The simultaneous assay of tenofovir and emtricitabine in plasma using LC/MS/MS and isotopically labeled internal standards

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ABSTRACT

An LC/MS/MS assay we published for tenofovir (TFV) plasma levels is a useful tool for monitoring the pharmacotherapy of HIV-positive individuals [T. Delahunty, L. Bushman, C.V. Fletcher, J. Chromatogr. B 830 (2006) 6–12]. A new combination therapy consisting of the TFV pro-drug (300 mg) and another reverse transcriptase inhibitor, emtricitabine (FTC, 200 mg) has become available in a convenient once-daily dosage form (Truvada). This widely used medication has prompted us to develop and validate a convenient assay to determine simultaneously TFV and FTC plasma concentrations. In view of their chemical similarity to the analytes, stable isotope internal standards (IS) were chosen. These consisted of TFV labeled uniformly with ^{13}C in the adenine moiety (Iso-TFV) and FTC labeled with ^{13}C and ^{15}N in the cytosine moiety (Iso-FTC). Trifluoroacetic acid was added to the patient's EDTA plasma (containing the IS) to produce a de-proteinated extract after high speed centrifugation. The extracts were directly injected into the mobile phase (3% acetonitrile/1% acetic acid, aq.) stream flowing at 200 μL/min. A Synergi Polar-RP, 2.0 mm × 150 mm, reversed-phase analytical column was used to achieve the chromatographic separation. Detection of the analytes was achieved by ESI positive ionization tandem mass spectrometry. The precursor/product transitions (m/z) in the positive ion mode were 288/176 and 293/181 ions for TFV and Iso-TFV, respectively and the precursor/product transitions (m/z) were 248/130 and 251/133 ions for FTC and Iso-FTC, respectively. When the analyte/IS abundance ratios were plotted against the specified concentrations, the linearity of the concentration curves were in the range 10 ng/mL to 1500 ng/mL for both analytes (250 μL plasma extracted), with a minimum quantifiable limit of 10 ng/mL for both analytes. The inter- and intra-day accuracy and precision for both TFV and FTC were within ± 20% at the LLOQ and ± 15% at the other QC levels. We have expanded the method originally designed for the assay of TFV alone to incorporate the simultaneous determination of the latter and FTC using stable isotope IS. This assay has been successfully used for the periodic monitoring of 678 HIV-positive patients being treated with the combination therapy.

1. Introduction

Tenofovir disoproxil fumarate (TDV, Viread) is a widely used nucleotide analog pro-drug administered to combat HIV infection. Since the disoproxil moiety is hydrolyzed following absorption to generate the viral reverse transcriptase inhibitor, tenofovir (TFV),

pharmacotherapy is monitored by quantitation of the latter in plasma. The TFV pro-drug is currently combined with another nucleoside reverse transcriptase inhibitor, emtricitabine (FTC) in a single tablet (Truvada) for the convenience of HIV-positive patients and to improve medication adherence. FTC is similar to the established drug, lamivudine (3TC) but is clinically more effective. Since these anti-viral drugs do not interact pharmacokinetically, and have moderate elimination half-lives in plasma, a once-daily combination therapy is possible [1]. Furthermore, the combination of TDF and FTC can result in a greater HIV RNA suppression than either drug alone [2]. TDF and FTC administration to pregnant women has also been shown to contribute to a reduced viral resistance to non-nucleoside reverse transcriptase inhibitor drugs after nevirapine treatment [3]. Since the LC/MS/MS method we originally validated [4] has proven to be a useful technique for monitoring TFV plasma concentrations in pharmacokinetic studies [5], we redesigned the original method to permit the simultaneous assay of TFV and FTC.

This new method utilizes stable isotope IS for both drugs whose chemical and chromatographic properties are negligibly different from that of the analytes. Our earlier TFV assay used adefovir [4] and others have used dideoxyuridine [6] and deoxy-fluorocytidine [7] for FTC as internal standards. However, these internal standards have different chromatographic and chemical properties from that of the analytes, which could potentially lead to errors in the calculation of analyte concentrations [8]. An earlier version of the method described herein which used 8-azido adenosine as the FTC IS has been published as an abstract [9]. Since this manuscript was originally submitted, three separate research groups have published LC/MS methods for the simultaneous assay of TFV and FTC without the use of stable isotope IS [10–12].

2. Materials and methods

2.1. Chemicals

TFV (MM: 287.2, 99.9% pure), the internal standards, Iso-TFV (MM: 292.2, 99% pure) and Iso-FTC (MM: 250.2, 98.9% pure) were purchased from Moravek Biochemicals Inc., Brea CA. FTC (MM: 247.2) was obtained from the NIH AIDS Research and Reference Reagent Program, cat# 10071, NIAID. The Iso-TFV contained adenine uniformly labeled with ^{13}C and the Iso-FTC was labeled in the urea portion of the cytidine moiety with ^{13}C (position 2) and ^{15}N (positions 1 and 3). No unlabelled analytes were present in these de novo synthesized IS. Trifluoroacetic acid, acetic acid, methanol, acetonitrile, (HPLC grade) and saturated ammonium hydroxide (14.8 M, A.C.S. certified) were obtained from Thermo-Fisher (Fairlawn, NJ). The ultra-pure DI water (DI water) was produced from a Barnstead Diamond Nanopure System. Human EDTA-plasma was purchased from Biological Specialty Corporation (Colmar, PA).

