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Development and Validation of an LC-MS/MS Assay for Tenofovir and Tenofovir Alafenamide in Human Plasma and Cerebrospinal Fluid

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Abstract

A liquid chromatography with triple quadrupole mass spectrometry method was developed and validated for the determination of tenofovir and tenofovir alafenamide concentrations in human plasma and cerebrospinal fluid. Tenofovir and tenofovir alafenamide were extracted from matrix by solid phase extraction. The dried extraction eluents were dissolved in water for LC-MS/MS analysis. Separation was achieved with a Phenomenex Synergi 4 μ m Polar-RP 80A column (50 $\times$ 2 mm) with a gradient elution of 0.1% formic acid in water and acetonitrile. The total run time was 5 minutes. Detection of analytes was achieved using electrospray ionization (positive mode) and triple quadrupole selected reaction monitoring. Standard curve concentrations ranged from 0.5 to 500 ng/mL for the plasma assay and 0.1 to 50 ng/mL for the cerebrospinal fluid assay. The intra- and inter-day accuracy and precision were less than 12% in low, medium, and high quality control samples for both matrices. The validated methods were applied to the analysis of plasma and cerebrospinal fluid samples of a patient undergoing tenofovir therapy which involved the switch from Stribild® (elvitegravir 150 mg/ cobicistat 150 mg/ emtricitabine 200 mg/ tenofovir disoproxil fumarate 300 mg) to Genvoya® (elvitegravir 150 mg/cobicistat 150 mg/ emtricitabine 200 mg/ tenofovir alafenamide 10 mg).

Graphical abstract

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Keywords

tenofovir; tenofovir alafenamide; plasma; cerebrospinal fluid; mass spectrometry

1. Introduction

The human immunodeficiency virus type 1 (HIV-1) accesses the central nervous system (CNS) early during infection and leads to HIV-associated neurocognitive disorder (HAND). HAND is a common disease complication that is thought to be due to neurotoxic events that result from HIV replication in perivascular macrophages and microglia and immune and glial activation as well as long-term antiretroviral therapy [1]. Antiretroviral drugs vary substantially in their distribution into protected compartments like the CNS. The CNS penetration effectiveness (CPE) estimation approach attempts to evaluate drug efficacy in the CNS, based primarily on drug concentrations in the cerebrospinal fluid (CSF) [1, 2]. Drug-specific CPE estimates of distribution and effectiveness range from 1 (below average) to 4 (much above average). Higher CPE values are associated with lower HIV RNA levels in CSF, better neurocognitive function, and better survival [2–4], although some studies had inconsistent findings. Therefore, the distribution of antiretroviral drugs into the CNS appears to be important for control of HIV replication.

Tenofovir disoproxil fumarate (TDF), the first approved oral prodrug of tenofovir (TFV), has been used in combination antiretroviral therapy of HIV-1 infection since 2001 and is also used to treat hepatitis B virus. TDF is generally safe and well tolerated however, clinically relevant adverse renal events and bone loss have been reported [5, 6]. Tenofovir alafenamide fumarate (TAF) is a new oral prodrug of TFV that is more stable in plasma than TDF and is primarily converted to TFV within lymphoid tissues by cathepsin A, non-enzymatic hydrolysis, and a phosphonamidase [7–9]. Once the drug is intracellular, TFV is then converted into the active form tenofovir diphosphate which inhibits the HIV-1 reverse

transcriptase. TAF exhibits enhanced distribution of TFV into peripheral blood mononuclear cells (PBMCs) and lymphatic tissue after oral administration in comparison to TDF at a 96% lower dose (TFV equivalents of 300 mg TDF in Stribild® vs. 10 mg TAF in Genvoya®) [9, 10]. TAF's ability to target the sites of HIV-1 reverse transcriptase, lower TFV renal exposure, and enhanced stability in plasma make it an ideal replacement for therapies that include TDF. The exposure of TFV in the CNS after TAF administration is not as well understood as with TDF administration.

Several analytical assays have been published for the determination of TFV concentration in human plasma [11–16] while few have discussed the measurement of TFV in CSF [17, 18]. TAF has been measured in human plasma [19, 20] with little method description or assay validation metrics described. Methods to measure TAF and TFV by LC-MS/MS have recently been reported with validation data in cell culture media [21] and in human plasma with method application to mouse blood [22]. There are no published LC-MS/MS methods to measure TAF in human CSF and therefore a validated assay to measure TAF and TFV in plasma and CSF is warranted.

