preparation stock (5 nmol/mL; 5000 fmol/μL) was prepared by diluting liquid reference standard appropriately in 70:30 methanol:ddH_2O. Purity and potency were taken into consideration prior to preparing as previously described [2]. Four levels of validation samples were prepared and diluted to 10 mL with (20 million hPBMC/mL) 70:30 methanol:ddH_2O lysate solution. Aliquots were prepared in labeled tubes and stored at −80 °C. Three levels of quality controls were prepared similarly to validation samples after the completion of the validation for routine sample use.

2.4. Determination of TFV-DP

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 was collected in three cell preparation tubes (CPTs). After gentle mixing, the tubes were centrifuged at 1500 × *g*, 20 min, 21 °C. After the top two layers were 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, transferred to a cryovial, and centrifuged at 400 × *g*, 10 min, 4 °C. The supernatant was removed and the cells were lysed with 0.5 mL cold 70:30 methanol:ddH_2O solution. After pellet disruption and vortexing, the lysate solution was stored at −80 °C until assayed.

2.4.2. TFV-DP isolation (quality controls, validation samples, and patient specimens)

TFV-DP was isolated from TFV and TFV-MP in the cell lysis media by anion exchange SPE and a salt gradient. Cell lysate (equivalent to 5 million cells) was applied to an ion-exchange SPE Waters QMA 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. Three (2 mL) 75 mM KCl washes and six (2 mL) 50 mM KCl washes, followed by centrifugation at 100 × *g*, 2 min, 21 °C, were applied to the cartridge. The nine washes eluted TFV and TFV-MP. The isolated TFV-DP 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

Isolated TFV-DP solution (2 mL of 1 M KCl) was dephosphorylated to TFV 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 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. Prior to incubation internal standard working stock (20 μL) was added to *all* the samples. The TFV standard curve was also incubated along with the unknowns, blanks, and quality controls.

2.4.4. Standard curve preparation

TFV standards were comprised of 20 μL TFV standard working stock, 20 μL internal standard working stock, 2 mL 1 M KCl, and 100 μL acid phosphatase working solution in order to make analogous to quality controls and patient specimens eluted from the QMA cartridge. The standards were processed identically with all other samples from the dephosphorylation step forward.

2.4.5. Desaltation and concentration of TFV

The dephosphorylated samples and standards were protonated, desalted, and concentrated from the KCl solution making it possible for tandem mass spectral detection. After incubation, freshly prepared 12% TFA solution (100 μL) was added to all the samples. The samples were applied to a prepared Oasis HLB SPE cartridge and centrifuged at 100 × *g*, 2 min, 21 °C. The cartridge was prepared by the addition of methanol (2 mL) and fresh 1% TFA solution (2 mL) followed by centrifugation at 100 × *g*, 2 min, 21 °C. One 1% TFA (2 mL) wash followed by centrifugation at 100 × *g*, 2 min, 21 °C successfully desalted the solution and isolated TFV onto the cartridge. TFV was eluted from the cartridge with methanol (2 × 500 mL). The sample was dried under nitrogen on a Zymark TurboVap at 37 °C for 45 min, and reconstituted in ddH_2O (100 μL).

2.5. LC–MS–MS conditions

The LC conditions were as follows: column temperature 35 °C, sample temperature 4 °C, isocratic flow rate at 0.200 mL/min, autosampler injection volume 40 μL, run time 5 min, and syringe draw rate 2.5 μL/s. The mobile phase was made by adding 10 mL aqueous acetic acid and 30 mL acetonitrile to 960 mL ddH_2O. The signal was achieved in positive ion mode with electron spray ionization (ESI) and selected reaction monitoring (SRM) detection. The MS source and quadrupole conditions were as follows: spray voltage 3200 V, nitrogen sheath gas 30 and auxiliary gas 10 (arbitrary units), capillary temperature 300 °C, chrom filter peak width 20 s, collision gas pressure 1.8 mTorr, scan width 1.0 *m/z*, scan time 0.1 s, collision energy 28 V, quadrupole resolution Q1:0.3 FWHM and Q3:0.7 FWHM (H-SRM), and source CID 0 V. The precursor/product *m/z* for TFV was 288/176 where the precursor/product *m/z* for IS was 293/181. The divert valve on the mass spectrometer was directed from 0 to 3.6 min to allow 100% methanol to flow into the ion source and then directed from 3.6 to 5 min to allow mobile phase to flow into the ion source for analyte detection. TFV and IS retention time was approximately 4 min.