2.2. Instruments

The HPLC autosampler/pump was a Surveyor (Thermo Fisher, San Jose CA). The analytical column was a Synergi 4 μm Polar RP HPLC Column pore size, 80 Å, 2.0 mm × 150 mm protected by a Polar RP guard column (Phenomenex, Torrance CA). The mobile phase consisted of 3% acetonitrile/1% acetic acid in DI water flowing at 200 μL/min. A second external LC pump was used to rinse the MS source with 100% methanol flowing at 0.5 mL/min (model 510, Waters Corporation, Milford, MA) while the LC flow was diverted from the source. The MS/MS detector was a TSQ Quantum, operating in the ESI, positive polarity mode (Thermo Fisher, San Jose, CA) and the data system was Xcalibur, version 1.3, also from Thermo Fisher.

2.2.1. Calibration, IS, and QC solutions

Two, separately weighted, 1.00 mg/mL stock solutions of TFV and FTC (Calibration Preparation stock, #1 and QC Preparation stock, #2) were prepared by dissolving approximately 1 mg aliquots of each drug, accurately weighed on a Mettler MX-5 micro balance (Mettler, Toledo, OH), in the calculated volumes of DI water and methanol, respectively. The TFV and FTC Stocks #1 and #2 were used for the preparation of working calibration standards and quality controls (QCs), respectively. Preparation stocks #1 and #2 were compared for accuracy prior to the preparation of working calibration standard solutions and QC's. Nine TFV/FTC combined working calibration standard solutions were prepared in 50:50 methanol/water (v/v) from the stock solution #1 using calibrated positive displacement pipettes to give a final plasma concentration range of 10–1500 ng/mL for each analyte. All stock solutions were stored at −20 °C in glass screw-top test tubes tightly sealed with Teflon-lined caps to prevent evaporation. The stock 1.00 mg/mL

internal standard (IS) solutions were prepared by dissolving the calculated quantity of the chemical in DI water (iso-TFV) or methanol (iso-FTC). A second preparation stock of the IS consisted of 10 μL (stock iso-TFV) and 50 μL (stock iso-FTC) per 3 mL of their respective solvents. The working IS solution was prepared daily by combining the appropriate volumes of the latter solutions in a 1:1 ratio. QCs were prepared from TFV/FTC preparation stocks #2. These consisted of High QC (1200 ng/mL; 80% of the highest calibration standard), Medium QC (300 ng/mL), Low QC (30 ng/mL; 3× the lowest calibration standard) and LLOQ (10 ng/mL; equivalent to the lowest calibration standard). They were prepared with blank human plasma and the appropriate volumes of the preparation stock #2 using volumetric flasks and the appropriate positive displacement pipettes. Aliquots (600 μL) were stored in screw-top Cryovial tubes at −20 °C.

2.3. Sample preparation

The preparation method for calibrators, QCs and patient samples followed that used for TFV alone (4). All plasmas used in this study were derived from EDTA treated human blood. Working calibration solutions, QCs and unknowns were allowed to equilibrate to ambient. Calibration standards for each run were prepared by pipetting 20 μL (calibrated positive displacement pipette) of each working calibration standard solution (methanol/water 50/50, v/v) to nine 250 μL aliquots of blank human plasma in 1.5 mL microcentrifuge tubes (SealRite, USA Scientific) resulting in a concentration range of 10–1500 ng/mL for the calibration curve. Aliquots (250 μL) of the plasma unknowns, blank and QCs were added to appropriately labeled 1.5 mL microcentrifuge tubes to which 20 μL of methanol/water (50/50, v/v) had been added to normalize the volume of unknown, blank and QC tubes to that of the calibration standard tubes. The working IS solution (20 μL) was added to each extraction tube using a calibrated positive displacement pipette and all were vortexed well. Trifluoroacetic acid (25 μL) was then added to all tubes, followed by prompt capping and vortexing. After standing at ambient for 15 min, all tubes were ultra-centrifuged in a Spectrafuge (Labnet International) at 13,000 rpm for 15 min. The de-proteinated extracts were transferred to labeled autosampler vials containing 400 μL capacity flat-bottom inserts (ChromTech, Apple Valley, MN) to which 20 μL of concentrated ammonium hydroxide (14.8 M) had been previously added. After capping and vortexing, the vials were loaded in the autosampler tray which was maintained at 4 °C.

2.4. LC/MS/MS procedure

2.4.1. Chromatography conditions

Ten microlitres aliquots of the extract were injected into the HPLC with the mobile phase (3% acetonitrile/1% acetic acid in DI water) flowing isocratically at 200 μL/min. Column temperature: 22–25 °C (ambient). Run time: 11 min. TSQ Quantum conditions: ESI, positive polarity. Spray voltage: 3200 V; capillary temperature: 300 °C; Gas Pressures: sheath (nitrogen); 30, Aux (argon); 10, Finnigan arbitrary units. Collision gas pressure: 1.8 mTorr; collision energy: 28 eV (TFV and Iso-TFV), 11 eV (FTC and Iso-FTC). Precursor/Product ion transitions were used for selective reaction monitoring (SRM). [M+H] ions detected (m/z): TFV; precursor 288, product 176. FTC; precursor 248, product 130. Iso-TFV; precursor 293, product 181. Iso-FTC; precursor 251, product 133. Quadrupole resolution: Q1 and Q3; 0.7 FWHM. Scan width: 1 m/z. Scan time: 100 ms. Source CID: 10 V.