The therapeutic use of TAF entails remarkably lower dose and therefore lower concentrations of TFV are expected. In this context, the aim of this study was to develop a highly sensitive method to measure TAF and TFV simultaneously in both plasma and CSF matrices. The validation and developed assay are also described using the United States Food and Drug Administration (USFDA) Guidelines for Bioanalytical Method Validation and the Crystal City V Workshop Report [23]. Lastly, the validated assay was applied to a study participant who switched from Stribild® (elvitegravir 150 mg/cobicistat 150 mg/emtricitabine 200 mg/ TDF 300 mg), to Genvoya® (elvitegravir 150 mg/ cobicistat 150 mg/emtricitabine 200 mg/ TAF 10 mg) is described.

2. Material and methods

2.1. Chemicals and reagents

Tenofovir alafenamide and *d*₅-tenofovir alafenamide were purchased from Toronto Research Chemicals (Toronto, Ontario, CA). Tenofovir monohydrate was obtained from the National Institute of Health, AIDS Research and Reference Reagent Program (Rockville, MD). Isotopic tenofovir [adenine-¹³C₅] was purchased from Moravek Biochemicals (Brea, CA). LC-MS OPTIMA grade solvents included water, acetonitrile, and methanol obtained from Fisher Scientific (Pittsburgh, PA). Ultra-pure grade (>99.9%) N₂ gas and Ar gases were obtained from Airgas (Buffalo, NY). Human plasma and CSF used for the method development, matrix studies, standard, and QC preparation was obtained from Biological Specialty Corp (Colmar, PA) and BioreclamationIVT (Westbury, NY) respectively.

2.2. Preparation of calibration standards and quality control (QC) samples

Each analyte (TAF, TFV, and internal standards) were dissolved in suitable solvent to obtain a 1.0 mg/mL stock solution; TAF and *d*₅-TAF were dissolved in acetonitrile while TFV and ¹³C₅-TFV were dissolved in water. Stock solutions were then diluted and mixed with methanol-water (50:50, v/v) to prepare intermediate stock solutions from which calibration

standards were prepared at 10× final concentration. The 10× standards were diluted 1:10 into blank matrix for each chromatographic run to create standards at concentrations of 0.5, 1, 10, 20, 100, 200, 400, and 500 ng/mL for plasma analysis and 0.1, 0.25, 1, 2, 10, 30, 42, and 50 ng/mL for CSF analysis. Four quality control samples (LLOQ, LQC, MQC, and HQC) were made by spiking with appropriate volumes of separate 1.0 mg/mL stock solutions into either plasma or CSF to achieve concentrations of 0.5, 1.5, 150, 375 ng/mL and 0.1, 0.3, 15, and 40 ng/mL, respectively. All stock solutions, calibration standards, and quality control samples were stored at −70°C, until further used.

2.3. Solid-phase extraction (SPE)

Sample preparation for plasma and CSF were done identically. For either analysis, calibration standards were prepared by adding working solutions (20 µL of 10× concentrated drug in 50:50 methanol:water) to 200 µL of blank matrix into a 1.5 mL polypropylene microcentrifuge tube. Internal standard working solution ($^{13}\text{C}_5$ -TFV and d_5 -TAF dissolved in methanol:water) was added to the samples. The samples were acidified with 200 µL of 4% phosphoric acid in water, vortexed, and centrifuged at $20,000 \times g$ for 5 min. The MCX 96-well µelution SPE plate was preconditioned with 200 µL of methanol followed by 200 µL of water. The supernatant of the acidified sample (400 µL) was loaded onto the SPE sorbent with vacuum assistance followed by a single wash step consisting of 200 µL water. The analytes were eluted with 100 µL of 2% ammonium hydroxide in methanol. The eluents were dried to completion under a stream of heated (40°C) N_2 and reconstituted with 100 µL of water.

2.4. Instrumentation

The samples were analyzed with a Waters Acquity UPLC system (Milford, MA) which included an ultra-performance binary pump, autosampler, column manager, and an Acquity TQD triple quadrupole mass spectrometer. Data acquisition and signal output were controlled by Empower 3 software v3471. Processed samples were placed in the autosampler maintained at 10 °C and 30 µL was injected.