2.6. Measurement and calculations

The chromatographs and calculated data were generated using Xcalibur™ software (version 1.3). Specifically, peak area ratios (PAR) were calculated by the software for standard curve, validation samples, and unknown samples. The resulting standard data were first fit to a weighted linear regression of 1/concentration for standard curve analysis. The standard curve parameters ($y = mx + b$, where y = PAR, m: slope, x: concentration, and b: intercept) were then used to determine the unknown and quality control sample concentrations from the peak area ratios already obtained for these samples. Unknown results in fmol/sample were then divided by the number of cells assayed to arrive at fmol/million cells. All unknown results were reported as fmol/million cells.

3. Validation of hPBMC sample analysis

Validation samples at four different TFV-DP concentrations were produced in 70:30 methanol:ddH_2O blank hPBMC lysate (5×10^6 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 validation sample concentration was 150 fmol, the medium validation sample concentration was 750 fmol, and the high validation sample concentration was 7500 fmol. The assay was validated for inter-day and intra-day accuracy and precision ($n = 5$ validation samples on 5 different days), conditional stability, recovery, and matrix and interference effects.

3.1. Inter-day average back calculated calibration standards

Each validation run contained singlet calibration standards with theoretical concentrations of 10,000, 5000, 2500, 1000, 500, 250, 100, and 50 fmol/sample. All eight standard concentrations were run in singlet on 5 separate days.

3.2. 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.

3.3. QMA recovery determination for TFV-DP

Optimization of TFV-DP isolation and recovery involved determining the correct number of washes to accurately remove TFV and TFV-MP and retain the maximum amount of TFV-DP on the QMA SPE cartridge. Initially, we attempted the same wash procedure as for our previously validated ZDV-TP isolation [13]. This ZDV-TP procedure validated with nine washes of 75 mM KCl. However, as ZDV-TP and TFV-DP are chemically different molecules, their isolation optimization proved different as well. The new TFV-DP optimized sequence was repeated three times to ensure reproducibility between runs. Known amounts of TFV-DP, TFV-MP, and TFV were loaded onto a prepped QMA SPE cartridge. Each 75 mM KCl (2 mL) and 50 mM KCl (2 mL) wash was eluted and saved for analysis on an LC system (method previously described [13]) The amount of phosphate moiety in each elution was recorded and compared to a theoretical 100% sample in the same elution matrix.

3.4. Enzyme incubation time determination

The incubation time necessary to produce the maximum amount of TFV from TFV-DP using acid phosphatase enzyme was optimized. A solution of TFV-DP (2500 pmol) and acid phosphatase preparation stock (4 units, pH 5) was incubated at 37 °C. At time points 30, 60, and 120 min, an aliquot of the solution was removed and was injected onto an LC system specifically established for TFV detection. Areas were back calculated to a standard curve of TFV and percentages of total expected TFV were recorded.

3.5. Selectivity

An experiment to test interference of concomitant medications was performed. Blank hPBMC 70:30 lysate and medium validation sample were spiked with high-level standard working stock solutions from the protease inhibitor, non-nucleoside, and nucleoside assay sets currently in use in the laboratory (10,000–20,000 ng/mL). The HIV drugs included in these sets were: indinavir, amprenavir, M8 (a nelfinavir metabolite), saquinavir, atazanavir, ritonavir, lopinavir, nelfinavir, delavirdine, efavirenz, nevirapine, lamivudine, didanosine, emtricitabine, stavudine, zidovudine, and abacavir. The blank and validation sample medium samples were extracted in duplicate.

3.6. Stability

Low and high TFV-DP validation samples were subjected to various conditions in order to test stability. First, 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, another set of low and high validation samples were removed from −80 °C storage and allowed to sit at room temperature for 24 h to test for room temperature stability. These validation samples were then extracted and run in triplicate and compared with an extracted control set ($n = 5$) 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 also tested. Previously extracted validation samples at low and high concentration ($n = 5$) were allowed to remain in the autosampler (4 °C) for a period of 5 days. These samples were compared with a freshly extracted control set ($n = 5$) of low and high validation samples.