2.4.2. Divert valve program

Methanol flowing externally at 0.5 mL/min from the Waters pump rinsed the source while the HPLC flow was diverted. The

HPLC eluate flow destination was switched alternatively between the detector and waste as follows. From 0 to 2 min: to waste. From 2 to 9 min: to detector. From 9 to 11 min: to waste.

2.5. Validation methods

An FDA Bioanalytical Method Validation publication was used as a guide for this validation [13].

2.6. Accuracy and precision

Calibration curves for this validation were run in separate analytical runs as previously described for TFV alone (4). They were created by plotting the peak area ratio of the TFV/Iso-TFV and FTC/Iso-FTC against the analyte concentration. A $1/X$ weighted linear regression equation of the type $y = bx + a$ was used. Back-calculated concentrations for each calibration level were determined as well as slope and coefficient of determination data ($n = 3$ for interday). Accuracy and precision of the method was determined by analysis of the QCs in 3 separate analytical runs with five replicates (see below). These results should be within ±20% at the LLOQ and ±15% at all other concentrations. Dilute specimens were also validated for accuracy as follows. A 3000 ng/mL highly concentrated sample was prepared for TFV and FTC. From this, 3-fold and 5-fold diluted samples were prepared and analyzed. Acceptable accuracy results should be within ±15%.

2.7. Stability

Stock solution stability included testing the stability of TFV and FTC in preparation solvents and storage at −20 °C. Both preparation stocks in their respective solvents (TFV, water; FTC, 50:50 methanol/water) were re-analyzed periodically (see below). The stability of the analytes in plasma during 3 freeze/thaw cycles was also tested using spiked plasma samples at concentrations of 30.0 and 600 ng/mL (TFV) and 30.0 and 800 ng/mL (FTC). Samples were removed from freezer, allowed to equilibrate to ambient and placed back into −20 °C storage. Room temperature stability was also assessed to account for potential shipping errors that may occur when plasma samples are in transit to the laboratory. The spiked plasma samples were allowed to equilibrate to ambient and held there for 6 days before being extracted and re-analyzed. The stability of the extracts in the autosampler at 4 °C was also tested to show that subsequent analysis after LC malfunction would not jeopardize the analysis. The long term stability of the analytes in proficiency plasmas (ACTG proficiency testing program, Rd 15, March 2005) was determined after storage at −80 °C. A comparison was then made to the nominal analyte concentrations of the tested samples (see below). Instability would be considered if a deviation from nominal of greater than 15% were observed.

2.8. Sensitivity and selectivity

Seven different blank plasma lots were checked for any erroneous positive or negative MS responses. Any positive response would need to have a peak area <10% of the LLOQ or the IS peak areas. These plasma lots were also used to generate separate calibration curves which were compared to determine if different plasma lots could affect the response of the calibrators. The possibility that concomitant drugs such as protease inhibitors (PI), other nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transciptase inhibitors (NNRTI) might have an effect on the TFV/FTC analysis was also assessed. Several antiviral drugs likely to be present in the plasma of HIV-positive patients at concentrations significantly higher than what would be expected in clinical practice were added to blank plasma. Aliquots of this plasma were used to prepare the 250 ng/mL calibrator and a blank. The following drugs were used: IDV, DLV, APV, NFV, M8, ATV, SQV, EFV, RTV and LPV (20,000 ng/mL for each drug) and 3TC, d4T, AZT, ABC and DDI (10,000 ng/mL for each drug). [Definitions of these drug acronyms can be found in the footnotes]. After analysis, a comparison was made to the nominal concentration. A difference of greater than 15% would be indicative of a potential effect from these concomitant drugs. Since SRM detection was employed, we did not investigate where these concomitant drugs eluted on the chromatogram.

2.9. Suppression effects

The possibility that the trifluroacetic acid and salts present in the extracts could by themselves result in a suppression of the MS signal was assessed as follows.

Set A: Five aliquots (250 µL) of mobile phase in microcentrifuge tubes were spiked with 20 µL of standard solution (300 ng/mL level) and 20 µL IS. Then 25 µL trifluoroacetic acid and 20 µL ammonium hydroxide were mixed in and the solutions transferred to vials for analysis as described above.

Set B: These solutions were prepared as described for Set A, except that 45 µL of mobile phase was used instead of the acid and base.

Sets A and B were analyzed and the results compared to assess suppression.

2.10. Matrix effects

The method used for testing matrix effects (ME) was a modification of that used by Matuszewski et al. [8] which involved the preparation and analysis at 3 concentration levels: 30, 300, and 600 ng/mL for TFV and 30, 300, and 800 ng/mL for FTC, constituted as follows.

Set 1: Analytes and IS were dissolved in the mobile phase at the appropriate concentration and analyzed (used for comparison).

Set 2: Post-extraction spike of analytes and IS into the appropriate volume of supernatant from extracted blank plasma, followed by analysis (used to calculate suppression and losses due to protein precipitation).

Set 3: Pre-extraction spike of analytes and IS into plasma followed by extraction as described above under sample preparation. (used to calculate overall process efficiency, PE).