2.4.1. Chromatography—Analytes were separated on a heated (40°C) Phenomenex Synergi 4µ Polar-RP 50 × 2 mm column with a 0.2 µm frit filter. The mobile phases consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate was 400 µL/min delivering 97% solvent A and 3% B for 0–1 min followed by a linear gradients to 80% B from 1–2.5 min and to 95% B from 2.5–4 min then set to the initial conditions from 4.01 to 5 min.

2.4.2. Mass spectrometry—Analytes were detected in positive electrospray ionization (ESI+) mode using tandem mass spectrometry with selected reaction monitoring (SRM). The spray voltage was set to 750V with desolvation gas set at 800 L/hr. The vaporizer temperature and source temperature were set to 400 °C and 150 °C, respectively. Collision gas (argon) pressure was set at 1.5 mTorr with a flow rate of 0.5 mL/min. The ion transitions, collision energies, and cone voltages are shown in Table 1 and were determined using Water's Intellistart software.

2.5. Assay validation

Each matrix (plasma and CSF) was independently validated for accuracy and precision to assure stability based on the recommendations of the USFDA Guidelines for Bioanalytical Method Validation.

2.5.1. Calibration and linearity—Calibration curves were constructed using eight samples run in singlicate each increasing concentrations of both analytes. For each standard curve, the peak-area ratios of the analyte to their corresponding stable isotope-labeled internal standard were calculated and plotted against the nominal analyte concentration. Calibration standards ranged from 0.5 to 500 ng/mL for TAF and TFV in the plasma assay and 0.1 to 50 ng/mL for TAF and TFV in the CSF assay. Calibration curves were constructed with linear $1/x^2$ weighted regression using the appropriate blank matrix (plasma or CSF). Blank matrix samples consisting of a double blank without analyte or SLIS and single blank which contained SLIS were injected directly after the highest calibrator on each run to monitor potential carryover.

2.5.2. Accuracy and precision—Accuracy and precision in both assays were determined by evaluating the resulting concentrations measured by the analysis of the quality control (QC) samples. The inter-day and intra-day accuracy and precision were measured using three QC concentration sets, a low, medium and high (LQC, MQC, HQC) of six prepared and frozen samples each analyzed on three different days. Accuracy and precision at the lower limit of quantification (LLOQ) were assessed at 0.5 ng/mL for plasma and 0.1 ng/mL for CSF and analyzed on the same chromatographic runs as the three QC concentrations. The calculated mean concentration relative to the nominal concentration was used to express accuracy (% bias). The relative standard deviation (RSD) was calculated from the QC values and used to estimate the precision. Accuracy and precision were determined acceptable when QC concentrations were less than 15% ($\pm 20\%$ at LLOQ).

2.5.3. Matrix effects—The Matrix effect (ME), process efficiency (PE), and recovery efficiency (RE) were tested for each analyte and their internal standards. Since the internal standards for both analytes are stable labeled isotopic standards (SLIS), a simple matrix analysis was performed which consisted of two studies.

First, a slope analysis [24] was carried out by spiking six lots of plasma or CSF at a low, medium, and high concentration within the standard curve range. The six lots of plasma and CSF were each obtained from separate individuals. The peak area ratio that resulted were regressed with their nominal concentration using equal weighting and a linear model. The slopes thus generated for each individual lot of matrix were compared. The slope precision (RSD) was calculated by the average slope generated from the six lots of matrix divided by the standard deviation times 100. If the slope precision was less than 5%, signifying that there was no concentration based effect within a matrix, then the second study was initiated.

Second, an abbreviated ME, PE, and RE were evaluated which mimicked the study as described previously [25]. Briefly, a single lot of matrix (plasma or CSF) was spiked at three concentrations encompassing the standard curve range in triplicate. Three sets of samples were prepared: set 1 included neat, un-extracted analytes in water, set 2 included matrix

samples spiked with analyte post-extraction, and set 3 included matrix samples spiked with analyte pre-extraction. The ME was calculated by dividing the response of post extracted samples by neat samples and multiplying by 100. RE was calculated by dividing the response of samples spiked with analyte before SPE by the response of samples spiked with analyte after SPE and multiplying by 100. The PE was calculated by dividing the response of samples spiked with analyte before SPE by the response of neat samples and multiplying by 100. ME should be between 85% and 115% to signify a lack of a matrix effect [26, 27]. RE and PE should be consistent over the three concentrations tested.