3.7. Matrix effects

For this LC–MS–MS application, the isotopic internal standard and the analyte of interest (TFV) co-eluted at approximately

Table 1
Inter-day average back calculated calibration standards

TDF-DP	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	Cal 8	Slope (×1000)	R^2
Theoretical concentration (fmol)	10000	5000	2500	1000	500	250	100	50	**	**
Mean	9976	4968	2478	1088	484	260	100	46.1	0.2902	0.9992
S.D.	115.40	68.71	63.36	29.71	19.01	15.76	6.36	2.08	0.0029	0.0005
%CV	1.16	1.38	2.56	2.73	3.93	4.66	6.36	4.51	0.9980	0.0542
%Dev	−0.24	−0.63	−0.88	8.78	−3.22	−4.43	0.09	−7.78	**	**
n	5	5	5	5	5	5	5	5	5	5

4 min. Matrix purity, matrix effect, recovery, process efficiency, cell number variability, and internal standard purity were tested. Although certain tests are not necessary if an isotopic internal standard is utilized, it was decided to perform all of the described matrix effect tests.

3.7.1. Matrix purity/specificity

Six different blank hPBMC lots were extracted and tested for environmental contamination and/or endogenous interferences.

3.7.2. Matrix effect (ME), recovery (RE), and process efficiency (PE)

A modification to the method of Matuszewski et al. [19] was used to test for ME, RE, and PE. Three different sets of samples were established.

Set 1: Unextracted analyte(s), no matrix (defines overall system and detector performance and effect of matrix on signal) (i.e. no extraction, TFV and IS spiked in ddH_2O).
Set 2: Post-extraction spike of analyte into extracted matrix (defines absolute and relative matrix effects) (i.e. blank hPBMC extracted then TFV and IS spiked post-N_2 dry).
Set 3: Pre-extraction spike of analyte into matrix then extraction (defines recovery and overall process efficiency) (i.e. TFV and IS spiked in blank hPBMC matrix then extracted).

Five different lots of hPBMC matrix were tested at low (500 fmol/sample), medium (2500 fmol/sample), and high (10,000 fmol/sample) concentrations for a total of 45 samples.

3.7.3. Cell number variability

In order to determine if cell number influenced final TFV-DP concentration, a mid-range TFV-DP level was spiked into different cell amounts of the same cell matrix lot (1, 2, 5, and 10 million cells) and extracted.

3.7.4. IS purity

When using an isotopic internal standard it is also necessary to determine if the isotopic internal standard contributes to analyte response. Blank hPBMCs were extracted according to the method protocol through the QMA SPE and IS was added prior to incubation with enzyme. The sample was extracted according to the method protocol through the HLB SPE. Blank hPBMC/IS samples were then monitored for TFV response.

4. Results and discussion

4.1. Inter-day average back calculated calibration standards

The TFV to IS ratio was plotted against concentration for each calibration curve. The experimental standard concentrations were back calculated using 1/concentration weighted linear regression curve (Table 1).

The experimental back-calculated mean was compared against the theoretical concentration and inaccuracy was calculated to be less than 9% across all the concentrations. Back-calculated standard precision statistics were represented by coefficient of variance (%CV) data. Overall, the %CV for all concentrations was less than 7%. The linear regression coefficient of determination (R^2) values for the five calibration lines were all $\geq$0.9982. Therefore, the five curves were linear through the calibration range tested (LLOQ to ULOQ). ESI tends to have a limited dynamic linear range compared to other detection methods, such as UV. As analyte concentrations increase, the curves typically become non-linear and plateau near the ULOQ. This ESI non-linearity was not observed for this method. 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.