Five different lots of plasma were used at each concentration level for Sets 2 and 3. Set 1 (mobile phase only) was performed in 5 replicates. Recovery and suppression were assessed by comparing the peak areas between the different Sets.

3. Results and discussion

The chemical structures of TFV and Iso-TFV are shown in Fig. 1(A). The nucleoside analog, TFV is a phosphonomethoxypropyl adenine derivative.

Fig. 1(B) depicts the structures of FTC and Iso-FTC. FTC is a dideoxyfluorothia derivative of cytidine, closely related chemically to 3TC (Lamivudine). The position of the isotopic elements in the structure of the Iso-TFV and the Iso-FTC molecules is indicated by their atomic mass in their respective structures. In Fig. 2(A) and (B) are depicted typical chromatograms showing the TFV and FTC response from an extracted calibration standard (TFV and FTC = 250 ng/mL) and their corresponding
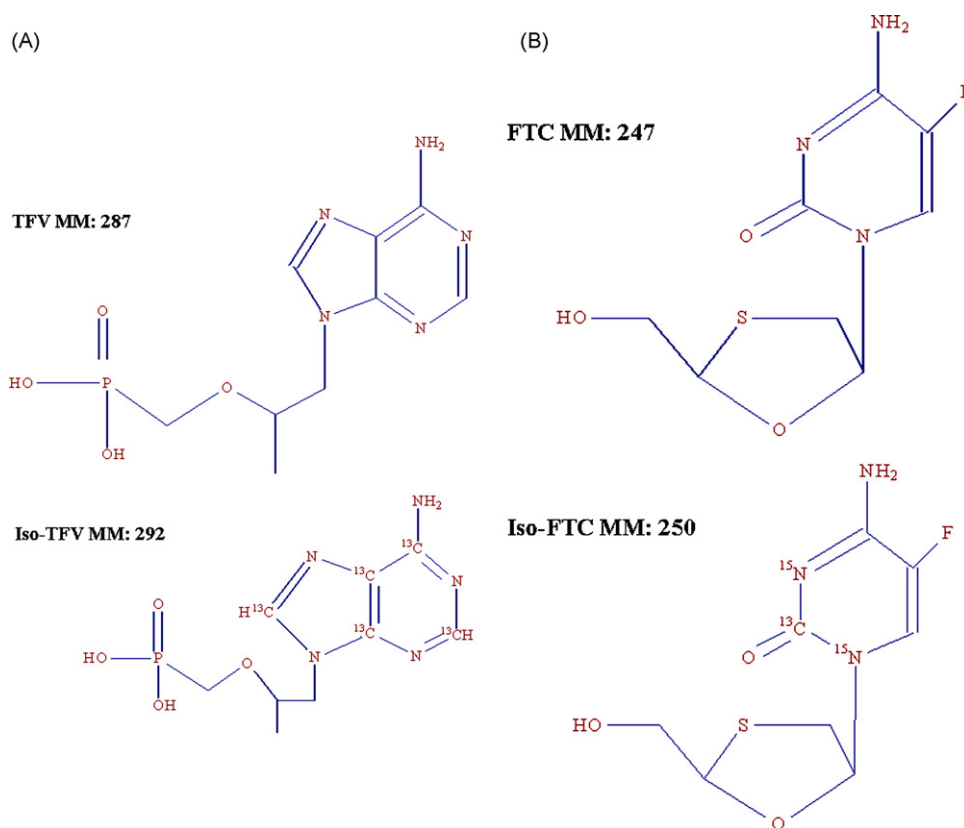
(A)   (B)

Fig. 1. (A) The chemical structures of TFV and Iso-TFV. (B) The chemical structures of FTC and Iso-FTC.

internal standard responses (Iso-TFV and Iso-FTC), respectively. The monitored m/z values for the respective precursor/products were: TFV, 288/176; FTC, 248/130; Iso-TFV, 293/181; Iso-FTC, 251/133. The peak patterns and retention times obtained when TFV and FTC precursor/products were specified were identical to that seen when the corresponding isotopic precursor/products were specified. Retention time variability within a run: TFV; mean ± SD, 3.51 ± 0.013 min; FTC; mean ± SD, 7.05 ± 0.039 min; N = 35.

Fig. 2(C) depicts the chromatogram obtained when a patient's plasma extract was analyzed for the drugs (TFV = 61.9 ng/mL, FTC = 475 ng/mL, IS's not shown). It should be noted that the peak area for FTC was always considerably greater than that of the TFV due to the greater response of the former in the MS source. The absolute abundances are plotted in the figures and the various peak areas are also shown in the legends for comparison. Fig. 2(D) depicts a typical chromatogram obtained when the LLOQ calibrator (10 ng/nL) was analyzed. The tracing obtained when blank plasma was analyzed using the specified precursor/product ions for TFV/FTC is also depicted in Fig. 2(D), illustrating the low abundance of the blank tracing relative to the LLOQ.