2.5.4. Stability—Analyte stability tests were conducted to evaluate the accuracy of determining TAF and TFV under different conditions at LQC and HQC concentrations in both matrices. Freeze-thaw stability was assessed for extracted matrix based QC samples that were subjected to three freeze-thaw cycles from -70°C for 24 hrs to RT for at least one hr prior to analysis. Bench-top stability was tested by analyzing matrix-based QC samples that were left out on the bench top at RT for one day (24 hrs for plasma and 20 hrs for CSF) before processing them for an analytical run. Autosampler stability was determined for processed samples stored at 10°C within the autosampler, which were re-injected (at 96 hrs for plasma and 72 hrs for CSF) and analyzed against the initial standard curve. Long term stabilities of TAF and TFV in plasma and CSF were tested by comparing the results of stored LQC and HQC samples against a freshly prepared calibration curve. Freeze-thaw, bench-top, autosampler, and long term stability samples were compared to the nominal concentrations of the LQC and HQC and percent bias was calculated. Stability was determined when the percent bias was within 15% of the nominal concentration.

2.5.5. Dilution study—Three replicate samples with over-the-curve concentrations (1500 ng/mL for TAF and TFV in the plasma assay and 150 ng/mL for TAF and TFV in the CSF assay) were diluted 1:5 in the appropriate blank human matrix and processed for analysis. The correct dilution factor (5 $\times$) was entered into the chromatography software and results were automatically calculated and corrected for the dilution factor.

2.6. Measurement of TAF and TFV in human plasma and CSF

The assay was applied to measure TAF and TFV concentrations in a human study subject's plasma and CSF as part of a clinical study (NCT02251236). The clinical study was approved by the University of California San Diego's Institutional Review Board (IRB). A consented subject had been taking a single tablet of Stribild® (EVG/COBI/FTC/TDF) once daily for at least three months and had undetectable plasma HIV-1 RNA (< 40 copies/mL). Blood was drawn pre-dose (0 hr), 2 hr, 4hr, and 6 hr after taking a dose of Stribild® containing 300 mg TDF. Collected whole blood was immediately placed on ice until centrifugation to separate plasma from the blood cells. Plasma was aliquoted and stored at -70°C until analysis. Within an hr of the 6 hr plasma collection time point, the subject had their CSF collected by lumbar puncture (~ 7 hr). The CSF samples were aliquoted and immediately placed in a -70°C freezer until analysis. The drug regimen was switched to Genvoya® and was taken once a day for 24 weeks. At the 24th week, the same procedure was performed to collect blood and CSF samples after Genvoya® administration. All samples were measured for TAF and TFV concentrations.

3. Results and discussion

This method was developed and validated to measure TAF and TFV in human plasma and CSF matrices. The method was validated with consideration of the FDA guidelines for bioanalytical method validation and was applied to a study subject in a short PK study who had taken TDF and later switched to TAF.

3.1. Method development

This method was developed and focused on being a highly sensitive assay for TFV and TAF. The dose of TAF that a patient would receive is much lower than its potential predecessor TDF (~30 times). Our previous assay to measure TFV in human plasma when patients took 300 mg of TDF ranged from 10 to 1000 ng/mL and processed samples by protein precipitation with trifluoroacetic acid. With manually optimizing certain settings on our triple quadrupole mass spectrometer, we were able to increase the signal intensity for TFV by approximately 20-fold. The sample processing procedure of SPE was developed in an attempt to concentrate the samples. TFV is a polar molecule and SPE is difficult to optimize without losing the less polar analyte TAF. Acidifying the samples with phosphoric acid prior to performing mixed mode cation exchange SPE gave the best results for both TFV and TAF allowing them to be analyzed from the same sample. After the samples were loaded onto the SPE cartridge, different wash solutions from varying acid content to washing with plain water. The best wash was with water yielding the highest recoveries for both analytes.

Chromatography of a method to measure TFV was similar to the described steps previously published [28]. Incorporating an acetonitrile gradient to elute TAF more expediently from the LC column shortened the chromatographic run time.