4.2. Accuracy and precision

The method precision and accuracy were tested both within each run (intra-assay) and between each run (inter-assay) for each TFV-DP validation sample concentration (Table 2). The greatest mean inter-assay percent deviation (%dev) was 6.1% for the LLOQ validation sample concentration. All non-LLOQ validation sample mean inter-assay %dev were less than 5%. The highest mean inter-assay %CV for TFV-DP was 12.85% for the LLOQ validation sample. All non-LLOQ validation sample mean inter-assay %CVs were less than 5%. The greatest mean intra-assay percent deviation was 20.77% for the LLOQ validation sample on Run 3. All non-LLOQ validation sample mean

Table 2
Accuracy and precision

	LLOQ	Low	Medium	High
Intra-assay statistics				
Theoretical concentration (fmol)	50.0	146	731	7305
1				
Mean	43.3	142	713	7130
S.D.	2.16	3.89	15.73	173.14
%CV	4.98	2.73	2.21	2.43
%Dev	−13.38	−2.41	−2.43	−2.40
n	5	5	5	5
2				
Mean	58.3	156	745	7222
S.D.	3.27	6.66	2.88	155.75
%CV	5.62	4.26	0.39	2.16
%Dev	16.56	7.10	1.86	−1.13
n	5	5	5	5
3				
Mean	60.4	161	755	7256
S.D.	4.43	3.67	12.42	51.19
%CV	7.34	2.29	1.64	0.71
%Dev	20.77	9.94	3.33	−0.67
n	5	5	5	5
4				
Mean	51.0	154	730	7186
S.D.	3.98	5.68	18.77	102.86
%CV	7.80	3.68	2.57	1.43
%Dev	2.02	5.72	−0.20	−1.62
n	5	5	5	5
5				
Mean	52.3	150	717	7217
S.D.	1.38	2.20	26.29	95.34
%CV	2.65	1.47	3.67	1.32
%Dev	4.51	2.72	−1.87	−1.21
n	5	5	5	5
Inter-assay statistics				
Mean	53.0	153	732	7202
S.D.	6.82	7.60	22.54	120.91
%CV	12.85	4.98	3.08	1.68
%Dev	6.10	4.61	0.14	−1.41
n	25	25	25	25

intra-assay %dev were less than 10%. The highest intra-assay %CV was 7.80% for the LLOQ validation sample on Run 4. All non-LLOQ validation sample coefficient of variance values were less than 5%. It was observed that the LLOQ validation sample showed the highest %dev and %CV values compared with the low, medium, and high validation sample percentages, as expected for an LLOQ.

4.3. QMA recovery determination

Rather than nine washes with the same concentration of KCl, as with ZDV-TP optimization, we varied the concentration and number of washes for TFV-DP and found that three washes with 75 mM KCl followed by six washes with 50 mM KCl most efficiently removed the unwanted analytes and retained the most TFV-DP on the cartridge. The amount of each analyte in each elution was recorded and compared with a theoretical 100% sample in that same elution matrix. After three 75 mM KCl and six 50 mM KCl washes, all TFV-DP remained on the cartridge. All TFV was eluted from the cartridge within the first three 75 mM KCl washes. TFV-MP continuously eluted with each additional 50 mM KCl wash. Moreover, it was shown that no TFV or TFV-MP was eluted in the 1 M KCl wash. All the TFV-DP was eluted in the 1 M KCl elution. This was proven by employing a second 1 M KCl wash that showed no TFV-DP present.

4.4. Enzyme incubation time determination

Incubation time was determined by back calculating areas to a 3-point standard curve of TFV and percentages of total expected TFV were recorded. It was determined that 1 h of incubation was sufficient to convert greater than 96% of TFV-DP to TFV. It was also shown that no increase in TFV yield was evident after 1 h. One hour of incubation was, therefore, chosen as the incubation time for the method.

4.5. Selectivity

The concentration of the medium validation sample spiked with HIV drugs was compared with theoretical. The mean %dev from theoretical was 5%. Blank hPBMC spiked with other HIV drugs showed no false positive or negative response for either TFV or IS. Therefore, no apparent interference from any of the HIV medications tested exists.

4.6. Stability

The experimental stability samples were compared with a freshly extracted control set ($n=5$) of low and high validation samples. Mean, standard deviation, %CV, and %dev were calculated for each low and high validation sample. From the mean values of these sets, a percent difference (treated versus control) was calculated for the low and high levels. We were willing to accept a 10% difference between mean treated and control results to be considered stable under the treated conditions. The greatest percent difference between the treated and control sample was −10.2% for that of low validation sample stored for 24 h at room temperature. Thus, we found TFV-DP stability in 70:30 methanol:ddH_2O lysate to be stable through three freeze/thaw cycles and at room temperature for 24 h. It was also found that the extracted samples, containing TFV were stable for at least 5 days in the autosampler (4 °C) in the ddH_2O reconstitution matrix. The quality control sample lot currently in use in the laboratory has been stable long term at −80 °C for approximately 8 months with mean %dev less than 6% from theoretical concentrations.