Calibration curves were obtained by plotting the peak area ratios of TFV and FTC, relative to the corresponding IS, against the assigned concentrations of the spiked plasma calibration standards (analytical range; 10–1500 ng/mL). Since the back calculated results for the validation showed good accuracy and precision, it was concluded that the TFV/FTC calibration curves produced by this method could be used to determine unknown drug concentrations in plasma. In addition, twenty-eight consecutive calibration analyses were performed on different days with freshly prepared calibration standards and the back-calculated values for each level recorded. The % CV at each level varied from 1.01 to 8.19 (TFV) and from 2.5 to 7.6 (FTC) while the % deviation from the theoretical

value varied from −3.79 to 6.09 (TFV) and from −2.5 to 7.6 (FTC). The % CV of the twenty-eight slopes was 11.1 for TFV and 12.2 for FTC and the lowest coefficient of determination (R^2) among the twenty-eight calibration curves was 0.9958 for TFV and 0.9955 for FTC.

3.1. Accuracy/precision

The assay was assessed for inter-day and intra-day accuracy and precision using the High, Medium, Low and LLOQ QCs. Tables 1 and 2 depict the within-run (N = 5) and day-to-day (N = 15) results obtained when the QCs were analyzed for TFV and FTC, respectively. Each level was run five times sequentially on three different days. Inter-day and intra-day accuracy was tested by calculating the percent deviation from the theoretical concentration. Precision was determined by calculating the coefficient of variation (%CV). As can be seen, the inter-day and within-run precision and accuracy were acceptable for TFV and FTC at each level for the five replicates. Overall, the assay was both accurate and precise between runs and within individual runs. The greatest mean inter-day percent deviation for TFV and FTC were 12.0% and 10.1% respectively for the LLOQ (10 ng/mL). All non-LLOQ QC levels had inter-day percent deviations which were less than 7% for both analytes. The within-run precision for the non-LLOQ QCs was less than 6% on each of the five days of the study (see Tables 1 and 2). The accuracy of measuring the analytes in diluted samples was also assessed. Aliquots of the 3000 ng/mL spiked plasma were diluted 5-fold (600 ng/mL) and 3-fold (1000 ng/mL) with blank plasma and analyzed along with the nine calibrators and two sets of QCs. The variation from nominal of the 5-fold and 3-fold dilution results were: TFV, −5.3% and −7.3%; FTC, 3.0% and −4.6%, respectively. This study indicated that should a result exceed the upper limit of the calibration curve, the remaining
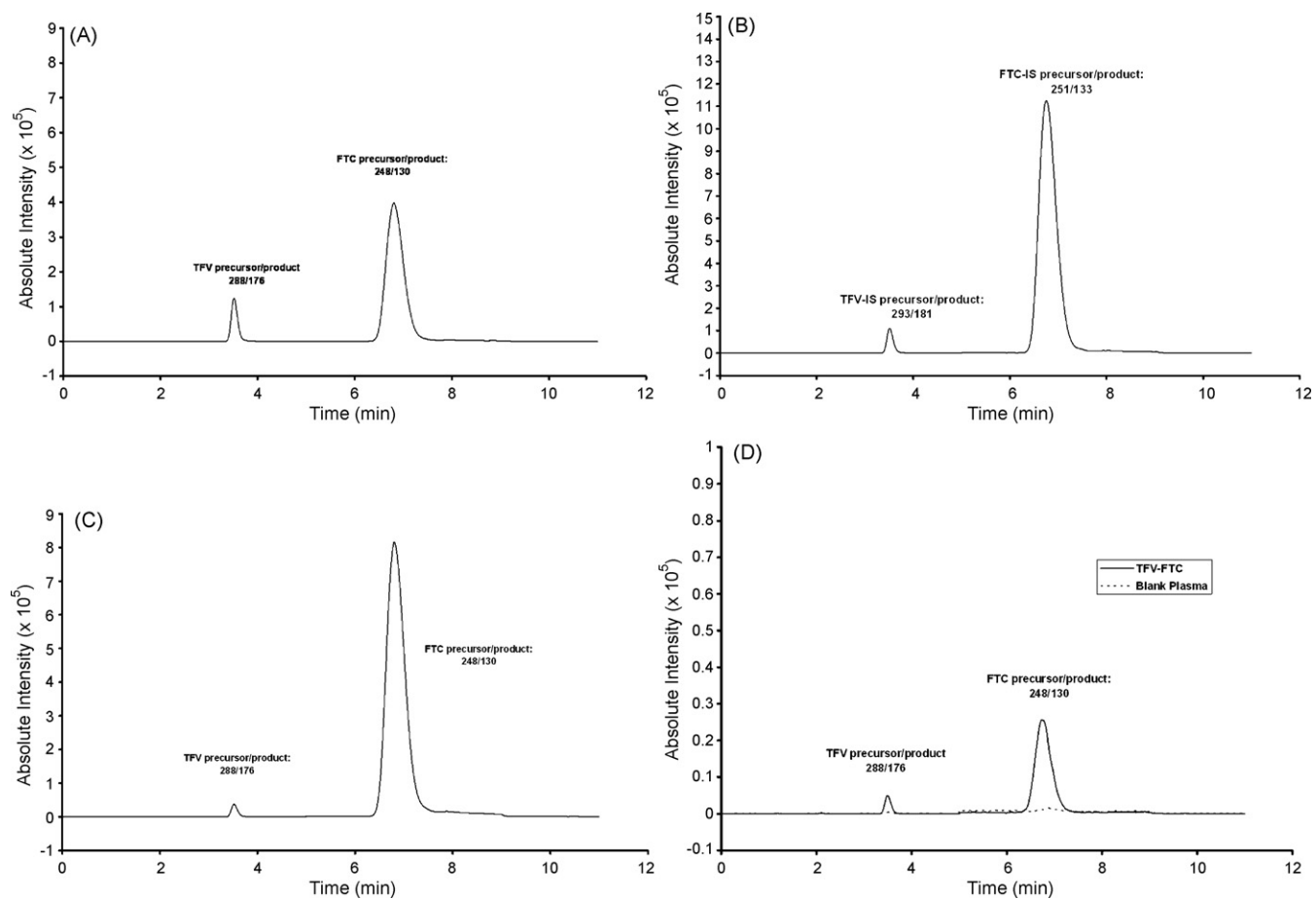
Fig. 2. (A) LC/MS/MS chromatogram of TFV and FTC in spiked plasma extract (250 ng/mL). Peak Areas: TFV, 1.16×10^6; FTC, 10.1×10^6. (B). LC/MS/MS Chromatogram of Iso-TFV and Iso-FTC Internal Standards. Peak Areas: Iso-TFV, 1.24×10^6; Iso-FTC, 33.1×10^6. (C). LC/MS/MS Chromatogram of TFV and FTC in Patient's Plasma Extract. Peak Areas: TFV, 0.347×10^6; FTC, 21.4×10^6. (D). LC/MS/MS Chromatograms of TFV and FTC in a Low Spiked Plasma (10 ng/mL) and a Blank Plasma (overlaid).