3.2. Chromatographic separation

The analytes and SLIS were separated on C18 Polar-RP reversed phase LC column with a mobile phase gradient of increasing acetonitrile. Representative chromatograms of blank plasma, plasma spiked at the LLOQ, blank CSF, and CSF spiked at the LLOQ are shown in Figure 1. The analytes were identified based on retention times and mass spectra of individual analytical standards. The retention times were approximately 0.66, 0.67, 2.38, and 2.39 min for TFV, $^{13}\text{C}_5$ -TFV, d_5 -TAF, and TAF, respectively. The peaks of interest were well separated and without interference from other compounds. A total run time of 5.00 min allowed for equilibration of the column to the initial gradient conditions.

3.3. Assay validation

3.3.1. Calibration and linearity—Eight calibration standards were used which had concentrations in the range of 0.5 to 500 ng/mL for the plasma assay and 0.1 to 50 ng/mL for the CSF assay. The calibration curves were generated using the response of peak area ratios (y) versus analyte concentration (x) and regressed with a linear $1/x^2$ weighted relation. Signal-to-noise between the lowest calibrator and blank matrix samples were greater than 5:1. The intra- and inter-day accuracy and precision were 10.6% for all levels of calibration standards and r^2 values were greater than 0.992.

3.3.2. Accuracy and precision—The intra-day and inter-day accuracy (% bias) and precision (RSD) for both TAF and TFV were determined at QC concentrations of 1.5, 150, and 375 ng/mL for the plasma assay and 0.3, 15, and 40 ng/mL for the CSF assay. Assay accuracy (% bias) for all analytes ranged from –6.28 to 6.65%, and precision (RSD) ranged from 1.23 to 8.52% as shown in Table 2. Accuracy and precision at the LLOQ concentration was less than 18.1% for both analytes.

3.3.3. Matrix effect—The use of SLIS for each analyte in an LC-MS/MS method can correct for matrix associated effect of ion suppression or enhancement [29]. The abbreviated slope analysis monitors the effect of matrix over a range of concentration. If a lot of matrix had a component that caused a matrix effect, the slope would be different than if the matrix was without that component. The practice of using a SLIS is that when a matrix effect arises, both analyte and SLIS would be effected the same way and therefore the peak area ratio would correct for the effect. The six lots of plasma had a slope range of 0.0216–0.0227 (RSD = 2.28%) for TAF and 0.404–0.426 (RSD = 2.27%) for TFV. The six lots of CSF had a slope range of 0.102–0.115 (RSD = 4.57%) for TAF and 0.198–0.216 (RSD = 3.42%) for TFV. With a slope precision < 5.0%, there appears to be no significant concentration based bias resulting from matrix and therefore a ME, RE, and PE assessment with a single lot of matrix for each plasma and CSF followed.

ME, RE, and PE were studied for both plasma and CSF matrices each at three different concentrations and the data is shown in Table 3. For plasma, TAF showed considerable ion enhancement (126–232% ME) however, the SLIS for TAF displayed a similar response and therefore, the peak area ratio showed the normalized ME ranging from 101–113%. TFV in plasma showed considerable ion suppression (42.3–55.4% ME) and low recovery (21.7–29.4% RE) which therefore resulted in a low PE of 9.19% to 15.3%. The same response was observed for the SLIS of TFV and therefore the peak area ratio ranged from 98.1–105% for all three assessments. Similar results were observed when the matrix was CSF. TAF showed minor ion enhancement (118–140%) and TFV showed the same ion suppression (58.9–64.5%). It is critical that a SLIS for both analytes be used to account for the observed ion suppression or enhancement. Both plasma and CSF assays incorporate the same SPE sample processing and chromatographic conditions, therefore it is not surprising that a similar outcome for each matrix was observed. The MCX SPE procedure yielded lower recoveries for TFV than TAF. Due to the differences in the chemical properties of TAF and TFV it was difficult to enhance the extraction of one analyte without negatively effecting the other analyte. The extraction procedure provided the best outcome for both analytes that gave drug concentrations within the therapeutic range desired.

3.3.4. Sample stability—The stability of TAF and TFV in matrix-based samples were evaluated at LQC and HQC concentrations under various conditions. Stability was assessed by comparing samples undergoing a stability test to the QC nominal concentration and considered stable if the percent difference was within 15% deviation. Freeze-thaw percent difference ranged from –14.8% to 13.6% (3.11% to 13.6% in plasma and –14.8% to 8.67% in CSF), bench-top stability percent difference ranged from –11.6% to 8.31% (–1.58% to 8.31% in plasma and –11.6% to 4.41% in CSF), autosampler stability percent difference

ranged from -7.56% to 12.0% (-5.16% to 12.0% in plasma and -7.56% to 10.4% in CSF), and long term stability ranged from -3.49% to 12.7% difference as shown in Table 4.