4.7. Matrix effects

4.7.1. Matrix purity/specificity

The extracted blank hPBMC ($n=6$ different lots) chromatograms' noise measured analogously to the noise depicted in blank water injections. No positive TFV or IS response was evident.

Table 3
Peak area data in Sets 1–3

	Precision (%CV), $n=5$								
	Analyte peak area			Internal standard peak area			Peak area ratio		
	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
Low									
Mean	186565	203083	167292	1281241	1461762	1309917	0.146	0.140	0.128
S.D.	8166	28361	8802	46977	240150	55134	0.005	0.005	0.004
%CV	4.38	13.97	5.26	3.67	16.43	4.21	3.09	3.27	3.14
n	5	5	5	5	5	5	5	5	5
Medium									
Mean	979399	1047420	925497	1358966	1546916	1391136	0.721	0.677	0.665
S.D.	24808	100347	17664	33383	151837	23657	0.007	0.006	0.006
%CV	2.53	9.58	1.91	2.46	9.82	1.70	1.02	0.90	0.91
n	5	5	5	5	5	5	5	5	5
High									
Mean	4067152	4318491	3623008	1405846	1555054	1384883	2.893	2.777	2.615
S.D.	10905	222611	167385	10663	48921	31551	0.025	0.096	0.075
%CV	0.27	5.15	4.62	0.76	3.15	2.28	0.86	3.45	2.86
n	5	5	5	5	5	5	5	5	5
Column	A	B	C	D	E	F	G	H	I

4.7.2. Matrix effect, recovery, and process efficiency

ME, RE, and PE were determined by comparing results from Sets 1–3 in different ways. Table 3 shows the compiled data of TFV areas, IS areas, and peak area ratios (PAR) for all five lots at all three concentration levels for all three experimental sets. Mean, standard deviation, and coefficient of variance were calculated across the five different lots. The %CVs were calculated for the peak areas and PAR. Set 2 showed the highest %CVs for both TFV and IS absolute values, nearing almost 17% for the low level. However, the PAR %CVs for all levels were less than 3.5%. This demonstrated that the isotopic internal standard was compensating for the absolute area variability regardless of concentration and matrix as expected when an isotopically labeled internal standard is employed.

Matrix effect was examined in Table 4. Set 2 was compared with Set 1. This calculation measured relative MS ESI source suppression or enhancement from the matrix. In this case, the calculation showed that the signal achieved in matrix was 108.85% (low), 106.95% (medium), and 106.18% (high) that of signal achieved in the absence of matrix. If isotopic IS were not used, or if the chosen IS did not correlate with TFV, this could be of potential concern. However, Column K showed similar values as Column J for the three concentrations. This demonstrated that the isotopic IS was equally enhanced as TFV. When ME was calculated using the PARs instead of absolute areas, the ME was 95.41% (low), 93.95% (medium), and 95.99% (high). Again, the internal standard perfomed as expected.

Recovery was also examined in Table 4, where Set 3 was compared with Set 2. This calculation measured recovery of

Table 4
Matrix effect (ME), recovery (RE), and process efficiency (PE)

Nominal concentration	ME		RE		PE	
	Analyte	IS	Analyte	IS	Analyte	IS
QL	108.85	114.09	82.38	89.61	89.67	102.24
QM	106.95	113.83	88.36	89.93	94.50	102.37
QH	106.18	110.61	83.90	89.06	89.08	98.51
Column	J	K	L	M	N	O

PAR

ME	RE	PE
95.41	91.93	87.71
93.95	98.25	92.31
95.99	94.20	90.43

Table 5
Slopes of the fitted line of low, medium, and high concentrations

Matrix lot #	Set 1	Set 2	Set 3
1	2.72E−04	2.92E−04	2.48E−04
2	2.87E−04	2.89E−04	2.67E−04
3	2.88E−04	2.85E−04	2.60E−04
4	2.63E−04	2.90E−04	2.62E−04
5	2.80E−04	2.91E−04	2.70E−04
Mean	2.78E−04	2.89E−04	2.61E−04
S.D.	1.06E−05	2.70E−06	8.47E−06
%CV	3.80	0.93	3.24