Table 1
Inter- and intra-assay accuracy and precision of TFV in plasma.

TFV						Intra-assay				
Sample #	LLOQ	Low QC	Middle QC	High QC	Run ID	Statistics	LLOQ	Low QC	Middle QC	High QC
1	10.9	27.2	289	1146	1	Mean	11.5	29.1	300	1181
2	11.2	29.3	293	1196		SD	0.6	1.2	9.2	20
3	12.0	29.0	301	1189		%CV	5.3	4.1	3.1	1.7
4	12.3	30.4	305	1187		%dev	15.0	−2.9	0.0	−1.6
5	11.1	29.7	312	1187		n	5	5	5	5
1	9.26	29.1	326	1251	2	Mean	10.4	28.6	321	1295
2	9.93	28.5	326	1287		SD	1.4	0.4	14.5	43
3	12.9	28.2	327	1286		%CV	13.8	1.5	4.5	3.3
4	9.77	28.2	330	1284		%dev	3.9	−4.7	6.9	7.9
5	10.1	29.0	295	1366		n	5	5	5	5
1	9.18	28.1	289	1148	3	Mean	9.31	28.0	299	1144
2	9.04	27.0	290	1102		SD	0.2	0.8	9.2	29
3	9.53	27.5	303	1153		%CV	2.3	2.8	3.1	2.6
4	9.53	28.6	308	1133		%dev	−6.9	−6.6	−0.2	−4.7
5	9.25	28.9	307	1182		n	5	5	5	5
Inter-assay statistics										
Theoretical concentration			10		30		300		1200	
Mean			10.4		28.6		307		1206	
SD			1.3		0.9		14.7		73	
%CV			12.0		3.2		4.8		6.0	
%dev			4.0		−4.7		2.2		0.5	
n			15		15		15		15	

Table 2
Inter- and intra-assay accuracy and precision of FTC in plasma.

FTC						Intra-assay				
Sample #	LLOQ	Low QC	Middle QC	High QC	Run ID	Statistics	LLOQ	Low QC	Middle QC	High QC
1	8.40	27.3	281	1089	1	Mean	8.76	28.4	287	1122
2	8.80	28.2	281	1145		SD	0.3	0.7	5.9	21
3	8.80	28.2	289	1129		%CV	2.9	2.6	2.1	1.9
4	9.10	29.0	287	1117		%dev	−12.4	−5.5	−4.5	−6.5
5	8.70	29.1	295	1131		*n*	5	5	5	5
1	8.50	26.6	302	1158	2	Mean	9.22	27.5	302	1189
2	8.89	27.8	306	1148		SD	1.4	0.6	13.7	41
3	11.7	27.6	308	1181		%CV	15.1	2.0	4.5	3.5
4	8.49	28.1	315	1211		%dev	−7.8	−8.2	0.7	−0.9
5	8.52	27.6	279	1249		*n*	5	5	5	5
1	9.42	27.2	280	1084	3	Mean	10.0	27.2	280	1083
2	10.3	27.7	273	1096		SD	0.3	0.44	5.0	19
3	10.0	27.5	277	1092		%CV	3.4	1.6	1.8	1.8
4	10.1	26.9	285	1050		%dev	0.0	−9.4	−6.7	−9.8
5	10.2	26.6	284	1093		*n*	5	5	5	5

Inter-assay statistics

	LLOQ	Low QC	Middle QC	High QC
Theoretical concentration	10	30	300	1200
Mean	9.33	27.7	289	1132
SD	0.94	0.7	12.8	53
%CV	10.1	2.7	4.4	4.7
%dev	−6.7	−7.7	−3.5	−5.7
n	15	15	15	15

plasma could be diluted up to 5-fold and re-analyzed to fit within the established parameters.

3.2. Stability of stock solutions

A comparison was made between freshly prepared stock solutions and those that had been stored at −20 °C. TFV (water) and FTC (MeOH) preparation stock 1.00 mg/mL solutions were shown to be stable for at least one year and 18 months, respectively. The percent differences in peak response varied no more than ± 6.1% between the freshly prepared and stored solutions (data not shown).