3.3.5. Dilution study—In the event that an unknown sample would have a concentration higher than the standard curve, a dilution study was performed to report accuracy and precision of a diluted sample. Percent bias was -2.44 to 5.78% for TAF and TFV in both matrices with precision (RSD) less than 5.48% for both analytes. Additionally an attempt was made to dilute the CSF samples 1:5 with water which resulted with percent bias of -61% for TAF and -57.1% for TFV. For CSF samples, it is important to dilute them with blank CSF to obtain accurate results. Blank CSF is more difficult to obtain than blank plasma, therefore dilution with water was tested for CSF samples for feasibility. This concludes that concentrations above the curve can accurately and precisely be determined when diluted 1:5 in appropriate blank matrix and that water should not be used to dilute CSF samples.

3.4. Application of the method

The developed and validated method was used to quantify and compare the concentrations of TFV and TAF in a human study subject when they were taking TDF (week 1) and TAF (week 24). TFV was measurable in plasma at all time points when either Stribild® or Genvoya® was taken. TFV concentrations were 5–20 times lower when Genvoya® was administered. TAF was only present in the plasma with concentrations of 23.5 ng/mL and 4.71 ng/mL at 2 hr and 4 hr post-dose in the week 24, respectively. TFV was measurable in the CSF at both weeks with 3.03 ng/mL at week 1 and 0.458 ng/mL at week 24. Table 5 shows the concentrations of TFV and TAF measured. TAF was less than 0.1 ng/mL in the CSF at 7 hrs after a dose of 10 mg TAF. The undetected TAF in the CSF at the 7 hr time point was consistent with its rapid metabolism and high intracellular TFV concentrations.

The plasma concentrations of TAF and TFV measured in the study subject are comparable to prior reports [19, 20]. The present study utilized the PK booster of cobicistat and the concentrations of TAF and TFV may only be best compared to studies with the same medications [30]. To our knowledge, this is the first study to report CNS exposure of TFV from an oral administration of TAF (10 mg) with the combination of elvitegravir, cobicistat, and emtricitabine. This study reports similar TFV concentrations in CSF after TDF (300 mg) oral administration [17, 18]. The comparative results of this study further prove the accuracy and usefulness of the LC-MS/MS assay that was developed and validated.

4. Conclusion

We developed and validated an LC-MS/MS method for determination of TFV and TAF in human plasma and CSF. The method is accurate and precise to measure the analyte concentrations down to 0.5 ng/mL for plasma samples and 0.1 ng/mL in CSF samples. The assay was validated with consideration of the USFDA Guidelines for Bioanalytical Methods and suitable for pre-clinical and clinical studies.

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Highlights

- An LC-MS/MS method for measurement of TAF and TFV in human plasma and CSF was developed.
- The method is fully validated to simultaneously measure TAF and TFV
- Lower limits of quantification of 0.5 ng/mL in plasma and 0.1 ng/mL in cerebrospinal fluid.
- The method was applied to patient plasma and cerebrospinal fluid samples.

Fig. 1. Representative chromatograms of blank human plasma and CSF at the lower limit of quantification (LLOQ). LLOQ concentrations were 0.5 ng/mL for TAF and TFV in plasma and 0.1 ng/mL for TAF and TFV in CSF.

Table 1

Optimized Selected Reaction Monitoring (SRM) parameters for TAF and TFV

Analyte	Q1	Q3	Collision (V)	Cone (V)
TAF	477.3	176.0	25 [*] /48	45
<i>D5</i> -TAF	482.3	176.1	25 [*] /48	40
TFV	288.2	176.0	30	40
¹³ C5-TFV	293.2	181.1	28	45

* Collision for plasma assay was half of the optimized voltage to reduce signal

Table 2
Intra- and inter-day accuracy (%bias) and precision (RSD) for LLOQ and quality controls