Table 6
hPBMC variation

Million PBMC	TDF area	IS area	Area ratio	Concentration
1	170953	342233	0.500	2011
2	196561	378780	0.519	2085
5	120952	224395	0.539	2161
10	75659	145286	0.521	2092
Mean			0.520	2087
S.D.			0.016	61.4
CV			3.1	2.9

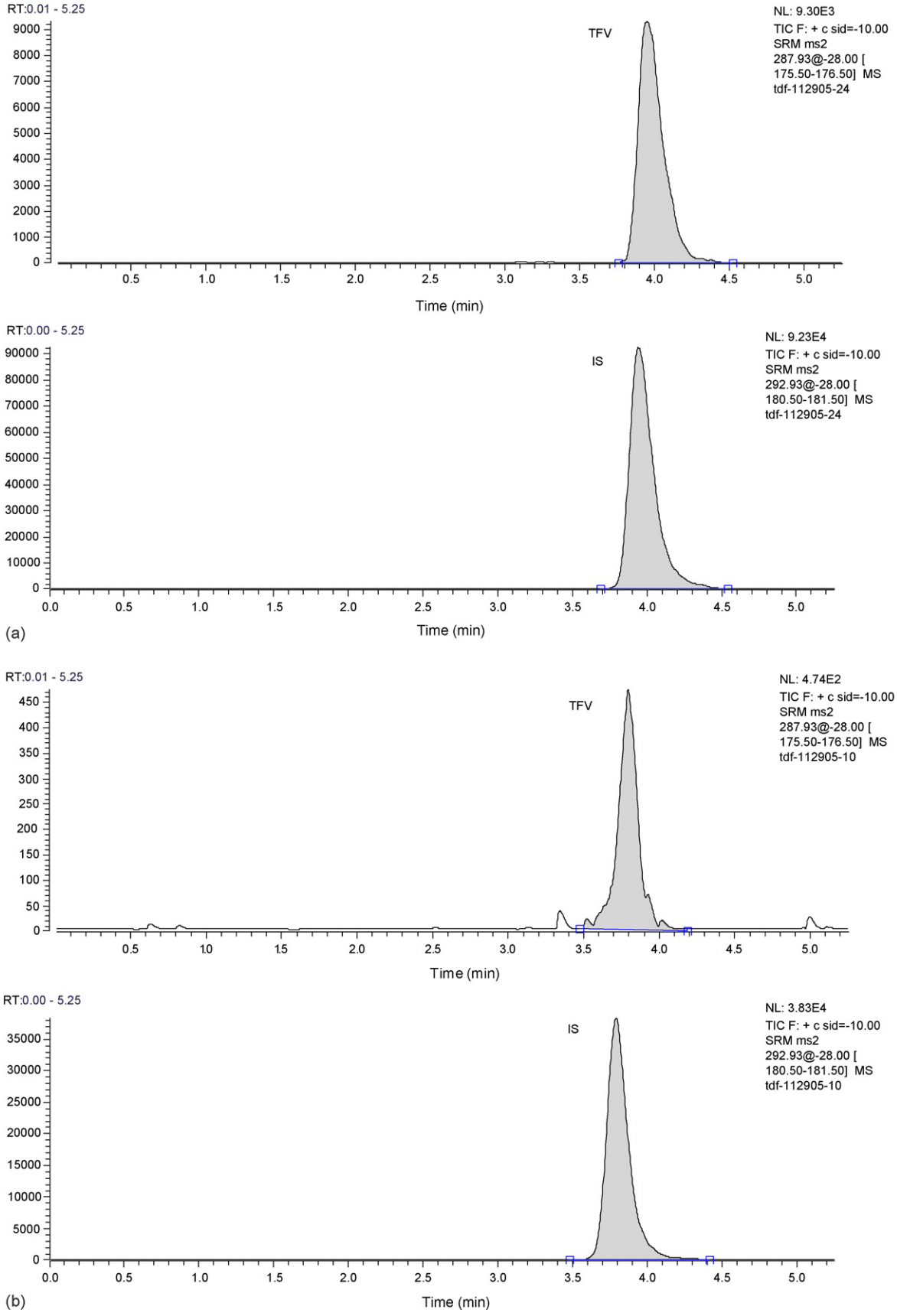


Fig. 2. (a) Typical patient chromatogram (81.73 fmol/million cells). (b) Chromatogram of lower limit of quantitation (50 fmol/sample). (c) Chromatogram of blank (no peak detected).