3.3. Stability in plasma and extracts

As can be seen in Table 3, both TFV and FTC were stable for three freeze–thaw cycles since the % deviation from the theoretical concentrations indicated that no decomposition had occurred. Similarly, the analytes were found to be stable in plasma when stored at ambient temperature for 6 days. (The % deviation from nominal for the low and high concentrations: TFV, 5.2 and 5.4; FTC, 5.1 and 0.1, respectively, *n* = 3, Table 3). This finding is consistent with reports by others illustrating the remarkable stability of these anti-viral drugs [4,6,10,12], also various Gilead Sciences publications, not listed. The stability of the extracted samples stored in the autosampler for two days was also assessed. As Table 3 shows, the % deviation from theoretical for the two levels (*n* = 8 and 9) was 2.30 and 2.17 for TFV and −4.00 and −5.58 for FTC, suggesting that there was no significant decline in the response ratio during that time. (The actual peak areas and retention times of the analytes did not drift from those of the corresponding freshly prepared extracts, data not shown). As depicted in Table 4, the TFV and FTC concentrations in samples stored at −80 °C for nearly 3 years were within acceptable limits of the nominal, indicating that both analytes were stable in plasma for this period when stored at −80 °C.

3.4. Sensitivity and selectivity

Since the CV at the 10 ng/mL level was <15% for both analytes (Tables 1 and 2), it was decided to make this the lowest

calibrator, although a lower concentration could probably have been achieved. This LLOQ was found suitable for the compliance and pharmacokinetic analyses performed at our institution. For comparison, the following LLOQs (ng/mL) were reported in recent publications: Gomes et al. [10]: TFV, 10; FTC, 25. D'Avolio et al. [11]: TFV, 15.6; FTC, 11.7. Nirogi et al. [12]: TFV, 20; FTC, 2. When the seven different plasma lots were used as described above, no positive or negative response for either the analyte or the IS at the appropriate retention times and SRM conditions was noted. After spiking the different plasmas with the nine calibration standards and the resulting calibration curves assessed, the variation between the area ratios at each TFV/FTC level was <5%. For example, at the 250 ng/mL level, the %CVs for TFV and FTC were 4.22 and 3.66, respectively (*N* = 7). Furthermore, the slopes of the calibration curves were similar for the different plasma lots (TFV: $4.25{-}3.90 \times 10^{-4}$; FTC: $1.65{-}1.30 \times 10^{-3}$), suggesting that plasma obtained from different individuals will not significantly affect the assay.

When 250 µL aliquots of the plasma spiked with the anti-viral drugs listed above were analyzed, no TFV or FTC was detected, indicating that none of the drugs listed were likely to give a false positive result. When this plasma containing the concomitant drugs was also spiked with the appropriate TFV/FTC working calibration solution at the 250 ng/mL level and included in a typical analytical run, the calculated concentrations of the analytes were within ±6% of nominal (250 ng/mL). It was therefore concluded that the presence of the concomitant anti-viral drugs did not affect the assay described here, a finding we expected due to the specificity of the precursor/product transitions. We also determined that the IS peak areas and retention times were not affected by the presence of concomitant drugs in the extracts.

3.5. Matrix effect

The presence of matrix effects in this assay was also investigated since suppression could theoretically cause undetected inaccuracies in the LC–MS–MS assay.

Table 3
Stability of TFV and FTC under various conditions.

3 F/T Cycles

TFV in plasma matrix

#				Low	High
	30	600	Theoretical concentration	30	600
1	33.2	644	Mean	35.5	652
2	39.5	630	SD	3.5	26.8
3	33.7	682	%CV	9.9	4.1
			%dev	18.3	8.7
			n	3	3

FTC in plasma matrix

#	Low		Low	High	
	30	800	Theoretical concentration	30	800
1	31.8	817	Mean	31.7	818
2	31.9	820	SD	0.3	1.7
3	31.3	817	%CV	1.1	0.2
			%dev	5.5	2.2
			n	3	3

144 h Room Temperature

TFV in Plasma Matrix

	Low	High		Low	High
	30	600	Theoretical concentration	30	600
1	32.1	639	Mean	31.6	632
2	31.3	621	SD	0.5	9.6
3	31.3	636	%CV	1.5	1.5
			%dev	5.2	5.4
			n	3	3

FTC in Plasma Matrix

	Low	High		Low	High
	30	800	Theoretical concentration	30	800
1	32.4	812	Mean	32	801
2	32.2	793	SD	1.3	10.1
3	30.0	798	%CV	4.1	1.3
			%dev	5.1	0.1
			n	3	3

2 Days in Autosampler 4 °C

TFV in extract

	Low	High
Theoretical concentration	30	1200
Mean	30.69	1226
SD	1.30	11.3
%CV	4.23	0.93
%dev	2.30	2.17
n	9	8

FTC in extract

	Low	High
Theoretical concentration	30	1200
Mean	28.8	1133
SD	0.53	6.77
%CV	1.83	0.60
%dev	4.0	5.58
n	9	8

Note: All concentrations are ng/mL.