Matrix	Analyte	Level	Nominal Conc (ng/mL)	Intra-day (n=6)		Inter-day (n=18)	
				% Bias	RSD	% Bias	RSD
Plasma	TAF	LLOQ	0.500	-12.5	12.6	18.1	6.09
		LQC	1.50	-3.11	8.52	1.97	8.17
		MQC	150	-2.30	3.78	-3.32	2.90
		HQC	375	-1.59	2.00	-1.49	3.22
	TFV	LLOQ	0.500	1.23	6.88	1.46	6.93
		LQC	1.50	5.89	3.09	1.25	5.58
		MQC	150	3.35	3.46	-0.851	4.53
		HQC	375	5.25	1.48	1.88	4.00
CSF	TAF	LLOQ	0.100	4.33	6.02	-5.89	13.1
		LQC	0.300	-6.28	7.24	-5.72	6.76
		MQC	15.0	-5.07	2.16	-1.25	3.96
		HQC	40.0	-4.43	1.43	-2.53	3.10
	TFV	LLOQ	0.100	-0.500	9.14	0.111	9.89
		LQC	0.300	2.17	5.72	6.65	7.09
		MQC	15.0	0.176	1.23	1.30	3.79
		HQC	40.0	0.652	2.18	0.803	4.37

Table 3

ME, RE, PE analysis for TAF and TFV in Plasma and CSF

Matrix	Drug	Level	Analyte Peak Area			SLIS Peak Area			Peak Area Ratio			
			ME	RE	PE	ME	RE	PE	ME	RE	PE	
Plasma	TAF	Low	232	94.7	220	230	87.5	202	101	108	109	
		Medium	199	88.6	177	176	88.6	156	113	100	113	
		High	126	96.0	121	114	95.5	108	111	100	111	
	TFV	Low	55.4	25.6	14.2	55.7	25.8	14.4	99.2	99.4	98.6	
		Medium	52.0	29.4	15.3	51.1	29.1	14.9	102	101	102	
		High	42.3	21.7	9.19	43.1	20.8	8.96	98.1	105	103	
	CSF	TAF	Low	140	96.2	135	132	92.5	122	106	104	111
			Medium	125	93.2	117	120	93.3	112	105	99.8	105
			High	118	93.8	110	112	96.9	109	105	96.8	101
TFV		Low	64.5	59.8	38.6	60.1	58.0	34.8	107	103	111	
		Medium	61.5	53.7	33.0	59.1	51.7	30.6	104	104	108	
		High	58.9	54.5	32.1	59.1	53.0	31.3	99.8	103	103	

Set 1 – neat samples (no matrix or extraction). Set 2 – matrix samples spiked with drug and stable labeled internal standard (SLIS) post extraction. Set 3 – matrix samples spiked with drug and SLIS pre-extraction. ME – matrix effect expressed as a ratio of set 2 divided by set 1 $\times$ 100. RE – recovery efficiency expressed as a ratio of set 3 divided by set 2 $\times$ 100. PE – processing efficiency expressed as a ratio of set 3 divided by set 1 $\times$ 100.

Table 4

Sample Stability

Matrix	Drug	Level	% deviation from nominal concentration			
			Freeze-thaw	Bench-top	Autosampler	Long-Term
Plasma	TAF	LQC	13.6	4.29	12.0	12.7
		HQC	4.50	-1.58	-5.16	2.92
	TFV	LQC	4.18	6.78	2.89	-3.49
		HQC	3.11	8.31	-0.267	1.24
CSF	TAF	LQC	-14.8	-11.6	-7.56	3.89
		HQC	-11.1	-10.0	-4.03	3.65
	TFV	LQC	8.67	-1.00	10.4	0.889
		HQC	2.28	4.41	3.56	2.45

Freeze-Thaw stability involved 3 cycles from -70°C to RT. Bench-top stability was at RT for 24 hrs (plasma) and 20 hrs (CSF). Autosampler stability was at 10 °C for 96 hrs (plasma) and 72 hrs (CSF). Long-term stability was at -70 °C for 6 mo (plasma) and 41 days (CSF).

Table 5

Plasma and CSF measurements of TAF and TFV

PK	Drug	Matrix	Hour	TFV		TAF	
				ng/mL		ng/mL	
Week 1	TDF 300 mg	Plasma	0	55.3	--	--	--
			2	195	--	--	--
			4	156	--	--	--
			6	136	--	--	--
			7	3.03	--	--	--
Week 24	TAF 10 mg	Plasma	0	10.1	--	--	--
			2	12.5	23.5	--	--
			4	14.3	4.71	--	--
			6	15.1	--	--	--
			7	0.458	--	--	--