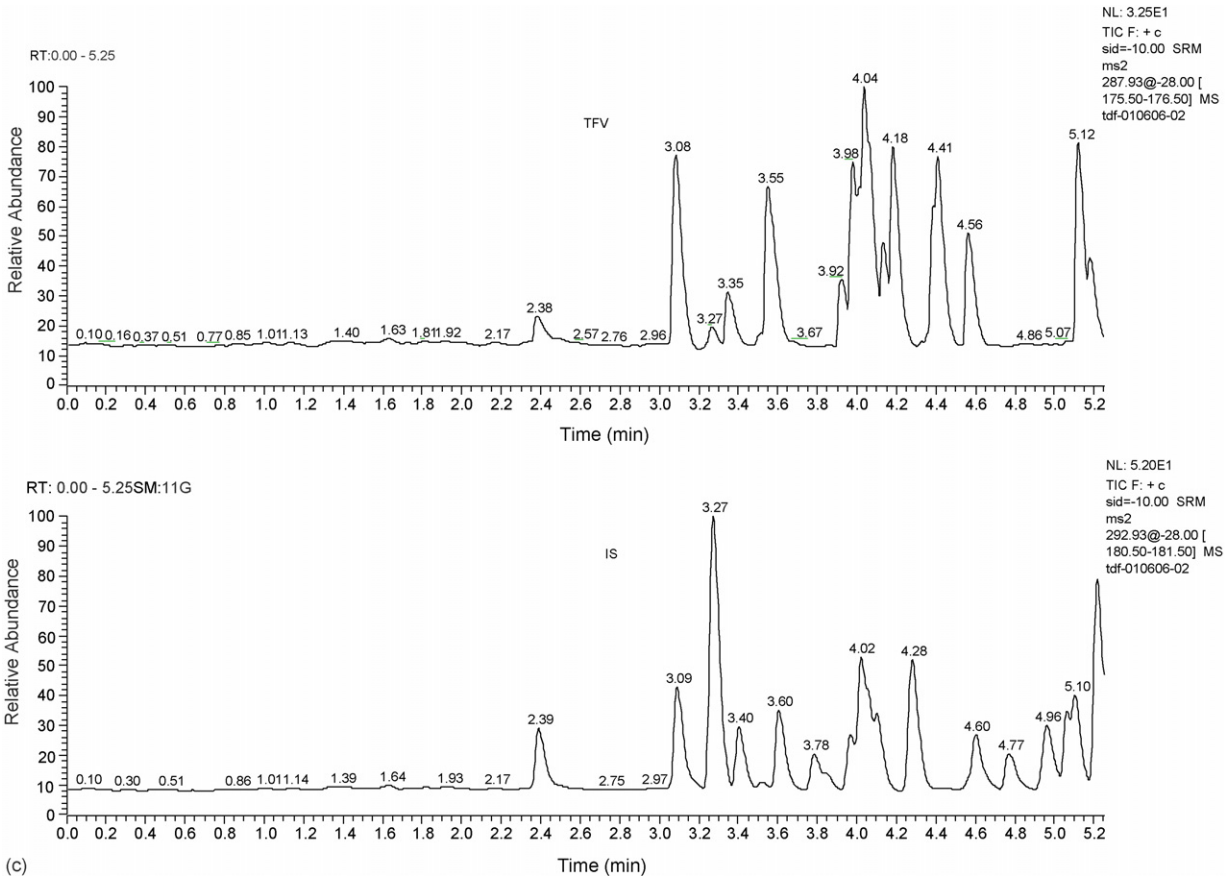


Fig. 2. (*Continued*).

analyte(s) through the extraction procedure. Recovery ranged from 82.38 to 88.36% across the three TFV levels.