3.5.1. Non-plasma effects

When the results of Set A and Set B (above) were compared, a 69% decrease in both TFV and Iso-TFV areas was detected, suggesting that the presence of trifluoroacetic acid and salts in the preparation results in a significant suppression. However, the FTC and its isotopic analogue, Iso-FTC, were only reduced by 16% in this experiment. This analyte-specific effect was presumably due to the early co-elution of the TFV and the acid and/or salts in the chromatogram.

3.5.2. Plasma effects

No significant variation in analyte areas was found between the 5 different plasma lots, suggesting that different endogenous plasma components did not affect the recovery. When Sets 1 and 2 were compared, the results showed that TFV and FTC abundances were suppressed by 73% and 17% at the three concentration levels, respectively. The abundances for the isotopic analogues were suppressed to the same degree as the analytes, illustrating the advantage of using stable labeled compounds as internal standards in this analysis. When Sets 2 and 3 were compared, the recoveries from the protein precipitation step were found to be 101.2% for TFV and 73% for FTC. The overall recoveries for TFV and FTC were 37.7% and 66%, respectively (Set 3 compared to Set 1). Even though the abundances underwent a large drop in PE, the peak area ratios were unaltered due to the fact that the analyte and analogue abundances were equally affected. As the results from Sets A and B demon-strate, the primary agents suppressing the TFV abundance were the acid and salts in the extracts. On the other hand the FTC abundances were mostly affected by losses during the centrifugation step.

3.6. Patient analysis

In one study, plasma samples from 228 HIV-positive patients receiving a daily dose of 300 mg TDF/600 mg FTC (Truvada) were analyzed periodically for drug concentration using the method described herein. The TFV concentrations ranged from 28.9 to 513 ng/mL ($N = 360$, median concentration, 92.6 ng/mL) and the FTC concentrations ranged from 41.9 to 2886 ng/mL ($N = 360$, median concentration, 373 ng/mL). The elapsed time from the morning dosing to phlebotomy varied from one to twenty-four hours for these random visits. In another protocol, HIV-positive patients were enrolled in pharmacokinetic studies which involved phlebotomy at various times up to 24 h after Truvada administration. A typi-cal pharmacokinetic curve is shown in Fig. 3 for a single patient from this study. Since this assay was designed, a total of 678 HIV-positive patient samples have been analyzed for TFV and FTC under the auspices of the ACTG.

The wide-spread prescribing of the single, once-a-day, Truvada combination tablet provided the incentive to develop a simple plasma assay for TFV and FTC which was sensitive, precise, accurate

Table 4
Long term stability of TFV and FTC in plasma.

1/9/2008 TFV	Nominal Rd 15	%Difference	1/9/2008 FTC	Nominal Rd 15	%Difference
363	387	−6.2	94.8	90.0	5.3
732	773	−5.3	447	450	−0.7
74.8	77.0	−2.9	853	900	−5.2
288	299	−3.7	524	550	−4.7
2301	2308	−0.3	101	100	1.0

Long term plasma stability of TFV and FTC at −80 °C. Proficiency testing samples ACTG PT Rd 15 (March 2005). Elapsed time: 34 months.
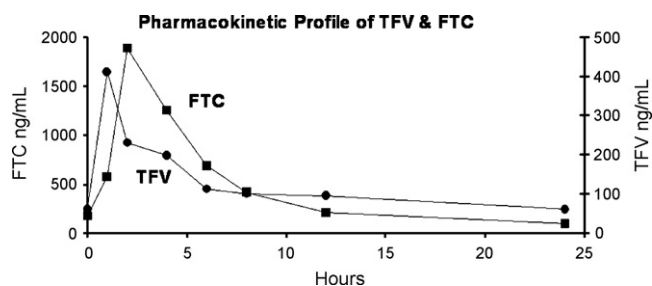
Fig. 3. A typical TFV/FTC pharmacokinetic profile.

and not very labor intensive. Since these drugs each have unique pharmacokinetic characteristics, it is desirable to monitor both in a single assay. A recent publication by Sparidans et al. [7], described a fast, sensitive LC/MS/MS method for FTC alone using deoxyfluo-rocytidine as the IS. However, in our view, the use of stable isotope labeled IS, chemically analogous to the analytes, is preferable in order to eliminate possible suppression effects which could potentially enhance or suppress the analyte/IS area ratio. Rezk et al. have published a simultaneous assay for TFV/FTC [6] using HPLC and ultra-violet detection. In this method, the plasma extracts were prepared using time-consuming solid phase extraction and evaporation with good recovery. An ion-pairing reagent was added to the mobile phase in order to achieve an adequate separation of the analytes. This method could be useful for those laboratories that are not equipped with MS/MS instrumentation. Gehrig et al. have recently characterized 18 anti-viral drugs from a single analytical run using LC/MS/MS [14]. The precursor/product ions used to monitor TFV and FTC were identical to those described herein. However, the utility of this method for the routine quantitation of these two analytes remains to be established.

3.7. Conclusion

We have expanded an LC/MS/MS method, originally designed for the assay of TFV alone, to incorporate the simultaneous determination of TFV and FTC using stable-isotope labeled analogues of both analytes as IS with similar chromatographic and chemical properties to the administered drugs, avoiding potential problems with matrix effects. Although potentially expensive to duplicate in other laboratories, this assay could well be considered the method of choice for pharmacokinetic studies involving HIV-positive patients being treated with the combined TDF and FTC therapy.

Acknowledgements

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