Finally, process efficiency was also examined in Table 4, where Set 3 was compared with Set 1. This calculation measured overall process recovery of the analytes, including in the calculation both enhancement (ME) and extraction efficiency (RE). This percentage ranged from 89.08 to 94.5% across the three TFV levels. This percentage ranged from 98.51 to 102.37% for the IS across the three levels.

We also investigated whether one lot of hPBMC ($n = 5$) differed in ME from another. The low, medium, and high concentrations for each lot were fitted to a 3-point linear unweighted regression to yield five separate lines (one for each hPBMC lot). Mean, standard deviation, and %CV data (Table 5) were calculated for these five lines from Sets 1–3. The resulting %CVs from Sets 1–3 were less than 3.8% indicative of no relative ME in relation to hPBMC lots and TFV quantitation with the methodology employed.

4.7.3. Cell number variability

The effect of the number of hPBMCs analyzed (1–10 million cells) was investigated. Mean, standard deviation, and coefficient of variance were calculated for PAR and concentrations (Table 6). The resulting coefficient of variance was 3.1% for PAR and 2.9% for concentration. Therefore, varying cell number did not change the final concentration of TFV-DP. This is important in the sense that our LLOQ can be optimized per million cells analyzed up to 10 million cells. We can conserve on lysate volume analyzed for other TP measurements or maximize cells tested to achieve an observable measurement above the LLOQ.

4.7.4. IS purity

It was shown that the stable labeled isotopic IS did not contribute any TFV response in the IS spiked hPBMC tested through the procedure.

5. Application to clinical samples

hPBMC samples from HIV-infected patients who were receiving the usual dose of 300 mg once daily TDF were analyzed for TFV-DP using the method described herein. See Fig. 2 for a typical patient chromatogram. The time elapsed from dosing time to citrate CPT blood draw (three tubes collected) varied from 0.5 to 48 h post-dose. We applied this method to 157 samples originating from 17 different patients. The samples were obtained from both initial intensive visits as well as single random visits throughout the study. We found a median concentration of 69 fmol/million cells. This compares well with the reported median TFV-DP concentra-

tion of 84.0 fmol/million cells observed in eight patients at 12 h post-dose and 87.2 fmol/million cells at 24 h in seven patients [12].

6. Conclusions

The standard curve responses for this assay were reproducible as well as significantly close to the theoretical values. The eight back-calculated concentrations of the standard curve could be used to accurately and precisely determine unknown sample concentrations used in this assay, including samples at the LLOQ level of 50 fmol/sample. This method was shown to be highly selective with no interferences from any of the HIV medications tested and no TFV response shown from different blank hPBMC sources. No endogenous interferences or environmental contamination were present in blank hPBMC for TFV or the IS retention times. Additionally, the internal standard was stable and pure throughout the entire extraction procedure.

It was also shown that low and high validation samples were stable under different temperature and time conditions both prior to extraction and post-extraction. Inclusion of an LC–MS–MS matrix effects supplement demonstrated with calculations of ME, RE, and PE that the internal standard worked as intended, by compensating for observed matrix and recovery effects. Cell number can vary from 1 million to 10 million hPBMC and not affect TFV-DP response, thus allowing for an LLOQ of 10 fmol/million cells if 5 million cells are analyzed.

Thus, the validation process has shown this to be a robust, stable, reliable, and efficient method for the quantitation of intracellular concentrations of the active, phosphorylated moiety of TFV in clinical samples.

The method described in this paper produced a median TFV-DP concentration of 69 fmol/million cells from 157 samples among 17 subjects, which is comparable to the median concentration of about 85 fmol/million cells reported by Hawkins et al. [12]. However, both these concentrations are lower than the median concentration of about 150 fmol/million cells reported by Pruvost et al. [11]. While an analytical basis for the reported differences in median TFV-DP concentrations by Pruvost cannot be ruled out, it is quite evident that further research is warranted to characterize the pharmacokinetics of TFV-DP. To this we would add that it is important to understand the pharmacologic mechanism(s) for undesirable patient responses with TDF such as, paradoxical CD4 lymphocytopenia, renal toxicity, and abrupt failure of triple NRTI regimens, so that rational strategies can be devised to avoid and manage these problems clinically. The analytical method described in this paper will facilitate such clinically important research.

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